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Edited by
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Advances in PARASITOLOGY

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Preface

The first review in this volume, by Anne-Laure Bañuls, Mallorie Hide and Franck Prugnolle from l'Institut pour la Recherche et Développement in Montpellier, France, considers the recent advances in knowledge of the genetics of species of the genus *Leishmania* and examines the ways in which these advances can help in the interpretation of the taxonomy and population structure of the organisms themselves and the epidemiology and pathogenicity of the infections they produce in humans.

In spite of some conflicting evidence, including the rare production of hybrids between some species of *Leishmania*, the authors conclude that the parasites have a predominantly clonal structure with occasional genetic exchange occurring, not necessarily by conventional sexual processes. The species are best regarded as agamospecies, and taxonomy below the level of the genus cannot therefore be based on the concept of biological species. As morphology has long been known to be inadequate for the separation of most species of *Leishmania*, the result has been taxonomic confusion and the probable creation of an unjustified number of species. The genetic heterogeneity of the genus *Leishmania*, apparently greater in the subgenus *Viannia* than it is in the other subgenus *Leishmania*, has influenced both the epidemiology and the pathogenicity of the parasites and needs to be borne in mind when seeking to elucidate underlying concepts in these two fields.

The review by Thaddeus K. Graczyk of the Bloomberg School of Public Health at Johns Hopkins University, Baltimore, USA and Bernard Fried of the Department of Biology of Lafayette College, Easton, USA, brings up to date recent scattered research on human waterborne helminth and protozoan infections. Trematodes

considered include the fasciolids, echinostomes and eye flukes. The schistosomes are not included as they have been recently reviewed in *Advances* (volume 61, 2006). Protozoans include *Cryptosporidium*, *Giardia*, *Cyclospora*, *Toxoplasma* and microsporidia. The biological features of the life cycles of some of the parasites are reviewed as are molecular methods of diagnosis in the environment.

"Russian Doll Killers" sounds like a good title for a movie but is, in fact, a fascinating and comprehensive review of gyrodactylid monogeneans written by three experts in the field: Tor A. Bakke from the Natural History Museum in Oslo, Norway, Jo Cable from the School of Biosciences, Cardiff University, UK and Philip D. Harris from the Schools of Education and Biology, University of Nottingham, UK. The gyrodactylid monogeneans are a large and diverse group of parasites, which are biologically extremely interesting and include oviparous and viviparous genera. They are also of great economic importance to the fish industry and can devastate fish stocks. The authors consider in detail many aspects of their biology ranging from morphology through taxonomy, host specificity and behaviour, evolution and host immunity to parasite pathogenicity. Epidemics of *Gyraulus salaris* on Atlantic salmon in Norway are considered and there is much of interest concerning strategies for control and the potential spread of parasites to new areas.

The final paper differs somewhat from our usual reviews, insofar as it deals more with the human host than with parasites. Michel Tibayrenc, who, like the authors of the first paper, is based at the French Institut pour la Recherche et Développement, discusses susceptibility/resistance to transmissible disease, including parasitic infections, from the viewpoint of the genetic make-up of the host rather than that of the parasite. He points out that resistance or susceptibility to infection, although based on the host's genome, is manifested phenotypically. The parasite exerts selection pressure on its host as much as the latter's immune response exerts similar pressure on the parasite, and the author makes an eloquent plea for an integrated study of this coevolutionary phenomenon. Much less is known about the interplay between the parasite's and the host's genes than about the other half of the equation, although some work has been done on identifying the loci involved in determining susceptibility or resistance to several

parasitic infections, including leishmaniasis, malaria, schistosomiasis and South American trypanosomiasis. The author draws mainly on his own experience of the latter condition, human infection with *Trypanosoma cruzi*, to illustrate his discussion.

J.R. Baker
R. Muller
D. Rollinson

A personal postscript by John R. Baker

The late Russell Lumsden, professor of medical parasitology at the London School of Hygiene and Tropical Medicine, took over the editorship of *Advances in Parasitology* from the founding editor, Professor Ben Dawes, in 1978 with volume 16, with Ralph Muller and I, then at the London School of Hygiene and Tropical Medicine and the Medical Research Council Biochemical Parasitology Unit in Cambridge, UK, respectively, as junior editors. Professor Lumsden retired from the post in 1981, and in 1994 (volume 34) the number of editors was restored to three by the appointment of David Rollinson, of the Natural History Museum in London, UK. After 30 years, and 49 volumes, I feel that the time has come, however reluctantly, to lay down my editorial red pen (possibly wielded rather prolifically, in the view of some contributors). I have greatly enjoyed my time as co-editor, and during its course I have learned a great deal of parasitology, much of which I have, needless to say, subsequently forgotten. My collaboration and friendship with Professor Lumsden and my subsequent colleagues has been wholly enjoyable and constructive. I have also benefited greatly from getting to know, and forming friendships with, the numerous authors with whose work I have been involved. It has also been a great pleasure to have worked with a series of development editors and, due to acquisitions, a series of publishers—successively, Academic Press, Harcourt Brace Jovanovich, Harcourt Brace (whatever happened to Jovanovich?) and, latterly, the publishing giant Elsevier. All of these press colleagues have been delightful to know and to work with. It is a truism, and therefore true, that without their efforts not a single volume of *Advances* would have reached the booksellers' shelves. I thank them all, and also our readers, without whom the series would soon have become defunct. I hope they have enjoyed and profited from reading the product as we, collectively, have enjoyed producing it. I am delighted to know that my place will be filled by Simon Hay, of the University of Oxford, UK, who has already proved his worth by guest editing two of our thematic volumes, those on Remote Sensing and Geographical Information Systems in Epidemiology (volume 47) and Global Mapping of Infectious Diseases (volume 62). The series remains in good hands and my wish and belief is that it may long continue to satisfy its readers.

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Leishmania and the Leishmaniases: A Parasite Genetic Update and Advances in Taxonomy, Epidemiology and Pathogenicity in Humans

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ABSTRACT

Leishmaniases remain a major public health problem today despite the vast amount of research conducted on *Leishmania* pathogens. The biological model is genetically and ecologically complex. This paper explores the advances in *Leishmania* genetics and reviews population structure, taxonomy, epidemiology and pathogenicity. Current knowledge of *Leishmania* genetics is placed in the context of natural populations. Various studies have described a clonal structure for *Leishmania* but recombination, pseudo-recombination and other genetic processes have also been reported. The impact of these different models on epidemiology and the medical aspects of leishmaniases is considered from an evolutionary point of view. The role of these parasites in the expression of pathogenicity in humans is also explored. It is important to ascertain whether genetic variability of the parasites is related to the different clinical expressions of leishmaniasis. The review aims to put current knowledge of *Leishmania* and the leishmaniases in perspective and to underline priority questions which 'leishmaniacs' must answer in various domains: epidemiology, population genetics, taxonomy and pathogenicity. It concludes by presenting a number of feasible ways of responding to these questions.

1. INTRODUCTION

The parasitic protozoa of the genus *Leishmania* Ross, 1903 are the pathogenic agents responsible for leishmaniases. These parasitoses are widespread on all continents except Antarctica (distribution maps are available on the World Health Organization website: http://www.who.int/leishmaniasis/leishmaniasis_maps/en/index.html).

Leishmaniases still constitute a major public health problem and the burden is increasing (Desjeux, 2001, 2004). In 2002, the World Health Organization (WHO) estimated the number of persons at risk to be around 350 million and the number of new cases to be 2 357 000 per year. Despite the huge amount of research conducted on these pathogens in numerous scientific fields since the beginning of the last century, recent studies have shown the reactivation of several foci—in Italy, China, Brazil and central Israel (Arias *et al*., 1996; Gradoni *et al*., 2003; Guan *et al*., 2003; Nasereddin *et al*., 2005)—and the emergence of new epidemic foci in northern and central Israel and Morocco (Jacobson *et al*., 2003; Al-Jawabreh *et al*., 2004; Guernaoui *et al*., 2005; Shani-Adir *et al*., 2005). Other studies indicate the undervaluation of the severity of these infections and of their socio-economical impact (Rioux *et al*., 1990; Desjeux, 2001, 2004). In addition, co-infection with *Leishmania* and human immunodeficiency virus is becoming more and more problematic in developing and industrialized countries (southern Europe) (Desjeux, 2001, 2004; Wolday *et al*., 2001). Nevertheless, these diseases are still considered as neglected diseases.

For all these reasons, research on these parasites is necessary to improve diagnosis and hence epidemiological study and also to support drug and vaccine development.

Despite considerable progress in cellular and molecular biology and in evolutionary genetics, we are far from understanding how these organisms act in natural populations. They have a complex life cycle, are present in very diverse ecological niches and can infect a wide range of hosts. Furthermore, *Leishmania* spp. can produce a great variety of clinical symptoms in humans. The genetic determinants responsible for this clinical polymorphism are still unknown, making this biological model complex from ecological, genetic and phylogenetic points of view. This review explores the advances in knowledge of *Leishmania* genetics in natural populations and tackles the problems of taxonomy, epidemiology and pathogenicity with the aid of genetic data.

The first objective of this review is to present an update of the genetics of *Leishmania* and to situate the biological phenomena in

the context of natural populations. The second objective is to explore the role of these parasites in the expression of pathogenicity in humans. An important issue to address is whether the genetic variability of the parasites is linked with the different clinical expressions of infection. The third objective is to integrate current knowledge of *Leishmania* and the leishmaniases.

2. GENERAL INFORMATION ON THE EPIDEMIOLOGY, TAXONOMY AND LIFE CYCLE OF *LEISHMANIA*

2.1. Life Cycle

During their complex life cycle, *Leishmania* parasites are exposed to different extra- and intracellular environments. These organisms are digenetic parasites with two basic life cycle stages: one extracellular stage within an invertebrate host (phlebotomine sand fly) and one intracellular stage within a vertebrate host (Figure 1, which is Plate 1.1 in the separate Colour Plate Section). Thus, the parasites exist in two main morphological forms, amastigotes and promastigotes, which are found in vertebrate hosts and invertebrate hosts, respectively.

2.1.1. Stages in the Invertebrate Host

The invertebrate hosts or vectors are small insects of the order Diptera, belonging to the subfamily Phlebotominae. They are commonly called phlebotomine sand flies (Figure 2, which is Plate 1.2 in the Colour Plate Section). Of the six genera described, only two are of medical importance: *Phlebotomus* of the 'Old World', divided into 12 subgenera, and *Lutzomyia* of the 'New World', divided into 25 subgenera and species groups. All known vectors of the leishmaniases are species of these two genera. Among the 500 known phlebotomine species, only 31 have been positively identified as vectors of pathogenic species of *Leishmania* and 43 as probable vectors (Killick-Kendrick, 1990, 1999). The sand fly species involved in the transmission of *Leishmania* vary from one geographical region to another but also depend on the species of *Leishmania* (see Killick-Kendrick, 1990, 1999). Like

mosquitoes, the female needs a blood meal for egg development and only the female is haematophagous.

Some phlebotomine species can support the growth of only those species of *Leishmania* with which they are infected in nature, such as *Phlebotomus papatasi* and *P. sergenti*; these species are considered to be restricted vectors (Pimenta *et al*., 1994; Kamhawi *et al*., 2000). By contrast, other phlebotomine species such as *Lutzomyia longipalpis* and *Phlebotomus argentipes* are permissive vectors since they are able to develop mature transmissible infections when infected with several *Leishmania* species (Pimenta *et al*., 1994; Kamhawi *et al*., 2000; Sadlova *et al*., 2003; Rogers *et al*., 2004).

Within the intermediate host, *Leishmania* develops as promastigote forms, elongated motile extracellular stages possessing a prominent free flagellum (Figure 3, which is Plate 1.3 in the Colour Plate Section). Nevertheless, a variety of different promastigote forms have been distinguished on morphological grounds (reviewed by Bates and Rogers, 2004).

2.1.2. Stages in the Vertebrate Host

As stated by Shaw (1997), perhaps one of the most remarkable accomplishments of *Leishmania* is that they successfully parasitize the mammalian cells that are responsible for killing invaders: the macrophages. *Leishmania* are extremely successful parasites and natural infections are found in many different orders of mammals (Lainson and Shaw, 1987): rodents, canids, edentates, marsupials, procyonids, primitive ungulates and primates. All these mammals are considered as potential reservoirs of the disease. Humans are possible hosts of these parasites, but in the majority of cases they are considered to be accidental hosts. Shaw (1997) pointed out that a *Leishmania* species is far more likely to become successfully adapted to a reservoir host on which the vector feeds regularly. Although susceptible to infection with many *Leishmania* species whose natural hosts range from edentates to rodents (Lainson *et al*., 1994), humans are not bitten regularly by the vector and they are not involved in the enzootic cycle. Similarly, the golden hamster (*Mesocricetus auratus*) is not a natural

reservoir of any *Leishmania* but it is experimentally susceptible to most of the species that infect humans (Sinagra *et al*., 1997; Bories *et al*., 1998; Saravia *et al*., 2005). This implies that mammalian host specificity may not be as restricted as the data on reservoir infections suggest. A few *Leishmania* species, such as *L. enriettii*, which has been found only in guinea pigs, may, however, present a higher degree of host specificity (Lainson *et al*., 1994).

In the vertebrate host, the parasite evolves into an amastigote form. Amastigotes are ovoid (2.5–5 μm diameter), nonmotile intracellular stages (Figure 4, which is Plate 1.4 in the Colour Plate Section). They do not have a free flagellum and are located in the parasitophorous vacuoles of the host's macrophages.

2.1.3. Life Cycle

The life cycle starts when a parasitized female sand fly takes a blood meal from a vertebrate host. As the sand fly feeds, infective promastigote forms (metacyclic promastigotes) enter the vertebrate host via the insect's proboscis. The promastigotes are then phagocytosed by macrophages in which they metamorphose into amastigote forms and reproduce by binary fission. They increase in number until the cell eventually bursts and then infect other phagocytic cells to continue the cycle (Figure 1).

2.2. Taxonomy

The classification of *Leishmania* was initially based on ecobiological criteria such as vectors, geographical distribution, tropism, antigenic properties and clinical manifestation (Bray, 1974; Lumsden, 1974; Pratt and David, 1981; Lainson and Shaw, 1987). However, biochemical and molecular analysis showed that pathological and geographical criteria were often inadequate and thus other criteria such as the patterns of polymorphism exhibited by kinetoplastic DNA (kDNA) markers, proteins or antigens came to be used to classify *Leishmania* (Chance *et al*., 1974; Kreutzer and Christensen, 1980; Simpson *et al*., 1980; Arnot and Barker, 1981; Miles *et al*., 1981; Pratt and David, 1981; De Ibarra *et al*., 1982; Handman and Curtis, 1982;

Wirth and Pratt, 1982; Anthony *et al*., 1985; Saravia *et al*., 1985; Barker *et al*., 1986; Le Blancq *et al*., 1986). A modern scheme of classification of *Leishmania* is shown in Figure 5.

All members of the genus *Leishmania* Ross, 1903 are parasites of mammals. The two subgenera, *Leishmania* and *Viannia*, are separated on the basis of their location in the vector's intestine (Lainson and Shaw, 1987). Rioux *et al*. (1990) used isoenzyme analysis to define species complexes within the subgenera.

Initially, species classification was based on various extrinsic criteria such as clinical, geographical and biological characteristics—for example, *L. guyanensis* (isolated in Guyana), *L. peruviana* (isolated in Peru), *L. infantum* (isolated from a child in Tunisia) and *L. gerbilli* (isolated from gerbils). Since the 1970s, intrinsic criteria such as immunological, biochemical and genetic data have been used to define species of *Leishmania*. Use of these molecular techniques led to the publication of a taxonomic scheme by the World Health Organization (WHO, 1990). New methods of detection, isolation and genetic identification resulted in a massive increase in the number of species described. Today, 30 species are known and approximately 20 are pathogenic for humans (see Figure 5).

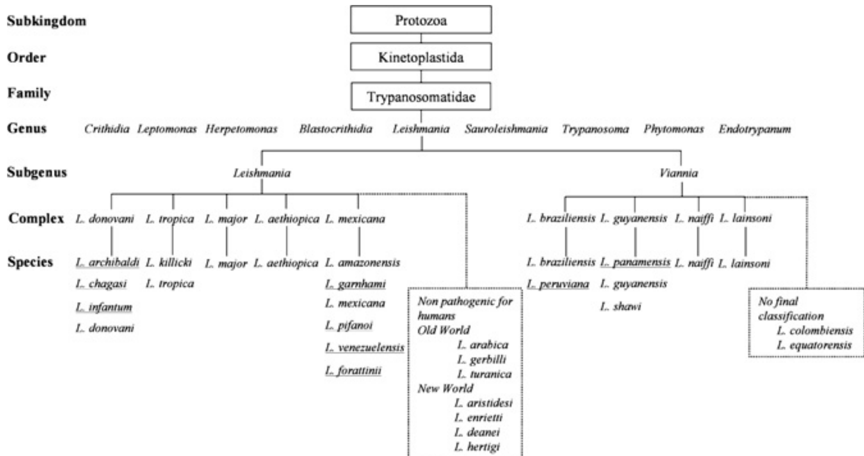

Figure 5 Taxonomy of *Leishmania*; underlined species are or have been questioned. (Based on the scheme published by the World Health Organization [WHO, 1990] with additions from the literature.)

These species generally present different epidemiological and clinical characteristics related to different genetic and phenotypic profiles. The validity of the classification scheme, considered by some workers as too arbitrary, has been questioned several times. Debate has centred on *L. panamensis*, *L. peruviana*, *L. chagasi*, *L. infantum*, *L. archibaldi*, *L. garnhami*, *L. pifanoi*, *L. venezuelensis* and *L. forattinii* (see Bañuls *et al.*, 1999b, 2000 and unpublished data; Mauricio *et al.*, 2000, 2001; Cupolillo *et al.*, 2001) (see Figure 5). Different studies have already clarified the status of some of these species; for example, *L. chagasi* is accepted as a synonym of *L. infantum* (see Mauricio *et al.*, 2000) and *L. peruviana* has been validated as an independent species (Bañuls *et al.*, 2000). The other species listed above are still under discussion.

2.3. Epidemiological and Clinical Profiles

2.3.1. Epidemiological and Ecological Diversity

The distribution area of the leishmaniases has been broadly subdivided into the 'New World' (the Americas) and the 'Old World' (Africa, Asia, Europe). *Leishmania* species are generally associated with one or other of the two subdivisions. All species of the subgenus *Viannia* were isolated in the 'New World', while those of the subgenus *Leishmania* were isolated from the 'Old World', except for species of the *L. mexicana* complex, *L. hertigi*, *L. deanei*—which are found in the 'New World' only—and *L. infantum/chagasi* and *L. major*, which are found in both the 'New' and 'Old Worlds'.

Leishmaniasis is a typical example of an anthropozoonosis. The majority of infections are originally zoonotic, although some cases are known of transmission of *L. donovani* from human to human. The different epidemiological cycles are (i) a primitive or sylvatic cycle (human infection is accidental, transmission occurring in wild foci), e.g. *L. braziliensis*; (ii) a secondary or peridomestic cycle (the reservoir is a peridomestic or domestic animal, the parasite being transmitted to humans by anthropophilic sand flies), e.g. *L. infantum*; and (iii) a tertiary, strictly anthroponotic cycle, in which the animal reservoir has disappeared (or not yet been identified) and the sand fly vectors

are totally anthroponotic, e.g. *L. donovani.* Nevertheless, many unknown factors remain. For example, the main animal reservoir of *L. braziliensis* is still unknown (Cupolillo *et al*., 2003). *L. tropica* was considered to be a strict anthroponosis, but several cases of canine infection have been described (Dereure *et al*., 1991a, b; Guessous-Idrissi *et al*., 1997).

One of the characteristics of the *Leishmania* cycle is that it is associated with various biotopes. For example, in the 'New World', the transmission cycles are mainly located in the Amazonian primary forest. Nevertheless, the foci are tending to become more domestic (Lainson and Shaw, 1987; OMS, 1990). For instance, *L. braziliensis*, described as a 'wild' zoonosis of the primary forest area, also occurs in settled areas resulting from deforestation and agricultural development and in periurban areas (Dedet, 1993). Thus, the cycle of a *Leishmania* species is not restricted to one specific environment, since a single species can be found in very different environments. Several examples can be cited such as *L. infantum* (syn. *L. chagasi*), which is present in the 'New' and 'Old Worlds' (Mauricio *et al*., 2000), and *L. guyanensis*, which is present in the Peruvian Andes and the Amazon rain forest in numerous South American countries (Lucas *et al*., 1998; Bañuls *et al.*, unpublished data). Therefore, a single parasite species can develop in different sand fly species; for example, *L. infantum* (syn. *L. chagasi*) has been isolated from very different vector species such as *Phlebotomus perniciosus* in the 'Old World' and *Lutzomyia longipalpis* in the 'New World' (Martin-Sanchez *et al*., 1994; Ferro *et al*., 1995), and the specific vector of *L. guyanensis* is *Lutzomyia umbratilis* (see Ferro *et al*., 1995), but it has also been isolated from *Lutzomyia peruensis* in the Peruvian Andes (Bañuls *et al.,* unpublished data). This environmental diversity also favours the ability of *Leishmania* to adapt to different vertebrate hosts; some species have been isolated from a large range of mammalian hosts (Rotureau, 2006).

Another complicating factor is the possible presence of several *Leishmania* species in a single leishmaniasis focus and thus sometimes in a single host (Lucas *et al*., 1998; Saravia *et al*., 1998; Martinez *et al*., 2002; Antoniou *et al*., 2004; Madeira *et al*., 2006; Rotureau, 2006). These various characteristics could have played an important

role in diversification of the *Leishmania* population and in speciation (Shaw, 1997).

2.3.2. Clinical Diversity and the Immune Response in Humans

(a) *Clinical expression in humans*. The hypothesis, based on epidemiological data, is that the majority of *Leishmania* species are adapted to a large range of hosts, and that the infections remain asymptomatic (Lainson and Shaw, 1987). On the other hand, in animals that are less well adapted, such as humans, infections can produce a wide range of diversified pathologies, from asymptomatic carriers and benign cutaneous lesions to more serious cases such as the visceral form. When humans are bitten by an infective sand fly, parasite inoculation can lead to the development of leishmaniasis but can also have no effect on health. The rate of asymptomatic carriers (infected individuals without clinical manifestations) is not accurately known, but different studies have suggested that it may be higher than expected. For example, in the Balearic Islands, *L. infantum* DNA was amplified by the polymerase chain reaction (PCR) from the blood of 22% of donors (Riera *et al*., 2004) and asymptomatic carriers have also been identified in Brazil (Costa *et al*., 2002), southern France (Le Fichoux *et al*., 1999) and India (Sharma *et al*., 2000). These data strongly suggest that asymptomatic human hosts could play an important role in transmission and could contribute to maintaining leishmaniasis foci.

Leishmaniases cause considerable morbidity and mortality. In humans, the disease occurs in at least four major forms: cutaneous, diffuse cutaneous, mucocutaneous, and visceral (Desjeux, 2004). Cutaneous leishmaniasis is frequently self-healing in the 'Old World' but, when the lesions are multiple and disabling with disfiguring scars, it creates a lifelong aesthetic stigma (Figure 6A) (Figure 6, A–D, forms Plate 1.6 in the Colour Plate Section). Its most severe form, recidivans leishmaniasis, is very difficult to treat, long-lasting, destructive and disfiguring. Diffuse cutaneous leishmaniasis occurs in individuals with a defective cell-mediated immune response (Figure 6C). Its severity is due to disseminated lesions, resembling those of lepromatous leprosy, which never heal spontaneously, and it is subject to relapse after treatment with any of the currently available drugs. Because of the

devastating consequences to the patient, it is recognized as a special public health problem. Mucocutaneous leishmaniasis, also known as espundia, causes extensive destruction of oro-nasal and pharyngeal cavities with hideously disfiguring lesions, mutilation of the face and great life-long distress for the patient (see Figure 6B). Visceral leishmaniasis, also known as kala-azar, is the most severe form (nearly always fatal if left untreated), characterized by undulating fever, loss of weight, splenomegaly, hepatomegaly and/or lymphadenopathies and anaemia (Figure 6D). It causes large-scale epidemics with a high fatality rate. After recovery, patients may develop a chronic cutaneous form called post-kala-azar dermal leishmaniasis (PKDL), which usually requires long and expensive treatment. PKDL is normally a sequel to kala-azar treatment, although some cases have been reported with no history of kala-azar. PKDL usually appears within two years of complete cure of the visceral infection, and commences with the appearance of mottling of the skin, resembling freckles. Five to fifteen percent of visceral leishmaniasis patients in India develop post-kalaazar dermal leishmaniasis, usually 1–2 years after apparent clinical cure (Salotra and Singh, 2006).

It appears that the different clinical forms are closely related to the adaptive immune response of the host, especially the equilibrium between cellular and humoral immunity. The nature of the pathogen, notably the species, seems to be a strong factor as well. Nevertheless, how *Leishmania* spp. cause human diseases and why the clinical symptoms are so variable remain enigmatic. These wide-ranging differences in clinical manifestations in human infection (virulence or degree of pathogenicity) have been discussed by Chang *et al*. (1999, 2003) and Chang and McGwire (2002).

(b) *The immune response in human leishmaniasis.* Since many individuals remain asymptomatic (see Section 2.3.2 (a) above), it is obvious that the natural immune response of humans can eliminate or control the parasites. As described above, macrophages are the first host cells to contact and be parasitized by *Leishmania*. They are key cells in the host immune defence (Basu and Ray, 2005). These cells, as well as dendritic cells, present the parasite antigens to T cell receptors, via the major histocompatibility complex (MHC) molecules (Klein and Sato, 2000; Zinkernagel and Hengartner, 2001). This

is the acquired immune response. This step is fundamental since it may influence the type of immune response (Dennert, 1974; Kisielow *et al*., 1975; Mosmann *et al*., 1986) depending on the cytokine context and on the *Leishmania* peptides presented. Thus, *Leishmania* parasites have evolved mechanisms to evade or interfere with antigen presentation processes, making it possible to partially resist the T cell-mediated immune responses (Antoine *et al*., 2004).

These escape strategies appear complex and various since, in humans, different patterns of immunological response are observed according to the clinical manifestation and exposure to different *Leishmania* species. Briefly, different T cell responses are observed among the different cutaneous forms of leishmaniasis: an absence of a Th1 response (rather than presence of Th2) in diffuse cutaneous leishmaniasis; a Th1 response in patients with self-healing lesions (Kemp *et al*., 1994; Ajdary *et al*., 2000; Kemp, 2000); and a mixed Th1/Th2 response with high interferon-γ (IFN-γ) levels in patients with mucocutaneous leishmaniasis (Carvalho *et al*., 1985; Melby *et al*., 1994; Louzir *et al*., 1998; Bacellar *et al*., 2002). In visceral leishmaniasis, a mixed Th1/Th2 response is observed with production of IFN-γ along with interleukin-10 (IL-10) (Ghalib *et al*., 1993; Kenney *et al*., 1998). However, individuals with asymptomatic or subclinical infections of visceralizing species of *Leishmania* show peripheral blood mononuclear cell (PBMC) proliferation and production of IL-2, IFN-γ and IL-12; in cured patients, both Th1 and Th2 clones producing IFN-γ and IL-4 have been isolated (Kemp *et al*., 1993).

3. EVOLUTIONARY GENETICS AND POPULATION STRUCTURE OF *LEISHMANIA*: IMPACT ON TAXONOMY AND EPIDEMIOLOGY

3.1. *Leishmania* Genome

3.1.1. Nuclear Genome

The *Leishmania major* genome project, begun in 1994, has had considerable success. The sequencing of *Leishmania major* is finished

(see the website http://www.sanger.ac.uk/Projects/L_major) and the sequencing of *L. braziliensis* and *L. infantum* is in progress (Ivens *et al.*, 2005). The determination of these *Leishmania* genome sequences will be a milestone for *Leishmania* research. The genome of *L. major* has been evaluated at 32.8 Mb distributed on 36 chromosome pairs. 'Old World' *Leishmania* species have 36 chromosome pairs (0.28–2.8 Mb) (Wincker *et al.*, 1996), whereas the 'New World' species have 34 or 35, with chromosomes 8 and 29, and 20 and 36, fused in the *L. mexicana* group and 20 and 34 in the *L. braziliensis* group (Britto *et al.*, 1998). The chromosomes are linear, between 200 and 4000 kb in length, and possess telomeres, but centromeres have not been identified (Lighthall and Giannini, 1992). Chromosome size variability is characteristic of some *Leishmania* species, even between homologous chromosomes (Blaineau *et al.*, 1991), which complicates the use of the karyotype for taxonomic studies.

From the *L. major* genome, 911 RNA genes, 39 pseudogenes and 8272 protein-coding genes (36% of which have a putative function) were predicted. The genome has a G + C content of 59.7% (Ivens *et al.*, 2005). *Leishmania* genes are often organized in tandem arrays or at least have two or more copies spread through the genome (Bard, 1989).

Sequence analysis revealed a density of one gene every 3.5 kb. Previous studies have established that 30% of the genome is composed of repeated elements, roughly half of which are telomeric/subtelomeric repeats, the rest being dispersed transposons, repeated genes and other simple repeated sequences. None of the protein-encoding genes of *Leishmania* studied to date contains introns, simplifying the identification of these genes in the genomic DNA. The first comparisons with other genomes revealed that gene order is highly conserved among the 30 *Leishmania* species, although interloci distances may vary (Ravel *et al.*, 1999). Nevertheless, a high level of polymorphism exists in nucleic acid sequences (Beverley *et al.*, 1987; Ravel *et al.*, 1999). Indeed, Beverley *et al.* (1987) estimated that nuclear DNA sequence divergence based on restriction patterns among the major lineages of *Leishmania* ranged from 13% to 25%, which is comparable to that observed between animal species that diverged 10–80 million years ago (Britten, 1986; Beverley *et al.*, 1987). This level of

divergence has been confirmed by Ravel *et al.* (1999) based on the comparison of *L. major* and *L. infantum* genomes. The complete genome sequencing of *L. braziliensis* and *L. infantum* will provide information about the true genetic divergence between these species.

Several studies suggest that *Leishmania* chromosomes are mainly diploid (Iovannisci *et al.*, 1984; Beverley, 1988), with some chromosomes being aneuploid (Bard, 1989). Until the mid-1990s, *Leishmania* was considered an aneuploid organism. Now diploidy of *Leishmania* is widely accepted (Wong, 1995). The ploidy level was challenged since gene amplification is a frequent mechanism by which *Leishmania* resists the action of cytotoxic or environmental pressures (Beverley, 1991). Nevertheless, Cruz *et al.* (1993) demonstrated that these relatively frequent phenomena transform *Leishmania* into facultative or transient aneuploids.

Even though the three classical RNA polymerases (pol I, II and III) have been detected in trypanosomatids (Cornelissen *et al.*, 1990), *Leishmania* exhibits an unusual combination of gene expression mechanisms (Donelson *et al.*, 1999). In trypanosomatids, including *Leishmania*, most gene transcription is polycistronic (Kapler and Beverley, 1989), followed by processing reactions including a coupling of *trans*-splicing and polyadenylation. Genes are initially transcribed into large polycistronic precursor RNAs 60 kb or more in length, which are cleaved into monocistronic mRNAs by the action of two intergenic RNA cleavage reactions, trans-splicing of a 39 nucleotides spliced leader to generate the 5′ ends of all mRNAs, and 3′ cleavage/polyadenylation to create the 3′ ends (Ullu *et al.*, 1993; Donelson *et al.*, 1999). Efficient 5′ trans-RNA splicing typically occurs at a short consensus sequence preceded by a polypyrimidine tract (Matthews *et al.*, 1994). Little is known about the mechanism of transcription initiation in trypanosomatids, and only a few promoters have been functionally analysed (Clayton, 2002). Different processes of protein modification occur within *Leishmania* (phosphorylation, glycosylation and lipidation for stabilization and/or activation). Several major modifications have been described and shown to be essential, namely glycosylphosphatidylinositol (GPI) anchor addition, acylation (including *N*-myristoylation and palmitoylation) and prenylation, all of which facilitate membrane attachment and/or protein–protein interactions.

Figure 7 Kinetoplast DNA (kDNA) of *Leishmania*. The kDNA is a network containing thousands of catenated DNA molecules (minicircles and maxicircles) (electron micrograph by Michèle M. Klingbeil, reproduced with permission).

3.1.2. Kinetoplast DNA

The kDNA is the mitochondrial DNA of the Kinetoplastida and constitutes 10–20% of the total DNA (Simpson, 1987). It is a network of concatenated circular DNA (see Figure 7), divided into two classes: the homogenous maxicircles (~25–50 molecules of 20 kb) and the heterogeneous minicircles (~0.8 kb), which have many (~10^4) copies. The maxicircle is the functional counterpart of the mitochondrial DNA even though its role in the editing of uracil residues into mRNA nucleotides has been demonstrated (León *et al.*, 1996). The minicircles encode guide RNAs (gRNA) for editing cytochrome oxidase subunit III mRNA (Sturm and Simpson, 1990). Also, Singh and Rastogi (1999) have described an unprecedented open reading frame in the variable region of one of the minicircle sequence classes of *L. donovani*, which is transcribed and translated to a protein product.

3.2. Molecular Tools for Epidemiology, Population Genetics and Phylogeny

The only way to detect the true genetic diversity of an organism is sequencing several entire genomes from different isolates of natural

populations. Since it is not yet possible to sequence the whole genome of *Leishmania* populations, in the last few decades, many different molecular methods based on DNA ‘fingerprints’ have been developed. Several molecular techniques are available to explore the genetic diversity of *Leishmania* populations. Only the main molecular techniques classically used for the study of genetic diversity in a natural population will be discussed here.

3.2.1. Multilocus Enzyme Electrophoresis and Multilocus Sequence Typing

For epidemiological purposes, the most useful taxonomic technique has long been isoenzyme analysis or multilocus enzyme electrophoresis (MLEE) (Miles *et al.*, 1980; Evans *et al.*, 1984; Rioux *et al.*, 1990; WHO, 1990). This technique is still considered the ‘gold standard’ for *Leishmania* species identification and genetic diversity studies. MLEE detects different alleles of housekeeping genes by scoring the electrophoretic mobility of the enzymes they encode. Mobility differences usually reflect differences in the charge (amino acid sequence) of proteins, and thus nucleotide differences in the genes encoding them. In MLEE data analysis, it was generally assumed that isolates exhibiting the same mobility for a given enzyme contain the same allele of the underlying gene. This technique can be performed on starch gel or acetate gel (see Figure 8 for an example of MLEE on acetate gel). A zymodeme consists of all the strains showing exactly the same profiles for all the enzymatic systems under study (the number of enzymatic systems used depending on the study). This technique remains one of most frequently used molecular techniques for *Leishmania* species identification and zymodeme characterization. Indeed, there are international databases in the WHO reference centres composed of worldwide zymodemes. Nevertheless, this technique has several disadvantages. First, it requires mass culture of *Leishmania* and use of a large amount of protein. Second, it is very time-consuming, labour-intensive, costly and technically demanding. Third, it has relatively poor discriminatory power since nucleotide substitutions that do not change the amino acid composition remain undetected, as do changes in the amino acid composition that do not

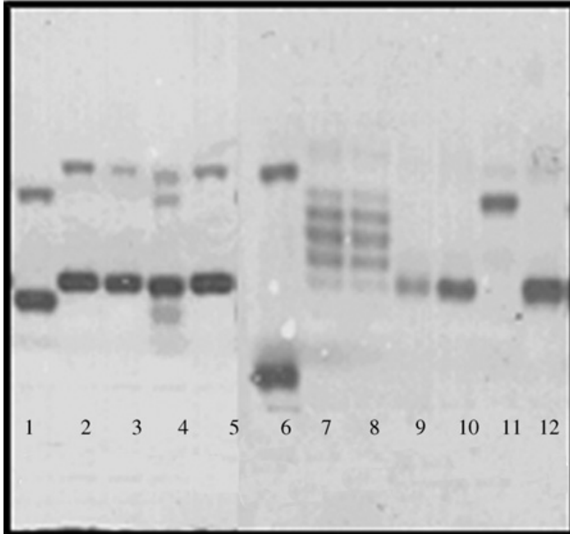

Figure 8 Multilocus enzyme electrophoretic (MLEE) profiles of nucleoside hydrolase substrate inosine on acetate plates from various species and hybrid strains of *Leishmania*. Lane 1: *L. amazonensis*; lanes 2, 3, 5, 10, 11 and 12: *L. braziliensis*; lane 4: mixed infection of *L. braziliensis* and *L. amazonensis*; lane 6: *L. infantum*; lanes 7 and 8: hybrids between *L. braziliensis* and *L. guyanensis*; lane 11: *L. guyanensis.*

modify the electrophoretic mobility. Thus MLEE is not well adapted for microepidemiology. For example, the most famous *L. infantum* zymodeme, MON1, is responsible for the majority of visceral leishmaniasis cases around the Mediterranean basin. This zymodeme was revealed to be more genetically heterogeneous and was shown to be polyphyletic with better resolving molecular markers (Hide *et al.*, 2001; Ochsenreither *et al.*, 2006). This technique was also confusing from a taxonomical point of view. For example, the distinction between *L. donovani* and *L. infantum* does not appear robust since it is based on only one enzymatic system, GOT (glutamate–oxaloacetate transaminase). Therefore, one zymodeme, MON30, was long identified as *L. infantum*, whereas recent analyses with various markers have shown that it is *L. donovani* (see Jamjoom *et al.*, 2004; Zemanova *et al.*, 2004). One of the advantages of this technique is its codominant character, which can easily identify heterozygous profiles and thus potential hybrids (see Figure 8). Furthermore, if the proteins

are highly polymeric, the distinction can be made between a heterozygous profile and a mixed infection, which is not the case for microsatellite markers, for example (see below).

MLEE was developed when the ability to sequence DNA rapidly could hardly be imagined and, although the method is now dated, the concept of indexing variation at multiple loci remains as valid as ever. A modification of this method, multilocus sequence typing (MLST), which takes advantage of the speed and simplicity of automated DNA sequencing, was described at the end of the 1990s and had many important advantages over the other methods available at the time (reviewed by Enright and Spratt, 1999). MLST is based on the well-tested principles of MLEE but identifies the alleles at each locus directly by nucleotide sequencing. As nucleotide sequencing reveals all of the variation at a locus, the number of alleles found per locus is much higher than with MLEE, and sequences of 450–500 bp internal fragments of only seven housekeeping genes are determined for each isolate. This length of DNA fragment was chosen as it can be sequenced accurately on both strands using a single pair of primers and, in most pathogens, it provides sufficient variation to identify many different alleles within the population (see the review by Enright and Spratt, 1999). This technique was first used for bacterial pathogens (Enright and Spratt, 1998; Maiden *et al.*, 1998; Feil *et al.*, 1999) and today it remains largely exploited and considered as the reference technique for several bacterial organisms (Bougnoux *et al.*, 2006; Perez-Losada *et al.*, 2006; Vivoni *et al.*, 2006) and has been very little used for *Leishmania*. Indeed, only one analysis similar to MLST has been performed very recently on the *L. donovani* complex (Mauricio *et al.*, 2006). These authors demonstrated that the technique could be applied directly to clinical samples or to small-volume cultures. Furthermore, it can be used to detect recombination indirectly and for population genetics studies (Mauricio *et al.*, 2006).

3.2.2. Molecular Karyotyping

Trypanosomatid chromosomes have been studied using pulse field gel electrophoresis (PFGE) and variations on this technique such as

orthogonal field alternate gel electrophoresis (OFAGE), since there is no evidence available for typical chromosome condensation during mitosis (Bishop and Miles, 1987; Lighthall and Giannini, 1992; León *et al.*, 1996). This technique allowed separation and visualization of the *Leishmania* chromosomes. A PFGE-karyotype is the simplest type of physical map. During continuous field electrophoresis, DNA above 30–50 kb migrates with the same mobility regardless of size. This is seen in a gel as a single large diffuse band. If, however, the DNA is forced to change direction during electrophoresis, different sized fragments within this diffuse band begin to separate from each other. With each reorientation of the electric field relative to the gel, smaller sized DNA will begin moving in the new direction more quickly than the larger DNA; thus, the larger DNA lags behind and is separated from the smaller DNA.

This technique also has been extensively used for genetic characterization of *Leishmania* populations (Blaineau *et al.*, 1992; Dujardin *et al.*, 1993a, b, 1994, 1995a, b, c, 1998; Katakura *et al.*, 1993; Bañuls *et al.*, 2000; Pacheco *et al.*, 2000; Guerbouj *et al.*, 2001). Karyotype variability has been shown to result mainly from chromosomal rearrangement, an event taking place at a rate different from the rate of point mutations (Wilson *et al.*, 1974). In the genus *Leishmania*, molecular karyotype displays extensive plasticity as well as conserved characters (Lighthall and Giannini, 1992). Two types of protocols were developed to study genetic polymorphism in *Leishmania* populations: size determination of chromosomal bands on ethidium bromide-stained OFAGE gels (Pagès *et al.*, 1989; Giannini *et al.*, 1990; Blaineau *et al.*, 1992) and size determination of chromosomes following hybridization with probes for genes such as the mini-exon gene or *psa*-2 or *gp*63 (Giannini *et al.*, 1986, 1990; Dujardin *et al.*, 1993a; Guerbouj *et al.*, 2001). Molecular karyotyping seems particularly appropriate for studying recent evolutionary divergence, including eco-geographical diversification, because of its rapid rate of evolution (resulting essentially from amplification/deletion of tandemly repeated genes). Despite this fast chromosome evolutionary rate, significant differences may be observed between major lineages, probably corresponding to major and less frequent rearrangements (fusion/fission, translocation) (Dujardin *et al.*, 2000).

3.2.3. Microsatellites

For more than 15 years, microsatellite markers have been extensively used to study the population genetics of various organisms (Goldstein and Schlotterer, 1999). Microsatellites (also known as short tandem repeats or STRs) are short stretches of DNA in which a motif of l–6 bp is repeated, often up to 60 times. They show substantial allelic diversity at a large number of loci and are distributed throughout the genome, inherited in a Mendelian fashion and co-dominant (Litt and Luty, 1989). Microsatellites are easy to clone (therefore banks can be established) and characterized, and they display considerable polymorphism because of variation in the number of repeat units. This high variability makes them the most useful molecular markers now available for use in genetic typing of individuals or of larger populations (Bruford and Wayne, 1993) and also for constructing high-resolution genetic maps to identify susceptibility loci involved in common genetic diseases (Hearne *et al.*, 1992). Zhu *et al.* (2000) reported that microsatellites were among the fastest evolving DNA sequences, with length mutation rates ranging from 10^{-2} to 10^{-5} per generation. The most likely mechanism for length variation is mutation in the repeat number due to slippage during replication (Schlotterer and Tautz, 1992; Strand *et al.*, 1993; Weber and Wong, 1993). It is thought that microsatellite markers occur within all eukaryotic organisms. If this is indeed true, then it is reasonable to expect that they are capable of playing a significant role in the study of parasite population genetics, as they have done in similar studies for vertebrates. Nevertheless, in *Leishmania* or *Trypanosoma cruzi* only a few genetic structure studies have made use of these markers. In the genus *Leishmania*, microsatellites have been under-exploited so far for population genetics or microepidemiology studies. Furthermore, the few studies published have used these markers mainly to examine allopatric samples. Nevertheless, these studies showed the advantages of microsatellite study for the population genetic approach in this genus. Several microsatellite loci have been reported in a few *Leishmania* taxa such as the *L. donovani* complex (Bulle *et al.*, 2002; Jamjoom *et al.*, 2002; Ochsenreither *et al.*, 2006), *L. tropica* (see Schwenkenbecher *et al.*, 2004, 2006) and the subgenus *Viannia*

(see Rodriguez *et al*., 1997; Russell *et al*., 1999). This technique has made it possible to reveal more intraspecific genetic polymorphism and higher frequencies of heterozygous loci than previously (Schwenkenbecher *et al*., 2004, 2006). Thus, their potential interest for intraspecific genetic study has been proved. Unfortunately, there has been no rigorous study of population genetics using these markers in the genus *Leishmania*. It is worth noting that these markers are not suitable for phylogenic studies of *Leishmania* at a supraspecific level since they are generally specific for only one species or one complex and they evolve too rapidly.

3.2.4. PCR– RFLP, RAPD and SCAR

Methods to genotype strains within parasite species using rapid PCR-based assays have provided valuable tools to study the genetic diversity of parasites. DNA amplification by PCR from low numbers of parasites in small sample volumes can overcome the limitations imposed by culture *in vitro* of clinical or field isolates, which may lead to selective growth and selection of strains. The PCR-based methods used most often for genotyping are PCR-restriction fragment length polymorphism (PCR-RFLP), random amplified polymorphic DNA (RAPD), and sequence-confirmed amplified region analysis (SCAR). These are rapid and simple tools which have been recently applied in molecular epidemiological studies. They provide useful information on the nature and extent of genetic diversity of parasite populations in a specific geographical location.

RFLP was long used to replace sequencing. PCR-RFLP remains in wide use today. This method detects minor variations in a gene where a single base substitution has either created or abolished a site capable of being digested by a specific restriction endonuclease. This methodology has been applied to various genes in the genus *Leishmania* such as the *gp63* coding sequences (Victoir *et al*., 1998; Mauricio *et al*., 2001), kDNA amplification (Ferroglio *et al*., 2006), the cysteine proteinase B gene (*cpb*) (Tintaya *et al*., 2004) and miniexon sequences (Marfurt *et al*., 2003). Recently, Rotureau *et al*. (2006) demonstrated that this technique, based on a small subunit and internal transcribed

spacer 1 of the rRNA genes, has many advantages over MLEE: (i) it differentiated the most medically relevant *Leishmania* species using only one enzyme (RsaI), (ii) it could be used directly on human biopsy specimens (sensitivity = 85.7%) and (iii) it revealed great diversity of several enzymes and thus could also be useful for taxonomic, ecological and epidemiological studies in space and time.

The PCR-RFLP method for genotyping relies on prior knowledge of the DNA sequence to be amplified. However, with RAPD, no DNA sequence knowledge is required, as only one relatively short oligonucleotide of arbitrary sequence is utilized in the PCR. This technique provides an effective method for obtaining genetic markers with all sorts of organisms (Welsh and McClelland, 1990; Williams *et al.*, 1990; Welsh *et al.*, 1991, 1992). The RAPD method identifies polymorphisms that are detected as DNA fragments, amplified by PCR, that are present in one but not in another individual or strain. Short oligonucleotides (about 10-mer) of arbitrary nucleotide sequences are often used as amplifying primers, although nonrandom longer primers have proved useful for certain purposes (Welsh *et al.*, 1991). Normally a number of short oligonucleotides are tested individually and those which result in reproducible banding patterns are used for genetic studies. Since the oligonucleotides are capable of binding to any complementary DNA, there is a high risk of DNA contamination. Using this technique, DNA 'fingerprinting' and genetic variation have been described for many parasites and have been extensively used for studying the structure of natural populations (Tibayrenc *et al.*, 1993; Mauricio *et al.*, 1999; Bañuls *et al.*, 2000). RAPD proved to be informative in differentiating *Leishmania* species and some intraspecific lineages (see, e.g., Hide *et al.*, 2001; Zemanova *et al.*, 2004). Furthermore, it is rapid and simple to use. Thus, RAPD is often used in molecular studies (Cuervo *et al.*, 2004; Indiani de Oliveira *et al.*, 2004; Martin-Sanchez *et al.*, 2004; De Castro *et al.*, 2005). The main drawbacks of this technique are that (i) bands of equal electrophoretic mobility may not be homologous, (ii) recognition of allelic variants of randomly amplified polymorphic DNA markers is difficult in the absence of crossing data and so it is normally impossible to distinguish homozygous from heterozygous genotypes at specific loci with this technique, and (iii) the technique is not very reproducible.

Genetic markers identified by sequence-confirmed amplified region analysis (SCAR) have been successfully applied to population genetic studies in fungi (reviewed by Taylor *et al*., 1999). These markers have two advantages: (i) they seem to represent a random sample of genetic variation in natural populations and (ii) they can easily be amplified by conventional PCR techniques using only minute quantities of even partially degraded DNA. These markers were initially amplified by PCR using random primers and subsequently analysed by single-strand conformation polymorphism and direct sequencing. They were used to assess levels of genetic variation, heterozygosity and genotype segregation in different strains of *L. donovani* (see Lewin *et al*., 2002). The markers are co-dominant since they can detect all possible allele combinations in a diploid organism and may, therefore, be very useful for population genetic analysis of *Leishmania.* This technique has also been used for genetic characterization of *Trypanosoma cruzi* (see Brisse *et al*., 2000). The authors concluded that the excellent correspondence of these PCR markers with the phylogenetic lineages, allied with their sensitivity, made them reliable tools for lineage identification and strain characterization in *T. cruzi*; this approach could be generalized to any species of microorganism harbouring clear-cut phylogenetic subdivisions. Despite all the advantages described, SCAR has not been extensively used for *Leishmania*. The main drawback of this technique is that the PCR fragments must be cloned and labelled radioactively. Thus, SCAR is time-consuming, labour-intensive, costly and technically demanding.

3.2.5. Kinetoplast DNA

Leishmania kDNA has mainly been used as a diagnostic tool to detect small amounts of parasite DNA in biological materials because of its highly repetitive character (see the reviews by Barker, 1989 and Wilson, 1995 for more detail). kDNA is a useful target for PCR due to the abundance of minicircles. The high degree of sequence heterogeneity makes kDNA useful for taxonomic and genetic purposes. Studies have shown that kDNA, and especially the minicircle sequences, have a significant degree of polymorphism together with

some highly conserved regions (McCann *et al.*, 1999; Franco *et al.*, 2000; Brewster and Barker, 2002). Based on these properties, kDNA is also used as a target to study genetic polymorphism at specific and intraspecific levels. Two types of approach have been developed: (i) PCR amplification of kDNA minicircles by specific primers followed by cleavage of PCR fragments by different restriction endonucleases (RFLP) (Kapoor *et al.*, 1998; Berzunza-Cruz *et al.*, 2000; Rodriguez-Gonzalez *et al.*, 2006); (ii) PCR amplification of kDNA minicircles using general kinetoplastid primers, which generates a polymorphic multi-banding pattern for all *Leishmania* species and other Kinetoplastida, followed by hybridization of the PCR products with specific kDNA probes (Brenière *et al.*, 1999). The banding patterns produced were generally very heterogeneous, making kDNA-PCR useful for differentiating closely related strains and for 'fingerprinting' individual strains. A combination of kDNA-PCR fingerprinting and hybridization with kDNA probes was found to be useful for both sensitive detection and direct identification of *Leishmania* species complexes (Brenière *et al.*, 1999). However, this technique is also labour-intensive and costly.

3.3. Population Structure of *Leishmania*: Evolutionary, Epidemiological and Taxonomic Consequences

3.3.1. Population Structure, Reproductive System and Recombination Events

Different evolutionary mechanisms such as migration, selection and genetic drift play a fundamental role in the genetic distribution in natural populations, but reproduction is the basic biological process influencing the population genetic structure. Identifying the reproductive system is all the more essential since it governs the allelic and genetic distribution in natural populations and conditions the stability of genotypes in space and time (Tibayrenc, 1995; Ayala, 1998; Spratt and Maiden, 1999). Therefore, this has important consequences from an epidemiological and medical point of view (strain typing, pathogenicity, vector specificity and susceptibility to drugs

and vaccines). For pathogenic microorganisms such as some bacteria and parasitic protozoa, the clarification of population structure provides unique insights into crucial public health issues, including the appearance and persistence of variants escaping immunity or the emergence of resistance to antibiotics (Tibayrenc and Ayala, 1991; Musser, 1996; Levin *et al*., 1999; Spratt and Maiden, 1999; Smith *et al*., 2000). Consequently, it appears incontestable that knowledge of the reproductive system is essential to exploit molecular epidemiological data fully. Prugnolle *et al*. (2005) explained that the study of population genetic structure is crucial for the understanding of microevolutionary processes (Nevo *et al*., 1984; Nadler, 1995). Furthermore, for small organisms and in particular parasites, the analysis of genetic variation at different hierarchical levels is often the only way to investigate natural population characteristics such as gene flow and breeding strategies (Nadler, 1995). Population genetic structure can also constitute a powerful tool to investigate epidemiological patterns (Paterson and Viney, 2000).

Pathogenic organisms including bacteria, parasitic protozoa and trematodes have a range of breeding strategies, but the most startling life-history trait of these organisms is that within a genus or even in a single species they are able to use several breeding strategies either (i) depending on the stage of the life cycle, e.g. *Plasmodium falciparum* (see Razakandrainibe *et al*., 2005), *Toxoplasma gondii* (see Ajzenberg *et al*., 2004) and trematodes (see Prugnolle *et al*., 2005) or (ii) independently of the cycle but as a function of environmental pressures (known or unknown), e.g. *Trypanosoma brucei* (see MacLeod *et al*., 2000), *Trypanosoma cruzi* (see Brisse *et al*., 2000) and *Neisseria meningitidis* (see Smith *et al*., 2000).

Leishmania also has complex breeding strategies and can implement various evolutionary processes. The first descriptions of *Leishmania* population structure were the subject of debate until the mid-1990s. These parasites were included in the clonal model by Tibayrenc *et al*. (1990). However, other authors have challenged this hypothesis based on pulse field gel electrophoresis (PFGE) data and have proposed that some *Leishmania* populations are potentially panmictic (Bastien *et al*., 1992; Blaineau *et al*., 1992). This hypothesis was also investigated using *L. peruviana*. The genetic markers used in the study gave

apparently conflicting results. OFAGE data showed no apparent departure from panmictic expectations, while RAPD results showed considerable linkage disequilibrium (different reproduction models can produce linkage disequilibrium such as self-hybridization, autogamy, consanguinity and clonality). Indeed, putative parental OFAGE genotypes and all possible offspring combinations were present in the same sample (Dujardin *et al.*, 1993b). Blaineau *et al.* (1992) described a similar apparent genetic equilibrium in chromosomal variability in a population of *L. infantum* from the Pyrenean valleys in France and they explained it by either random genetic exchange or a high rate of recurrent chromosomal mutations. Accumulation of chromosomal rearrangements might explain the apparent discrepancy between OFAGE and RAPD results and this phenomenon has been extensively described. It is mainly the result of amplification/deletion of repeated sequences (Iovannisci and Beverley, 1989; Victoir *et al.*, 1995) and a very fast molecular 'clock' (Dujardin *et al.*, 1998). However, even if such mechanisms might theoretically generate all the observed combinations, it is still not clear whether they could lead to frequencies that meet panmictic expectations. A last hypothesis to account for apparent panmixia in OFAGE data might be the occurrence of pseudo-recombination in *Leishmania* populations. This could happen without sexual exchange, in three steps. First, rearrangement might occur in one chromosome of a pair of homologues, as described elsewhere (Iovannisci and Beverley, 1989; Dujardin *et al.*, 1994; Wincker *et al.*, 1996). Secondly, this parasite might become tetraploid (totally or for this chromosome alone) under stress conditions, as shown by experiments *in vitro* (Cruz *et al.*, 1993). Return to diploidy might generate apparent patterns of segregation (or linkage equilibrium if considering two chromosomes) of chromosomal variants without affecting the genetic loci characterized by RAPD. These phenomena have also been demonstrated *in vitro* since the molecular karyotype of an *L. peruviana* strain changed during long-term cultivation (Dujardin *et al.*, 2007). Thus, the hypothesis proposed was that *Leishmania* presented a basically clonal population structure with the occurrence of pseudo-recombination events. These evolutionary processes could explain the divergence which existed in the literature concerning the clonality or sexuality of *Leishmania*. Concurrently with

these phenomena of intrachromosomic recombinations, Victoir *et al.* (2005) suggested mechanisms of intragenic recombination. From a study of the organization of the gene *gp63* (or *msp*, *major surface protein*) the authors provided evidence of a mosaic structure in *Leishmania*. This type of structure is normally encountered in a sexual species (Kapur *et al.*, 1995; Maiden, 1998; Putaporntip *et al.*, 2002) and is generally attributable to reciprocal crossing over and segmental gene conversion, as reported in *Trypanosma brucei* by Victoir *et al.* (2005). In *Leishmania*, gene amplification, which is a common phenomenon (Iovannisci and Beverley, 1989; Inga *et al.*, 1998; Kebede *et al.*, 1999), and the linkage of *gp63* genes with subtelomeric regions, which are known to be especially turbulent (Inga *et al.*, 1998; Wickstead *et al.*, 2003), might be responsible for these mosaic structures.

Concerning interindividual recombinations, some studies suggest genetic exchange in *Leishmania* despite the lack of evidence for a sexual stage. For example, in the 'New World', hybrids between *L. braziliensis* and *L. peruviana*, and *L. guyanensis* and *L. braziliensis*, have been described (Da-Cruz *et al.*, 1992; Belli *et al.*, 1994; Dujardin *et al.*, 1995a; Bañuls *et al.*, 1997) and in the 'Old World' hybrids have been reported between *L. major* and *L. arabica* (see Evans *et al.*, 1987; Kelly *et al.*, 1991) and between *L. infantum* and *L. donovani* (Hide *et al.*, 2006 and in press). Furthermore, Kreutzer *et al.* (1994) demonstrated by quantitative microspectrophotometry that nuclear fusion or sexual reproduction takes place in the intracellular amastigote form. This has been confirmed by Youssef *et al.* (1997) in amastigotes and promastigotes stained using the Feulgen procedure followed by determination of nuclear DNA content using computerized image analysis. Nevertheless, this is the only evidence obtained *in vitro* of possible sexual events. It is worth noting that molecular data suggest that, after a hybridization event, hybrids are clonally propagated in a natural population, e.g. the *Leishmania* sample from Huanuco (Dujardin *et al.*, 1995a) or hybrids between *L. braziliensis* and *L. guyanensis* from Ecuador (Bañuls *et al.*, 1997). To date, the hypothesis is that *Leishmania* has a clonal population structure with the occurrence of genetic exchanges that are not frequent enough to disturb the propagation of clones stable in space and time. Nevertheless,

much remains to be elucidated since contradictions remain in the model such as the absence of excess heterozygosity and the lack of a clear structure in individualized lineages at an intraspecific level, elements that would normally be expected in a clonal model (unpublished observations). In Section 5 (p. 53) recommendations are provided to advance knowledge of the population structure and develop a reproduction model of these organisms.

3.3.2. Genetic Processes and Evolutionary Significance

As described above, *Leishmania* species must adapt to varied and heterogeneous environments during their life cycle. Efficient biological mechanisms are essential for the long-term and short-term survival of the parasite. It seems evident that the different processes described above—asexual multiplication, gene amplification, occasional interindividual recombination, and intrachromosomic and intragenic recombination—might be evolutionarily advantageous for *Leishmania.*

First, it is postulated that the asexual model contributes to parasite fitness and that sexual recombination would not be necessary for the production of a large repertoire of genotypes (Ayala, 1998; Victoir and Dujardin, 2002). Furthermore, the evolutionary consequences of this model are the propagation of independently evolving clonal lineages, with or without rare genetic recombination between them, significant genetic diversity between the lineages and an association between genes (linkage disequilibrium) (Ayala, 1998). According to the model proposed by Lythgoe (2000), *Leishmania* may have developed asexual mechanisms for generating a large range of genotypes in order to ensure parasite fitness in various environmental conditions.

Second, concerning the occasional sexual recombinations, Awadalla (2003) detailed the evolutionary significance of recombination in pathogenic species. Analysis of different models, from obligatory sexual species to optionally asexual and sexual species, indicates that optional sex endows these taxa with an alternative mechanism, other than mutation, to generate new multilocus genotypes with different types of fitness (Awadalla, 2003). This hypothesis was confirmed by

Otto and Lenormand (2002), who also postulated that most of the evolutionary hypotheses for sex stem from the idea that sex generates greater variability because chromosomal segregation and recombination break down genetic associations. The different authors agree that genetic exchange enhances the diversity of the species' repertoire and thus might influence the maintenance of antigenically distinct strains, which has been clearly demonstrated in *Trypanosoma brucei* (see Gupta *et al*., 1996; Hastings and Wedgwood-Oppenheim, 1997). Another hypothesis to explain the occasional occurrence of sexual recombination has been proposed for the pathogen yeast *Candida albicans*, the prevailing mode of reproduction of which in natural populations is also clonal, with rare sexual events (Nebavi *et al*., 2006). With these organisms, mating can occur only between specific genotypes (homozygous at a particular locus, called mating-type locus or MTL), and this is extremely rare or absent (Nebavi *et al*., 2006). The same hypothesis can also be applied to *Leishmania*, i.e. the ability to recombine only between specific genotypes, since to date very few hybrids have been reported, almost always between the same species (Darce *et al*., 1991; Belli *et al*., 1994; Bañuls *et al*., 1997).

Third, gene amplification and gene duplication are both biological mechanisms which can modulate gene expression to enable rapid adaptation to a changing environment (Victoir and Dujardin, 2002). Stiles *et al*. (1999) and Victoir and Dujardin (2002) showed that, in natural conditions, gene expression can be regulated through several mechanisms, including increase in gene expression by gene amplification. In *Leishmania*, the process of gene amplification is well documented: (i) tandem repetition of genes encoding housekeeping products required in abundance such as tubulin, rRNA and mini-exon (Spithill and Samaras, 1987; Inga *et al*., 1998; Kebede *et al*., 1999); (ii) amplification of short or long chromosome segments in the form of linear or circular DNA (Ouellette and Papadopoulou, 1993); and (iii) ploidy changes in the entire chromosome or genome (Cruz *et al*., 1993). Furthermore, expansion such as duplication and contraction of loci is also a common feature in wild *Leishmania* populations. Victoir and Dujardin (2002) described two types of tandem repeats: (i) tandem repeats that encode housekeeping genes such as rDNA and mini-exon genes for enhancing gene overexpression (Inga

et al., 1998; Kebede *et al.*, 1999), and (ii) tandems corresponding to genes involved in host–parasite relationships, such as those for gp63, promastigote surface antigen (PSA-2) and cysteine proteinase (Victoir *et al.*, 1995; Mottram *et al.*, 1997; Jimenez-Ruiz *et al.*, 1998). Victoir and Dujardin (2002) suggested that gene overexpression is also probably important for gp63, PSA-2 and cysteine proteinase (CP). Nevertheless, they proposed that, considering the role of these genes as an interface between the parasite and a heterogeneous external environment, it is probable that gene repetition offers additional benefits (Victoir and Dujardin, 2002). These duplication events would allow the organism to 'try out' mutations on the additional gene copies at low risk: the normal copies of the gene would compensate for deleterious mutations, whereas successful mutations could be expanded. This has been documented as a major source of variation in several organisms (Flavell, 1982). Another hypothesis suggested by the structure, function and differential expression of *cp* tandem repeats in *Leishmania* is that this multicopy gene represents an archival library of variant genes (M. Hide *et al.*, unpublished data). Indeed, many different types of parasites escape host immunity by switching gene expression between variants stored within each genome (Frank, 2002) such as *Trypanosoma brucei* and *Plasmodium vivax*. Del Portillo *et al.* (2001) have demonstrated that *P. vivax* has an extensive family of *vir* gene variants whose products are immunogenic. However, they observed that the parasites expressed only a small subset of these genes in an infected erythrocyte. The differential expression of these variants in the host serum bore the hallmarks of antigenic variation from a large archival library (Frank, 2002). Experiments conducted by Mottram *et al.* (1997, 1998), Rafati *et al.* (2001) and Denise *et al.* (2003) supported the hypothesis that the *cpb* gene corresponds to an archival antigenic library by demonstrating that the *cpb* gene variants are immunogenic and differentially expressed.

3.3.3. Taxonomic Consequences and Species Definition

The species concept has long been a matter of debate which is far from resolved (see De Meeûs *et al.*, 2003 for a review). Whatever the

concept, nature hardly ever conforms to human-made definitions, and even the most operational species definition, in terms of classifying life, will never be able appropriately to describe the process of life (De Meeûs *et al.*, 2003). Nevertheless, scientific investigation would be impossible without these categories, and there is an indispensable need for rigorous and clear nomenclature to permit efficient communication between the scientific and medical professions.

Considering clonal organisms, the first problem noted was that they could not be defined on the basis of the biological species concept (BSC) defined by Mayr (1963) (see review by De Meeûs *et al.*, 2003 for further details). These authors clearly stated that this concept could be applied only to organisms reproducing exclusively by biparental sexual reproduction and excluded all organisms able to crossbreed between more or less distant lineages, as it excludes clones and cannot apply to strict 'selfers' (self-fertilizing populations). Other species concepts have been designed but De Meeûs *et al.* (2003) explained that they all fail by trying to fit living things into fixed categories even though life itself is not fixed.

Thus, since the genetic data suggest that *Leishmania* species correspond to agamospecies (a group of individuals in which reproduction is almost exclusively by asexual means) (Cain, 1954), the species definition still remains arbitrary and is based on a mix of intrinsic and extrinsic characteristics considered together. Thus, in *Leishmania*, the notion of species is particularly confusing and today there is still no firm consensus on species definition. The drawback of this is the description of an exaggerated and unjustified number of species (see above and Figure 5). Furthermore, with the development of molecular tools with ever-increasing powers of resolution, there is a risk that a non-codified use of molecular data will lead to an uncontrolled and useless increase in the number of species of *Leishmania*.

Different analyses have clearly shown that the species status of some taxa is questionable or not taxonomically valid (see Figure 5). The status of several species has been explored, including *L. peruviana*, *L. panamensis*, *L. infantum* and *L. chagasi.* The results supported the species status of *L. peruviana* (see Bañuls *et al.*, 2000) and *L. infantum* (M. Hide *et al.*, unpublished data), while *L. panamensis* cannot be clearly distinguished from *L. guyanensis,* and *L. chagasi*, as

already demonstrated by Mauricio *et al.* (2000), cannot be distinguished from *L. infantum* (see Hide *et al.*, 2001). These studies demonstrate that there is a crucial need for a clear definition. Thus, in order to limit the increasing number of unjustified species, we propose the adoption of guidelines to define the species status of *Leishmania* parasites (see Section 5.1.1, p. 54).

3.3.4. Genetic Diversity in Leishmania*: What Does it Mean Epidemiologically?*

As described above, the genus *Leishmania* is characterized by ecological, epidemiological and clinical complexity. The presence of these organisms throughout the world, except in Antarctica, and their ability to infect a large range of mammalian hosts and sand fly species, show that *Leishmania* has the ability to adapt and survive in very diverse environments. Environmental changes and biotic interactions can impose strong selection pressures on various life-history traits and thus influence their degree of genetic diversity.

As shown by genome comparison (see above), all the published data have revealed a high level of global heterogeneity in the genus *Leishmania* (see Beverley *et al.*, 1987; Rioux *et al.*, 1990). At this level of phylogenetic divergence, slowly evolving markers such as ribosomal ribonucleic acid genes are indispensable to build up a reliable picture of the various phylogenetic subdivisions within this genus. A very high level of genetic divergence is also observable at the subgenus level; it is at the species complex level that genetic diversity decreases considerably. Furthermore, the genetic and phylogenetic data demonstrated that, at any given taxonomic level, different taxa can reveal different levels of genetic heterogeneity. For example, the *Leishmania* subgenus appears much more genetically heterogeneous than the *Viannia* subgenus (A.-L. Bañuls *et al.*, unpublished observations). Similarly, all the species complexes do not present the same degree of genetic diversity. For example, the *L. braziliensis* complex and *L. mexicana* complex appeared more polymorphic than the *L. guyanensis* or *L. donovani* complexes (Cupolillo *et al.*, 1995, 1998; A.-L. Bañuls, unpublished data). Other authors have also observed decreasing degrees of genetic variation among the following

Leishmania species from the 'Old World': *L. tropica*>*L. aethiopica* >*L. major*>*L. donovani* (see Schonian *et al*., 2001).

At the intra- and inter-specific levels, the evaluation of genetic diversity is the basis for all population genetics and taxonomic studies and therefore for understanding the organism's evolution. From an applied research point of view, it is an essential step in understanding the epidemiology of the disease, defining the most efficient diagnostic tools, and selecting and elaborating pertinent and efficient vaccine targets (Bañuls *et al*., 1999a, 2002). Moreover, the genetic heterogeneity level provides information on life-history traits since it is directly linked to the recent or ancient emergence of species in a country or in an area and to the selection pressure stemming from environmental factors (variety of hosts, diversity of vectors, presence of several *Leishmania* species or other organisms such as *Trypanosoma cruzi*). Although several studies have discussed the polymorphism observed in natural populations of different *Leishmania* species (Cupolillo *et al*., 1998, 2003; Mauricio *et al*., 2001; Schonian *et al*., 2001), until now there has been little information available on the genetic variability of the parasites and the correlation with ecoepidemiological features of the disease (Guerbouj *et al*., 2001; Cupolillo *et al*., 2003; Zemanova *et al*., 2004; Garcia *et al*., 2005) and with the evolution of the parasites (Ibrahim and Barker, 2001; Victoir *et al*., 2005). The data available from natural population studies show differential degrees of polymorphism, first among *Leishmania* species (as described above) and second within a single species among geographical location or host (vector or reservoir) diversity.

The *Leishmania donovani* complex, *L. infantum* and *L. donovani*, can be considered in the light of the first point. *L. donovani* revealed much more polymorphism than *L. infantum* despite a wider geographical distribution for the latter (Ibrahim and Barker, 2001; M. Hide *et al.*, unpublished data). This does not seem to be related to either pathogenicity (see below) or epidemiology (*L. infantum* is an anthropozonootic parasite, while *L. donovani* is considered to be mainly anthroponotic). Phylogenetic studies suggest that *L. infantum* is a descendant of an *L. donovani* lineage and thus *L. infantum* may have appeared more recently than *L. donovani* (see Ibrahim and Barker, 2001; Hide and Bañuls, 2006). This evolutionary event can

explain the genetic differences between these two species. Furthermore, the homogeneous environment in Europe (considering reservoir hosts or vectors) can also explain the limited genetic divergence seen in *L. infantum.*

An interesting example of the second point is *L. braziliensis.* This species has been described as the most heterogeneous species of *Leishmania* (Chouicha *et al*., 1997; Ishikawa *et al*., 2002). Nevertheless, *L. braziliensis* populations from different regions have shown a relationship between the level of similarity among the parasite populations and their geographic range (Macedo *et al*., 1992; Gomes *et al*., 1995; Cupolillo *et al*., 2003). Furthermore, recent data have also indicated that the considerable variability detected among these parasites is more probably related to the sand fly vector(s) and/or animal reservoir(s) involved in the transmission cycles (Ishikawa *et al*., 2002; Cupolillo *et al*., 2003), in agreement with the accepted argument that pathogens that produce many different genetic variants are more prone to infect multiple hosts (Woolhouse *et al*., 2001). In the case of *L. braziliensis*, the principal mammalian hosts in the sylvan cycle have not been identified, but there is evidence that dogs, horses and donkeys may act as this parasite's reservoir hosts (Grimaldi and Tesh, 1993; Cupolillo *et al*., 2003). These relationships between genetic polymorphism and geographical distribution and the range of reservoir hosts and vectors can be observed for many species such as *L. guyanensis* (see Saravia *et al*., 1998; Bañuls *et al*., 1999a, b) and *L. donovani* (see Kamerbeek *et al*., 1997; Zemanova *et al*., 2004). One of the less polymorphic species is *L. peruviana* (see Bañuls *et al*., 2000). This can be explained by different factors: (i) its geographical distribution, restricted to the east Andean region of Peru and (ii) the low number of different reservoir hosts and vector species (Llanos-Cuentas *et al*., 1999; Caceres *et al*., 2004). Another important point is the hypothesis that this species is a descendant of *L. braziliensis*. These two species are genetically very close (Bañuls *et al*., 2000), suggesting that *L. peruviana* appeared recently.

This demonstrates that, in order to understand the evolution and the various epidemiological traits of *Leishmania*, research efforts must not be limited to only one strain of only one species of *Leishmania* (see Bañuls *et al*., 1999a, 2002). The environmental factors

affecting each *Leishmania* population must also be investigated since each focus can contain different hosts, different vectors and different *Leishmania* species; also, other microorganisms may be present. Furthermore, because coevolutionary interactions can be highly divergent across time and space, it is also important to quantify and compare the phylogeographic variation in both host and parasite throughout their geographical range (Huyse *et al*., 2005).

4. *LEISHMANIA* GENETICS AND PATHOGENICITY: WHAT'S NEW?

Clinical manifestations of *Leishmania* infection are determined by a combination of factors: the host's genetic make-up and immune status, features of the parasite, and factors associated with vectors. First of all, infections by *Leishmania* may remain inapparent, indicating that the parasites are well adapted to almost all their vertebrate hosts (Lainson and Shaw, 1987). As described above, epidemiological studies have revealed that infected humans may remain asymptomatic or manifest more or less severe pathological processes (Ho *et al*., 1982; Badaro *et al*., 1986; Zijlstra *et al*., 1994; Le Fichoux *et al*., 1999; Sharma *et al*., 2000; Costa *et al*., 2002; Bucheton *et al*., 2003; Riera *et al*., 2004).

The objectives of this section are (i) to show that parasites play a role at both specific and intraspecific levels in determining the degree of pathogenicity and (ii) to analyse the advances in genetic knowledge concerning this role. We also detail the modern tools available for studying the parasitic factors involved in virulence and pathogenicity and the advances resulting from their use.

4.1. Clinical Outcomes in Humans and *Leishmania* Taxonomy

Specific clinical manifestations are generally associated with particular *Leishmania* species, but different clinical forms of disease can be encountered within a given species. *Leishmania* species display

preferential tropisms (viscerotropism, dermotropism and mucocutaneous tropism) and all the resulting diseases can progress from infection to the symptomatic phase and then to host death or recovery. Cutaneous leishmaniases are generally relatively benign, even though they may develop into serious and complex clinical disease such as diffuse or mucocutaneous leishmaniasis. In contrast, visceral leishmaniases are fatal in the absence of treatment.

4.1.1. Leishmania *Species and Clinical Symptoms in Humans*

The main clinical varieties of leishmaniasis in humans and the *Leishmania* species involved are as follows:

(a) Cutaneous leishmaniasis (CL), usually caused by species of the *Viannia* subgenus, members of the *Leishmania tropica* complex, *L. major*, *L. aethiopica* and all species of the *L. mexicana* complex; CL may however result from infection by any of the *Leishmania* species that infect humans (reviewed by Murray *et al.*, 2005).
(b) Mucocutaneous leishmaniasis (MCL), or espundia (Escomel, 1911), usually caused by *L. braziliensis*, but also more rarely by *L. panamensis* and *L. guyanensis* (Saravia *et al.* 1985; Naiff *et al.*, 1988; Santrich *et al.*, 1990; Saenz *et al.*, 1991; Osorio *et al.*, 1998).
(c) Diffuse cutaneous leishmaniasis (DCL), produced by *L. aethiopica* or *L. amazonensis* (reviewed by Lainson and Shaw, 1998).
(d) Visceral leishmaniasis (VL) (kala azar), usually produced by species of the *L. donovani* complex (Boelaert *et al.*, 2000).
(e) Post-kala-azar dermal leishmaniasis (PKDL), caused by *L. donovani* following cure of the initial visceral leishmaniasis (reviewed by Zijlstra *et al.*, 2003).

Nevertheless, these are not absolute categories and there is considerable overlap in clinical manifestations caused by the various species of *Leishmania*. For instance, cases have been described of visceral disease caused by *L. amazonensis* (see Almeida *et al.*, 1996; Aleixo *et al.*, 2006) and *L. tropica* (see Magill *et al.*, 1993; Lemrani *et al.*, 2002; Alborzi *et al.*, 2006). Other examples have been published of patients with mucosal leishmaniasis due to infection by *L. donovani*

or *L. major* in Sudan and Tunisia (El-Hassan *et al*., 1995; Kharfi *et al*., 2003). Similarly, a form of disseminated leishmaniasis following infection with *L. guyanensis* has been observed in French Guyana (Couppie *et al*., 2004).

4.1.2. Particular Cases: Hybrids and Mixed Infections

Other factors may complicate the clinical picture: the existence of hybrids as described above (Section 3.3.1, p. 24) and mixed infections with different *Leishmania* strains. Examples of hybrids include those between *L. braziliensis*, which can produce cutaneous or mucocutaneous lesions in humans requiring care, and *L. peruviana*, responsible for dry benign cutaneous lesions that heal spontaneously. Hybrids between these two species have been isolated from patients in Peru with either mucocutaneous lesions or benign lesions typical of *L. peruviana* infection (Dujardin *et al*., 1995a). These hybrids strains were thus capable of producing the different pathologies characteristic of each species.

Mixed infections by different *Leishmania* species have been described in only a few cases (Ibrahim *et al*., 1994; Strelkova *et al*., 2001; Martinez *et al*., 2002; Bastrenta *et al*., 2003; Antoniou *et al*., 2004), although molecular epidemiology studies demonstrated that many foci exist in which several species circulate simultaneously (Lucas *et al*., 1998). It is hypothesized that the number of mixed infections is underestimated because of selection during parasite culture. This was confirmed by a study conducted in Bolivia (Bastrenta *et al*., 2003). Mixed infections present a problem for *Leishmania* diagnosis, prognosis, and for understanding the real role played by the parasites in pathogenicity in humans.

It is also important to consider cases of co-infection of *Leishmania* with other pathogens. This is relatively frequent according to the literature, and various pathogens such as *Mycobacterium tuberculosis* (see Wang *et al*., 1999; Delobel *et al*., 2003), *Trypanosoma cruzi* (see Bastrenta *et al*., 2003), *Salmonella* and *Schistosoma* (see Djidingar *et al*., 1997) and of course human immunodeficiency virus (HIV) (for reviews see Deniau *et al*., 2003; Desjeux and Alvar, 2003; Molina *et al*., 2003; Rabello *et al*., 2003) have been described in association

with *Leishmania*. In some cases, these co-infections can produce unusual clinical forms of leishmaniasis (Colebunders *et al.*, 1999; Calza *et al.*, 2004).

4.1.3. Diverse Pathogenic Properties at Intraspecific Level

As discussed above, each species is generally associated with one type of disease in humans. However, this rule is not absolute and some variations in clinical outcome may exist within species. One hypothesis to explain these infrequent manifestations is the patient's physiological status. Thus, a particular clinical progression may occur in immunocompromised patients. This was observed for instance in an Italian patient infected with *L. infantum* and undergoing haemodialysis, who displayed mucosal leishmaniasis (Maggi *et al.*, 2002). Another current example is the case of HIV–*Leishmania* co-infection. Unusual forms of leishmaniasis have been reported in HIV-positive patients, due to the alteration of the natural immune response caused by the action of the virus (Echevarria *et al.*, 1993; Miralles *et al.*, 1994; Durand *et al.*, 1998; Bosch *et al.*, 2002). As shown here, it may sometimes be difficult to know whether the parasite is responsible for these infrequent forms.

Some species such as *L. braziliensis* or *L. infantum* possess the intrinsic potential to produce various symptoms in humans such as cutaneous or mucocutaneous symptoms for *L. braziliensis* and cutaneous or visceral leishmaniasis for *L. infantum*. However, with both species the cutaneous form is more frequent than mucocutaneous or visceral progression (see, e.g., Rioux *et al.*, 1986, 1990; Jones *et al.*, 1987; Campos, 1990; Pratlong *et al.*, 2004; Campos-Ponce *et al.*, 2005). About 10% of all patients infected with *L. braziliensis* and having a primary cutaneous lesion will develop a metastatic mucosal infection at some time (Campos, 1990). Different disease outcomes of infection with *L. amazonensis* have also been reported: three of six patients showed cutaneous lesions, one had mucosal lesions, and two had diffuse cutaneous leishmaniasis (Lucas *et al.*, 1998). It remains unknown whether the parasite plays a role in the expression of the disease at an intraspecific level.

4.2. Experimental Data: Variations in Pathogenic Potential Among and Within Species

4.2.1. Animal Models

Animal models are largely used for immunobiological studies to understand and characterize host–parasite interactions during infection. Both the host and the *Leishmania* species are determinants in the progression of the infection.

Thus, animal models such as mice, hamsters and non-human primates respond differently depending on the *Leishmania* species involved (McMahon-Pratt and Alexander, 2004; Wilson *et al*., 2005). For example, the *Leishmania* subgenus *Viannia* (which is found predominantly in Latin America) fails reliably to infect mice, whereas the 'Old World' species such as *L. infantum* and *L. major* do so regularly (McMahon-Pratt and Alexander, 2004). This is also true at an intraspecific level, since different genetic strains of mice may show different responses to different *Leishmania* species. Mice strains C57BL/6, C57BL/10 and B10.D2 are susceptible to *L. donovani* infection but resistant to *L. major*, while DBA/2 is resistant to *L. donovani* and susceptible to *L. major* (see Wilson *et al*., 2005).

Differences in disease expression and immune response were observed depending on the *Leishmania* species, but also depending on the strains used for infection. Genetic and immunological studies using animal models have shown that various members of the *Leishmania* genus differ in various aspects of their relationship with the host immune system. In mice infected with *L. major* (responsible for cutaneous leishmaniasis), a dichotomy was shown in the host's immune response, determined by the type of cytokines and CD4+ T cells involved (McMahon-Pratt and Alexander, 2004). Infected mice can develop either self-limiting cutaneous infection or progressive disease depending on their genetic background. With *L. donovani*, responsible for visceral leishmaniasis, it was not possible to establish a clear dichotomy in the immune response because of the existence of a mixed Th1/Th2 response in mice, as has been described in humans (Kurtzhals *et al*., 1994; Khalil *et al*., 2005).

Other experiments have demonstrated that different *Leishmania* strains of several species can produce different immune responses and disease expression. In BALB/c IL-4-deficient mice, different *L. major* strains can induce either a non-healing or a healing infection (Kopf *et al.*, 1996; Noben-Trauth *et al.*, 1996). Other authors have also demonstrated that different *L. major* strains can generate different immunological responses in mice (Hondowicz and Scott, 1999). Another example concerns *L. tropica* strains isolated from dermotropic and visceral infections in humans and then used to infect mice and hamsters (Lira *et al.*, 1998). This experiment showed differences in disease progression that may reflect the parasite tissue tropism and pathogenic potential displayed by these strains in their human hosts. All these observations suggest a role for parasite-related determinants in the clinical spectrum of disease. Similarly, Gangneux *et al.* (2000) used C.B-17 SCID and congenic BALB/c mice to examine *L. infantum* strain pathogenicity independently of host genetic factors. While parasite loads were significantly higher in immunodeficient than in immunocompetent mice, the kinetics of infection during long-term follow-up were similar, suggesting that intrinsic parasitic factors also influence the outcome of *L. infantum* infection. Furthermore, Garin *et al.* (2001) concluded that parasite virulence in *L. infantum*-induced visceral leishmaniasis was clonal and dominant in nature, having explored the biodiversity of different *Leishmania* strains previously known to express different virulence phenotypes in mice. The fact that different human parasite isolates produced different infection patterns in a given mouse model also suggested that parasite-related factors play an important role in the resistant versus susceptibility status and in the type of immune response elicited in the infected host (Kebaier *et al.*, 2001). There is, thus, good evidence that, in addition to host factors, parasites also influence the progression of infection.

4.2.2. Experiments In Vitro

Studies *in vitro* of parasite dynamics have been conducted to compare virulent and avirulent strains at inter- and intra-specific levels. A study of growth behaviour *in vitro* of promastigotes of *L. braziliensis* (responsible for MCL), *L. peruviana* (responsible for benign CL) and putative hybrids between *L. braziliensis* and *L. peruviana* by Dujardin

et al. (1995a) found that hybrids and *L. peruviana* presented similar growth characteristics and a growth capacity (growth rate and cell density at the stationary phase) significantly lower than that of *L. braziliensis* (see Torrico *et al*., 1999).

We compared the dynamics *in vitro* of *L. infantum* promastigotes in relation to the clinical characteristics (visceral or cutaneous) of the infection in humans (M. Hide *et al*., unpublished data). Promastigote concentrations of nine *L. infantum* strains (four VL and five CL strains) were estimated on a daily basis using a FACScan flow cytometer until population extinction. All the experiments were repeated three times to check reproducibility and used a rigorous statistical protocol. A mathematical model was developed to analyse the raw data involving the whole dynamics of promastigote growth including exponential and stationary phases (Choisy *et al*., 2004). Analysis revealed that VL and CL strains could be discriminated by characters related to the exponential phase, i.e. the procyclic promastigotes; those from viscerotropic strains grew faster than those from dermotropic ones and needed a smaller initial inoculum. These experiments also showed different biological characteristics related to strain tropism. Since the dynamics were obtained without any interaction with host cells, these results suggested that the strains themselves played a role in disease progression. Our results were in agreement with other studies. Infectivity in the murine model (Sulahian *et al*., 1997) and in macrophages (Louassini *et al*., 1998) has been shown to be higher for *L. infantum* visceral strains. Louassini *et al*. (1998) also compared the growth rate of one CL strain and one VL strain and found that the maximum percentage of metacyclic promastigotes was produced earlier, and was higher, for the VL strain than for the CL strain.

It seems clear that the clinical outcome of the disease in humans is multifactorial. Despite the complex clinical picture, parasite biology and genetics seem to be very important in determining the clinical outcome.

4.3. Relation Between Pathogenic Diversity in Humans and *Leishmania* Genetic Polymorphism

The genetic mechanisms which lead to the expression of the disease in hosts are of course complex since they result from the interactions

between parasites and hosts. The majority of genetic studies on pathogens have the primary objective of identifying whether there is a genetic association between virulence and/or pathogenicity of strains and the disease outcome in humans and identifying the markers involved directly or indirectly in the different clinical forms. The parasite factors described in the literature as related to disease outcome can be divided into three categories: (i) indirect genetic markers of pathogenicity, (ii) factors called invasive/evasive determinants (Chang and McGwire, 2002), and (iii) so-called pathoantigenic determinants (Chang and McGwire, 2002).

4.3.1. Indirect Genetic Markers

Indirect genetic markers include genes or loci not directly involved in virulence or pathogenicity but associated with clinical polymorphism. They have been and continue to be widely explored. This type of comparison is warranted because of the population structure of these organisms, i.e. a reproduction model with infrequent genetic exchange between different lineages (Tibayrenc, 1996) (see Section 3.3.1., p. 24). The population structure and the frequency of genetic exchange condition the importance of these genes or loci as epidemiological or clinical markers. Infrequent genetic exchange implies linkage disequilibrium (non-random associations of genotypes occurring at different loci) and thus a correlation between genetic and phenotypic markers. This strongly suggests the possibility of finding some genotypes associated with clinical or biological phenotypes (Tibayrenc, 1996). Other reproduction models can also produce this type of linkage disequilibrium such as self-fertilization, homogamy or consanguinity. Different molecular tools such as the 'gold standard' method for species identification MLEE (multilocus enzyme electrophoresis) (Rioux *et al.*, 1990) or RAPD (random amplified polymorphic DNA) (Cuervo *et al.*, 2004; Indiani de Oliveira *et al.*, 2004), PFGE (pulse field gel electrophoresis) (Dujardin *et al.*, 1993a; Guerbouj *et al.*, 2001), RFLP (restriction fragment length polymorphism) (Victoir *et al.*, 1995; Cuervo *et al.*, 2004) and recently microsatellites (Russell *et al.*, 1999; Bulle *et al.*, 2002; Jamjoom *et al.*, 2002) or real-time PCR

(Schulz *et al.*, 2003; Quispe Tintaya *et al.*, 2005) were used extensively to analyse the *Leishmania* genome regarding clinical and epidemiological data. The results showed that these genetic markers were able to distinguish the different species or reveal intraspecific polymorphism. However, only a few of them were found to be associated with clinical phenotypes at the intraspecific level. For example, some zymodemes of *L. infantum* (all the stocks belonging to the same zymodeme have the same MLEE patterns) were associated exclusively with dermotropic strains and others with strains mainly isolated from visceral forms of the disease (Rioux *et al.*, 1985; Harrat *et al.*, 1996; Aoun *et al.*, 2000). Other investigations studying different genetic markers also showed a correlation between clinical polymorphism and genetic data in *L. infantum* (see Guerbouj *et al.*, 2001) and *L. braziliensis* (see Schriefer *et al.*, 2004). Similarly, a relation between MLEE, RAPD and PFGE data and severity of lesions in patients was found in *L. peruviana* by Dujardin *et al.* (1993b, 1998) and Bañuls *et al.* (2000). Unfortunately, all these associations were relatively weak and neither provided a clear understanding of the role of parasites in the outcome of the disease nor allowed the use of these tools as prognosis markers. Interestingly, except for the data described above, many studies based on different markers have failed to find an association with clinical diversity. Thus, different PCR multilocus approaches revealed that, within *L. braziliensis*, there was no specific genetic difference between parasites isolated from cutaneous and mucosal lesions (Garcia *et al.*, 2005). Microsatellite analysis of *L. braziliensis* (E. Waleckx *et al.*, unpublished data) could not distinguish between strains causing the various clinical expressions observed in humans.

4.3.2. Parasite Factors Involved in Pathogenicity and Virulence

For these reasons, recent studies have concentrated on parasitic genetic factors and genetic markers directly related to virulence or on genetic markers that make it possible to apprehend them. With recent progress in molecular biology, a variety of tools is available to study these markers from genes to proteins, their functions in cells and their involvement in interactions between hosts and parasites.

Direct genetic factors have been explored via genome manipulations which can be classified into two groups: forward and reverse methods. The first is used to identify genes involved in a given phenotype and the second provides a better understanding of gene function by manipulating it (for a review see Beverley *et al.*, 2002). Based on these methods, many genetic and genomic tools have been used for the study of *Leishmania*, e.g. expression vectors, gene knockout and mutagenesis (Clayton, 1999). Furthermore, many genes have been cloned and their function inferred from homology with genes of other organisms, the location of the corresponding proteins, or their expression in heterologous systems. Later, transfection methodology analysed gene function within the organisms themselves. Since then, it has become possible to create mutants, overexpress foreign proteins in the parasites, knockout genes and even switch off essential functions.

The technique of RNA interference (RNAi; a strategy for reducing RNA expression) and experiments based on sexual crosses and positional cloning approaches (to define phenotypes of interest) cannot be used for *Leishmania*. These organisms, unlike *Trypanosoma brucei*, are RNAi-negative (Robinson and Beverley, 2003) and genetic exchange between different genotypes appears to be infrequent (see above) and impossible to produce experimentally (Panton, 1991). These tools have largely been used for *Trypanosoma brucei*, which is RNAi-positive (Motyka and Englund, 2004) and with which experimental crosses are easily performed *in vitro* (Gibson *et al.*, 1995; Turner *et al.*, 2004).

With the development of these molecular tools, numerous potential *Leishmania* virulence and pathogenicity factors have been discovered. To classify them, Chang and colleagues have proposed a model for *Leishmania* virulence. Their model is based on the classification of parasite factors into invasive/evasive determinants and pathoantigenic determinants, as outlined above (Chang and McGwire, 2002; Chang *et al.*, 2003). Invasive/evasive determinants of *Leishmania* are crucial for infection, but produce no pathology in the host, whereas *Leishmania* pathoantigenic determinants elicit antibodies at high titres and thus host immunopathology is the principal cause of clinical symptoms (Chang and McGwire, 2002). This is not to say that patients with

leishmaniasis do not produce any antibodies against the invasive/evasive determinants, but that the titres against these determinants are insignificant in comparison to those produced by pathoantigenic ones. It should be noted that the distinction between these two groups is somewhat unclear and must not be considered inflexible. This classification depends on knowledge acquired about each type of marker and therefore could be questioned in the future. Section 4.3.2(c) (p. 49) details these modern tools and the usefulness of gene expression and protein studies in *Leishmania* for identifying new genes and proteins involved in virulence or pathogenicity.

(a) *Invasive/evasive determinants*. The invasive/evasive determinants (Chang and McGwire, 2002) relate to parasitic mechanisms that are necessary to establish infection, such as (i) *Leishmania*–macrophage attachment, (ii) entry of *Leishmania* into macrophages, (iii) intramacrophage survival, and (iv) differentiation and intracellular multiplication of *Leishmania* amastigotes. They are not responsible for the symptoms of the disease. These determinants therefore refer to all molecules that help *Leishmania* successfully to establish in the host such as glycosylphosphatidylinositol (GPI), glycosylphospholipid (GIPL), lipophosphoglycan (LPG), leishmanolysin (GP63), cysteine proteases (CPs) and others. These molecules have been widely studied, especially LPG, GP63 and CPs.

LPG is the dominant surface molecule of promastigotes involved in many of the processes essential to the survival of *Leishmania* within hosts, including binding, migration and release of the parasite in the sand fly midgut, but it is also involved in the modulation of resistance to lysis by the host's complement, resisting the innate immune system and entering macrophages. It is almost completely absent from amastigotes (McConville *et al*., 1992; Pimenta *et al*., 1992; Cunningham, 2002; Olivier *et al*., 2005). LPG differs among *Leishmania* species, particularly in its sugar composition, in the sequence of branching sugars attached to the repeat galactose–mannose–phosphate backbone, and in the cap structure (Soares *et al*., 2002). The involvement of LPG in virulence has been demonstrated during *L. donovani* and *L. major* infection (McNeely and Turco, 1990; Spath *et al*., 2000), whereas Ilg (2000) showed that it was not required for *L. mexicana* infection. In addition to stage-specific and interspecies variability,

some analyses have shown an intraspecies polymorphism in lipophosphoglycan structure (Mahoney *et al.*, 1999). This diversity may be related to the parasite's adaptation to the sand fly species rather than to the clinical diversity observed in humans.

GP63 is another important surface molecule, an ecto-metalloprotease particularly abundant in promastigotes and released by this stage (McGwire *et al.*, 2002). Like LPG, GP63 is down-regulated in the amastigote form (Schneider *et al.*, 1992). These molecules may be involved in evasion from humoral lytic factors and in attachment of parasites to macrophages followed by their entry into these phagocytes (Yao *et al.*, 2003). GP63 protein is encoded by a multigene family repeated in tandem. Genetic and structural diversity were extensively studied and showed a high degree of both inter- and intra-specific polymorphism (Roberts *et al.*, 1993; Steinkraus *et al.*, 1993; Espinoza *et al.*, 1995; Victoir *et al.*, 1998; Guerbouj *et al.*, 2000). Like LPG, this protein seems to be subjected to strong host selection pressure in both vector and mammalian host; however, no link was found between the genetic or phenotypic diversity of GP63 and the intraspecies clinical polymorphism of strains (Guerbouj *et al.*, 2001).

There has been increased interest in cysteine proteases (CPs) because of the key roles some of them play in infection and expression of the disease, making them potential targets for new chemotherapy. Within the genus *Leishmania*, three types of CPs have been found: CPA and CPB, homologous with mammalian cathepsin L, and CPC, which is homologous with mammalian cathepsin B (Mottram *et al.*, 1997, 1998; Sajid and McKerrow, 2002); *cpa* and *cpc* are single-copy genes, whereas *cpb* is a multicopy gene. CPs seem to play crucial roles in host–parasite interactions, particularly in facilitating survival and growth of parasites in mammals by destruction of host proteins, aiding nutrition, evasion of the host immune response and promoting survival within host macrophages (Alexander *et al.*, 1998; Rosenthal, 1999; Mundodi *et al.*, 2002; Mottram *et al.*, 2004). CPA and CPB are involved in immune system-modulating activities such as the inhibition of IL-12 production by macrophages, degradation of MHC class II molecules in the parasitophorous vacuole and induction of IL-4 production (Mottram *et al.*, 2004). Inoculation of CP-deficient *Leishmania* mutants to mice demonstrated the involvement of these

proteins in virulence and pathogenicity. For example, an *L. mexicana* strain deficient in the *cpb* array showed reduced virulence in BALB/c mice (Mottram *et al*., 1996; Alexander *et al*., 1998). In addition, Zadeh-Vakili *et al*. (2004) showed that a recombinant hybrid protein consisting of CPA and CPB could elicit a protective immune response against *L. major* in the BALB/c mice model. Recently, Campos-Ponce *et al*. (2005) demonstrated a statistical correlation between gene organization of *cpb* in an *L. infantum* population and strain tropism (cutaneous or visceral). Rafati *et al*. (2005) demonstrated the importance of CPB, and C-terminal extension, in particular, as a target of the immune response. The first trials of a vaccine in dogs using CPA and CPB 'cocktails' gave interesting results but further study is needed (Rafati *et al*., 2005; Poot *et al*., 2006). Finally, the copy number of *cpb* genes in the tandem array is variable among *Leishmania* species. *L. mexicana* contains nineteen *cpb* genes (Mottram *et al*., 1997) which differ in length, substrate specificities, COOH-terminal extension and expression during the *Leishmania* life cycle (Mottram *et al*., 1997, 1998; Brooks *et al*., 2001), whereas the *L. donovani* complex seems to have roughly five *cpb* genes (Mundodi *et al*., 2002). Recently, we showed that the *L. donovani* complex contained one specific gene in the *cpb* cluster (named *cpbE* for *L. infantum* and *cpbF* for *L. donovani*) which is absent from the *L. mexicana* and *L. major* genome (Hide *et al*., 2006; Hide and Bañuls, in press). However, any involvement of this *cpb* cluster in the viscerotropic character of the *L. donovani* complex has yet to be demonstrated.

This list is far from exhaustive; other important molecules include PSA (GP46), an abundant surface glycoprotein of the promastigote form (Symons *et al*., 1994; Beetham *et al*., 2003).

Miniexon genes can also be considered as fundamental parasite factors since they are present in all trypanosomatids, including *Leishmania*, and their corresponding transcripts play a major role in the maturation of mRNA in these parasites through the process known as *trans*-splicing (Kooter *et al*., 1984). To demonstrate the role of these genes, Zhang and Matlashewski (2004) introduced an *L. donovani* cosmid library into *L. major* to select for *L. donovani* sequences which might increase *L. major* virulence in BALB/c mice. They identified a region of the *L. donovani* genome which increased virulence in

both visceral and cutaneous sites and was divergent from the corresponding region of the *L. major* genome, which supports the argument that amplification of miniexon genes is associated with increased virulence. Antoniazi *et al.* (2000) observed that more virulent *L. major* strains (such as LV39 and Friedlin V9) contained longer stretches of miniexon gene arrays than less virulent strains (e.g. LT252), which contains a 44-kb miniexon sequence. However, in the same study these authors showed that an additive miniexon gene array could reduce the virulence of LV39. They hypothesized that there was a subtle imbalance in the transcriptional level of a gene, or class of genes, crucial to parasite development in the vertebrate host cell. They suggested that it is possible to imagine that miniexon imbalance would play a more universal role affecting regulation of different factors by altering the maturation process of these transcripts, leading to attenuation of virulence.

(b) *Pathoantigenic determinants.* The second group of factors comprises *Leishmania* pathoantigenic determinants (Chang and McGwire, 2002; Chang *et al.*, 2003). As described above, this group includes all the molecules described in the literature capable of inducing host immunopathology as the principal cause of clinical symptoms. Thus, all *Leishmania* antigens eliciting antibodies at high titres compared to those raised against invasive/evasive determinants can be classified in this category. These pathoantigenic determinants are all conserved structural or soluble cytoplasmic proteins, which are often complexed with other molecules to form subcellular particles (Chang and McGwire, 2002; Chang *et al.*, 2003). Moreover, they have been found to contain immunogenic B cell epitopes. The list of candidate molecules is based on data obtained from kala-azar patients with visceral leishmaniasis (VL) (Requena *et al.*, 2000): it includes cytoskeletal components (e.g. kinesin and tubulins), chaperones (e.g. HSP60, 70, 83, STI 1), ribosomes (e.g. eIF, PO, P2a, b), nucleosomes (e.g. H2A/B, H3, H4), glycosomes (e.g. TPI), etc. These molecules clearly differ from those present in strains isolated from cutaneous leishmaniasis.

For example, the unique 117 bp repeat, encoding for a 39-amino acid peptide (recombinant products = rK39) in the *Leishmania* kinesin-like gene, is expressed by the amastigotes of visceralizing *Leishmania* species (*L. donovani*, *L. chagasi*) and not by dermotropic

species (*L. major*, *L. amazonensis* and *L. braziliensis*) (Burns *et al.*, 1993). Sera from VL patients contains high titres of antibodies specific to this 39-amino acid peptide, called anti-rK39 (Singh *et al.*, 1995). This antigen has been successfully used for serodiagnosis of active VL cases.

Another example is the A2 protein, known to have an influence on the outcome of the disease (Zhang *et al.*, 2003). A2 is an important gene for *L. donovani* virulence but is not expressed in *L. major* (see Zhang and Matlashewski, 2001; Zhang *et al.*, 2003). *L. donovani* strains deficient in A2 protein are attenuated with respect to survival in the viscera of infected mice (Zhang and Matlashewski, 1997, 2001). Although *L. major* does contain *A2* genes, they are not expressed and lack multiple repeats in the protein-coding regions and therefore represent non-expressed pseudogenes (Ghedin *et al.*, 1998; Zhang *et al.*, 2003). This study suggests that the inability of *L. major* to express A2 has contributed to its tropism for cutaneous infections (Zhang *et al.*, 2003). Furthermore, Ghedin *et al.* (1997) demonstrated that high levels of anti-A2 antibodies also occur in cases of acute VL. All these data suggest that A2 should be considered as a patho-antigenic determinant.

To date, the interactions between these molecules and the human immune system and the method of activation of specific antibody production remain unknown. All these molecules are localized in amastigote cytoplasm and are thus beyond the reach of their specific antibodies (Chang and McGwire, 2002; Chang *et al.*, 2003). However, their potential contributions to immunopathology are apparent. Commenting on a study on protective immunity in *Leishmania* by Probst *et al.* (2001), Chang and McGwire (2002) and Chang *et al.* (2003) suggested that some *Leishmania*-specific T cell epitopes may exist and may cause additional immunopathology.

(c) *Usefulness of modern tools for gene expression and protein analysis*. Thanks to the *L. major* genome project (http://www.sanger.ac.uk/Projects/L_major and http://www.genedb.org/genedb/leish/index.jsp), many new genetic tools are being developed for the post-genomic analysis of *Leishmania*. These include large-scale sequence annotation and database building, microarray analysis and proteomics. Until now, these modern tools have been used mostly to

explore differences between the development stages of *Leishmania*, but exploration of pathogenic diversity at the inter- and intra-species level has also recently begun.

Microarray or genechip analysis can be based on genomic DNA or cDNA. DNA microarray is a high-density arrangement of immobilized samples of nucleic acid on a glass slide. Hybridization with fluorescent cDNAs synthesized from RNA samples of interest permits the evaluation of gene expression on a genomic scale (Rathod *et al.*, 2002; Stears *et al.*, 2003). The use of DNA microarrays for the analysis of biological samples is becoming a mainstream tool for exploring the transcriptional regulation of gene expression (Li and Lazar, 2002) and can rapidly screen new vaccine candidates. The array elements react specifically with labelled mixtures, producing signals that reveal the identity and concentration of each labelled species in solution. These attributes provide miniature biological assays that allow the exploration of any organism on a genomic scale. This technology can be used for new gene discovery, a global genetic perspective on biological processes important in parasite survival and host–parasite interactions of *Leishmania* parasites. Despite polycistronic transcription, RNA transcript abundance can be measured using microarrays, and this tool was validated by the observation of genes that are known to be upregulated at the procyclic promastigote stage (β-tubulin, histones and ribosomal proteins) and at the metacyclic promastigote stage (*HASP*/geneB, *SHERP*/geneD, *META1* and *HSP70*) (Beverley *et al.*, 2002). Previous studies have examined differential gene expression in *Leishmania* (reviewed by Duncan *et al.*, 2004) and most microarray studies have investigated *L. major*. Differential gene expression in *L. major* procyclic and metacyclic promastigotes has been studied by Saxena *et al.* (2003). This study confirmed the stage-specific expression of several known genes and identified a number of novel genes that are up-regulated in either procyclics or metacyclics (Saxena *et al.*, 2003). At the same time, Almeida *et al.* (2002, 2004) generated microarrays using PCR-amplified inserts from an *L. major* cDNA library. Despite the lack of transcriptional regulation that polycistronic transcription in *Leishmania* dictates, the data provided evidence for a high level of post-transcriptional regulation of RNA abundance during the

developmental cycle of promastigotes in culture and in the lesion-derived amastigotes of *L. major* (Almeida *et al*., 2002). Furthermore, the results from a study of *L. donovani* genomic microarray led to the discovery of new genes (Duncan *et al*., 2004). Holzer *et al*. (2006) analysed the *L. mexicana* transcriptome (the set of all messenger RNA [mRNA] molecules, or 'transcripts') by microarray using *L. major* probes and showed that interspecies hybridization with microarrays can be used to analyse closely related *Leishmania* species. In another study, cDNA microarrays were used to compare *L. donovani* at day 5 and *L. major* promastigotes at days 3, 5, 7 and 10 in culture. This analysis identified 147 unique genes among 1092 (13.5%) that were significantly upregulated in amastigotes (Almeida *et al*., 2002). Almeida *et al*. (2004) compared cDNAs from *L. major* promastigotes at days 3, 7, 10 of culture and lesion-derived amastigote libraries. Four hundred and thirty unique (i.e. non-redundant) stage-specific genes were identified. A higher percentage of stage-specific gene expression was observed in amastigotes (*c.* 35%) than in metacyclics (*c.* 12%) for both cDNAs and open reading frames (ORFs). It is worth noting that cDNAs provided a richer source of regulated genes than currently annotated ORFs from the *Leishmania* genome (Almeida *et al*., 2004). A similar study conducted by Akopyants *et al*. (2004) compared promastigote, metacyclic and amastigote stage microarrays based upon random 1 kb 'shotgun' DNA fragments. They obtained a set of genes that included most of those previously identified in the literature as differentially regulated as well as a number of novel genes. The microarray method has also recently been used to screen novel vaccine candidates against murine *Leishmania major* infection (Stober *et al*., 2006).

Concerning the differential pathogenic potential of *Leishmania* strains, in a recent study using highly sensitive gene expression microarray technology, Salotra *et al*. (2006) identified genes that are differentially expressed in *Leishmania* parasites isolated from PKDL patients in comparison with those from VL. Sequence analysis revealed that these genes showed significant homology with gp63, gp46, putative amastin, a putative reductase and a possible calpain-like protein.

Proteomics is the study of the proteome, i.e. all the proteins that can be synthesized by the cell. The proteomic approach is based on

the separation of proteins by two-dimensional (2D) gel electrophoresis. This separates the proteins in one dimension by their electrical charge and in the second dimension by their size. The technique can be used to compare the proteome of strains showing different characteristics such as virulent versus avirulent, pathogenic versus non-pathogenic, or sensitive versus resistant, etc. For example, proteomics using 2D polyacrylamide gels is used for researching drug targets and studying resistance mechanisms (Drummelsmith *et al.*, 2003). The different protein spots obtained can be punched out and analysed by mass spectrometry.

To further the molecular knowledge of virulence and resistance, proteomics has been widely used with *Leishmania*. The production of large-scale proteomic data sets has been made possible by technological advances in mass spectrometry, combined with the availability of complete *L. major* genome sequences; proteomic analysis of *L. major* is available on the internet (http://www.cri.crchul.ulaval.ca/proteome/). This technology is also used to aid understanding of intracellular survival and pathogenesis of *Leishmania.*

Several studies have compared procyclic promastigote, metacyclic promastigote and amastigote stages. For example, these stages have been compared for *L. donovani* (see Thiel and Bruchhaus, 2001; Bente *et al.*, 2003), *L. infantum* (see El Fakhry *et al.*, 2002) and *L. mexicana* (see Nugent *et al.*, 2004). These studies defined changes in the protein pattern during the *Leishmania* cycle. For example, Bente *et al.* (2003) visualized a total of approximately 2000 protein spots in *L. donovani*; of these, 31 proteins were present only in promastigotes. They also observed a decreased abundance of four proteins late in amastigote differentiation. From these analyses, the authors used mass spectrometry and peptide mass 'fingerprinting' to analyse 67 protein spots, identified as 41 different proteins known from databases and eight hypothetical proteins. They showed that most of the stage-specific proteins fell into five groups of functionally related proteins: (i) stress response (e.g. heat, oxidative stress), (ii) cytoskeleton and cell membrane, (iii) energy metabolism and phosphorylation, (iv) cell cycle and proliferation, and (v) amino acid metabolism. They also confirmed the critical role of HSP90 in controlling life cycle differentiation. Similarly, a combined proteomic and transcriptomic study of stage

differentiation in *L. infantum* by McNicoll *et al.* (2006) suggested that post-transcriptional control at translational and post-translational levels could play a major role in differentiation of *Leishmania* parasites.

In a study of interspecies polymorphism, Papadopoulou *et al.* (2004) have tried to compare 2D gels derived from *L. major* and *L. donovani* in order to reveal species-specific proteins. While several spots (*c.* 30%) in both species were at the same position on the 2D gel, the variability between these two species was too great to have confidence that a protein migrating differently was species-specific. Thus, the proteomic approach may not lead to the discovery of species-specific proteins. At an intraspecific level, Walker *et al.* (2006) compared the soluble proteomes of *L. guyanensis* promastigotes from the laboratory clone MCL (mucocutaneous leishmaniasis) phenotype (ranging from highly metastatic through to infrequently metastatic) and the CL (cutaneous lesion) phenotype (non-metastatic) using 2D electrophoresis. They found two abundant protein spots specifically associated with MCL phenotype clones and two others exclusively expressed by CL phenotype clones. Identification of these spots by biological mass spectrometry (LC-ES-MS/MS) and bioinformatics revealed that MCL phenotype clones express distinct acidic and neutral isoforms of both elongation factor-1 subunit beta (EF-1β) and cytosolic tryparedoxin peroxidase (TXNPx) (Walker *et al.*, 2006). Both polypeptides are active in resistance to chemical and oxidant stress, providing a basis for further elucidation of the importance of antioxidant defence in the pathogenesis underlying MCL. Currently, we are using this approach to identify proteins associated with pathogenesis by comparing virulent and avirulent strains of the same species (especially dermotropic and viscerotropic strains of *L. infantum*) (M. Hide *et al.*, unpublished data).

5. WHAT GENETIC RESEARCH IS NECESSARY TO ADVANCE KNOWLEDGE OF LEISHMANIASIS?

The objective of this last section is to put current knowledge about *Leishmania* and leishmaniases in perspective. We will underline

important questions to which 'leishmaniacs' must find priority answers in various domains: epidemiology, population genetics, taxonomy and pathogenicity/virulence. We will also suggest a number of feasible ways of responding to these questions.

5.1. The Remaining Gaps and How to Fill Them

In the previous sections, we have attempted to detail the advances in epidemiology, taxonomy, population genetics and clinical diversity of *Leishmania*. Important advances have been made, but many important questions must still be answered.

5.1.1. Population Structure, Taxonomy and Epidemiology

As we have seen above, *Leishmania* population structure is complex since these organisms have a 'clonal structure' but are also able to make use of different genetic processes such as recombination, pseudo-recombination, gene amplification, etc. (see Section 3.3.1, p. 24). Nevertheless, a number of points conflict within this model. A clonal model implies an excess of heterozygous genotypes and a population structure with individualized lineages at specific and intraspecific levels. On one hand, the published genetic studies on *L. infantum* and *L. donovani* (see Mauricio *et al*., 2006; Ochsenreither *et al*., 2006) and the genetic data obtained in our laboratory with *L. infantum* and *L. braziliensis* (V. Rougeron *et al.*, unpublished data) suggest a deficit in heterozygous genotypes and no clear structuring at an intra-specific level in sympatric populations (A.-L. Bañuls *et al.*, unpublished observations). Similarly, even though *Leishmania tropica* revealed a higher degree of heterozygosity (Schonian *et al*., 2001; Schwenkenbecher *et al*., 2004), the populations displayed a deficit of heterozygous genotypes (Schwenkenbecher *et al*., 2006). This suggests that unknown factors remain and that further studies are necessary to clarify *Leishmania* population structure and the mating system of each species. Study of the genetic structure in natural populations is crucial for different reasons: (i) it obviously provides a better understanding of the connections by migration between different populations; (ii) it is fundamental for the understanding of *Leishmania*

mating systems, since experimental crossings are not feasible; (iii) the population genetics approach constitutes one of the best ways to understand the epidemiology; and (iv) knowledge of the reproduction model is the foundation for defining *Leishmania* species and therefore for building a solid taxonomy. However, the amount of information on parasite biology that can be retrieved using population genetics depends on some very important aspects that are still too often neglected in molecular epidemiological studies. We should like to emphasize their importance here. First, the sampling design is a crucial if not the most important aspect. Too often, unfortunately, the sampling design is not appropriate or adequate to answer the research question under investigation. Obviously, it depends on the question but some general advice can be given. As much as possible, (i) populations must be sampled hierarchically, from local to regional; (ii) the sampling must be exhaustive, i.e. both symptomatic and asymptomatic patients should be sampled; (iii) whenever possible, strains in animal reservoirs or in vectors must be sampled from the same places as those sampled in humans; and (iv) the detailed location of each sample must be known.

Another important aspect in population genetics is the use of powerful and well-adapted molecular markers. Until now, imperfect markers such as MLEE (with low resolving power), RAPD or PFGE have usually been employed in studying *Leishmania* population genetics. While the use of these markers contributed interesting information regarding the epidemiology and the biology of *Leishmania* species, other markers such as microsatellites could be more efficient in describing their genetic structure, especially their mating system. In comparison to RAPD and PFGE, which are dominant markers (homozygotes are not distinguishable from heterozygotes), microsatellites are co-dominant (homozygotes are distinguishable from heterozygotes), a very important difference. With microsatellites (or other co-dominant markers), it is possible to access the genetic variability within individuals (the observed heterozygosity) and to compare it to the expected variability (the expected heterozygosity given the allelic frequencies). Comparison of these two variables may, for instance, provide very valuable information on mating systems. F_{IS} is the parameter measuring the difference between both variables (see Wright,

1951; Weir and Cockerham, 1984; see also Box 1 in the review by Tibayrenc, 2007, on p. 395 of this volume). No difference between observed and expected heterozygosity translates into $F_{IS} = 0$ and means that individuals sexually reproduce at random in the population (panmixia). Departure from $F_{IS} = 0$ ($F_{IS} > 0$ or < 0) is indicative of a deviation from panmixia. For instance, self-mating may lead to $F_{IS} > 0$ (deficit in heterozygotes), while in very strong or strict clonality one may expect excesses in heterozygotes [$F_{IS} < 0$; De Meeûs and Balloux (2004)]. Finally, choosing the appropriate parameters is important in describing genetic structure. *F*-statistics (e.g. F_{IS} and F_{ST}) and their estimators are advantageous when describing the distribution of genetic variability at different hierarchical levels. We have just seen that F_{IS} different from 0 is indicative of departure from panmixia within populations. $F_{ST} > 0$ is indicative of differences in allelic frequencies between different populations. This parameter depends on the effective population size and the rate of migration occurring between populations. A value of $F_{ST} > 0$ is indicative of low connectivity between populations (i.e. a low effective migration rate). As such it may be used to describe the patterns of migration among different *Leishmania* populations, at different scales.

From a taxonomic point of view, as described above, until now there have been no clear criteria for species definition. Therefore, species description still remains totally arbitrary and is often based only on the identification of genetic or epidemiological variants. It is for this reason that the status of several species remains under debate and must be elucidated: *L. garnhami*, *L. pifanoi*, *L. venezuelensis* and *L. forattinii*. Several species have been reconsidered, such as *L. chagasi*, *L. peruviana*, *L. panamensis* and *L. infantum*. Several recent studies have agreed that *L. archibaldi* has no taxonomic value (Jamjoom *et al.*, 2004; Zemanova *et al.*, 2004; Kuhls *et al.*, 2005). The subgenus to which *L. colombiensis* and *L. equatoriensis* should be assigned is not clear (Cupolillo *et al.*, 1998; Fernandes *et al.*, 1999). Clarifying the species status of these taxa would avoid both multiple descriptions of the same organism under different names and confusion on the clinical or epidemiological properties by misidentification. Therefore, there is a crucial need for a clear and stable definition of taxa and especially of species of *Leishmania*, to

permit efficient communication between scientists of various disciplines and medical doctors. In this context, we (Bañuls *et al.*, 2002) have proposed that any new species of *Leishmania* should be based on the clearly distinct phylogenetic approach of Tibayrenc's discrete typing unit (Tibayrenc, 1998), which includes specific medical and/or epidemiological characteristics. This definition rests on the phylogenetic species concept (PSC) described by Cracraft (1983), combined with medical or epidemiological criteria. In any case, the prerequisite to rigorous species description is knowledge of the mode of reproduction and the population structure.

In epidemiology, we have emphasized that foci of leishmaniasis vary greatly. A single species can be found in different foci, in various hosts, and transmitted by different vectors, while different species can be transmitted in a single focus by the same vectors and can have the same reservoir hosts. This shows how extraordinarily adaptable *Leishmania* species are. For these reasons, clear knowledge of the overall characteristics of a species requires a solid taxonomy and consideration of the different populations throughout the distribution area of this species with its environmental diversity. Studies of this nature will do away with limited specific descriptions resulting from the exploration of a single epidemiological environment; it is necessary to investigate any given species in various foci in which the other components differ within these foci (vectors, host, environment, etc.), and to consider all the resulting data. This approach is indispensable to developing an integrated study (see Section 5.3, p. 67), the main objective of which is to take account of all the factors involved in the transmission of the disease.

5.1.2. Pathogenic Properties of Leishmania *at an Intraspecific Level*

In Section 4 (p. 35), we detailed the different studies conducted on pathogenic diversity. The clinical diversity observed in leishmaniasis stems from complex interactions between host factors (genetics, immunity, nutrition, etc.), parasite factors and environmental factors. The part played by *Leishmania* in the clinical diversity observed in human infections, especially that between cutaneous and visceral leishmaniasis, has to date been related mainly to the particular

Leishmania species involved. However, although numerous genetic factors implicated in virulence and pathogenicity have been identified at both the interspecific and intraspecific levels, we cannot strictly associate a clinical phenotype with a particular genotype, mutated gene, differential expression or any other single factor. For all these reasons, the role of parasite factors in clinical diversity must be studied by isolating the effects of the host or the environment, i.e. by concentrating on the intraspecific level. This approach also requires an integrative approach, at epidemiological and ecological levels (see below) as well as at the genomic, transcriptional and proteomic levels in the parasites themselves. For example, we are currently comparing dermotropic and viscerotropic strains of *L. infantum* with regard to their gene expression, proteomics, antigen polymorphism, potential epitope variation and comparative genomics (see below) in order to gain knowledge of the evolutionary events that have led to this dichotomy (Hide *et al.*, unpublished data).

Currently, the microarray (see Section 4.3.2(c), p. 49) is the major tool used to compare gene expression between the developmental stages of a strain. It could also be used to identify genes regulating transcript abundance in *Leishmania* strains isolated from different clinical forms of infection with a given species. This idea was first explored by Salotra and Singh (2006), who compared the regulation of surface proteins in *L. donovani* strains isolated from patients with either PKDL or VL. Similarly, the proteomic approach is currently being used to study stage differentiation by El Fakhry *et al.* (2002), Nugent *et al.* (2004), McNicoll *et al.* (2006) and Walker *et al.* (2006) and drug resistance mechanisms by Drummelsmith *et al.* (2003).

Much remains to be discovered with the aid of these markers but we are convinced that other tools, developed for use with other organisms, could be usefully employed to advance our knowledge of *Leishmania*.

5.2. New Genetic Approaches for *Leishmania*

Comparative genomics and single nucleotide polymorphism genotyping are two existing tools that could be applied to *Leishmania* research.

5.2.1. Comparative Genomics

The comparative genomics approach has already been tested for other parasites such as *Plasmodium* by Carlton *et al.* (2002) and has recently begun to be used on the Kinetoplastida by El-Sayed *et al.* (2005). Thanks to the complete sequencing of *Trypanosoma cruzi*, *T. brucei* and *L. major*, El-Sayed *et al.* (2005) have revealed a conserved core proteome of approximately 6200 genes in large syntenic polycistronic gene clusters (gene arrays conserved on chromosomes among species). Many species-specific genes, especially large surface antigen families, occur at nonsyntenic chromosome-internal and subtelomeric regions. Retroelements, structural RNAs and gene family expansion are often associated with syntenic discontinuities that—together with gene divergence, acquisition and loss, and rearrangement within the syntenic regions—have shaped the genomes of each parasite (El-Sayed *et al.*, 2005). Comparative genomics has thus provided very interesting data on these genomes; for this reason we will now discuss this approach which we hope will soon be applied to the comparison of *Leishmania* genomes.

The availability of genome sequences and their comparison among species is a revolution in biological science that is greatly accelerating our knowledge of species biology and evolution. Comparative genomic approaches have now been used for a number of different purposes concerning pathogens. Giving an extensive description of the tools used and the questions that could be addressed by comparing genomes of different species, and especially *Leishmania* species, would deserve a complete review in itself. We will thus make only a brief summary of the basic principles of comparative genomics, and indicate how these tools could be used to understand better both the biology and the evolution of pathogens.

(a) *What is comparative genomics?* Comparative genomics is the analysis and comparison of the genomes of different species. The major principle of comparative genomics is very simple: the two (or more) genomes under examination once had a common ancestor (Figure 9). Therefore, every nucleotide base present in the genome of each species is the result of both nucleotide composition of the original ancestral genome and the action of evolution on the genome of

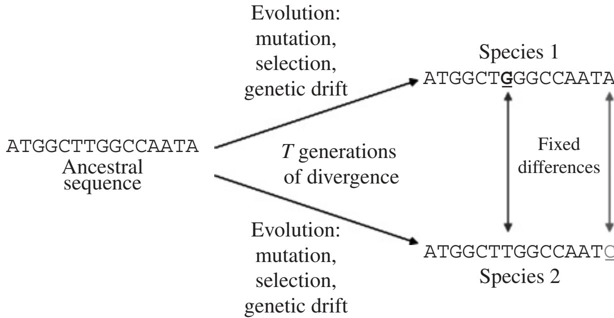

Figure 9 Schematic representation of the divergence of two base sequences after a speciation process which occurred *T* generations ago. Each resulting species has one fixed mutation (indicated by arrows).

each species since their divergence. In this context, evolution can be regarded as a combination of three major forces that independently affect and modify the composition of the genome of each species: mutation, selection and genetic drift. Mutations are artefacts generated randomly and recurrently in the genome of living organisms. These mutations can be of different types, affecting only one base pair in certain cases (i.e. one nucleotide change), or a larger region of the genome (by insertion or deletion) in other cases. Once a mutation has occurred in the genome, its future is then determined by the two other processes: selection and genetic drift. Selection pressure can act upon mutations in two different ways: purifying selection tends to eliminate mutations from the population when the mutation has a deleterious effect, i.e. when it reduces the fitness of the individuals that carry it, while positive selection increases the frequency of the mutant gene up to fixation (100% frequency) because of its positive effect on the individual's fitness. Genetic drift refers to the gradual drift over time of gene frequencies in a population due to random sampling effects in the formation of successive generations. In a narrower sense, genetic drift refers to the expected population dynamics of alleles, which are predicted eventually to become fixed at zero or 100% frequency in the absence of other mechanisms such as selection affecting the distribution of the alleles. Genetic drift therefore tends to increase or decrease the frequency of a mutation in the population, but only by chance. Because it is caused by random sampling effects,

it depends on the effective population size and is higher in small populations. The future of a mutation affected solely by drift is thus randomly determined. Neutral mutations (i.e. mutations that have no effect on fitness) are affected only by genetic drift. Mutations that affect fitness (negatively or positively) are influenced by both selection and, especially in small populations, genetic drift.

Because of mutation, genetic drift and selection, the two diverging species will tend to fix different mutations in their genomes and will therefore tend to accumulate differences over time. By analysing these differences between genomes, comparative genomics provides a means of understanding and reconstructing what happened during the evolution of the two species. Depending on the question addressed, many different features can be compared among genomes: sequence similarity/dissimilarity, gene location, the length and number of coding regions (exons) or noncoding regions (introns) within genes (it is worth noting that *Leishmania* lacks introns), the amount of noncoding DNA in each genome, the number of duplicated genes and highly conserved regions and so on.

In any case and whatever the question addressed, the first step of comparative genomics is generally to identify, in the genome of both (or all) species, the regions of similar ancestry and to align them, which requires the use of several specific and basic tools (homology research using BLAST processes and sequence alignment using ClustalX, or Bioedit software, for example).

(b) *What can be learnt about gene function?* Comparing genomes can help to reveal and analyse gene function. Information on sequence similarity among genomes of different species is a major resource for finding functional regions and for predicting what those functions are (Fraser *et al.*, 2000; Gutierrez, 2000). One of the best examples is, for instance, the improvement in identification of protein-coding genes. Various methods that incorporate interspecies similarity into gene prediction are now being used to analyse large genomes. The basic idea is very simple. Since common sequences of active genes are commonly under the influence of strong negative selection, comparing genomes is expected to unveil new sets of sequences corresponding to previously unidentified genes, thus expanding the complete gene catalogue for each organism. Several of the

novel genes predicted in mammals using this method have been verified experimentally, adding many new genes to the mammalian set (International Mouse Genome Sequencing Consortium, 2002).

Functional assignment of genes comes primarily from biochemical experimentation. However, comparative genomics can also help to resolve gene function. For instance, the regions involved in an organism's pathogenicity can be identified by comparing its genome with that of a related, non-pathogenic, organism. The pathogenicity genes will, *a priori*, be located in a region that greatly differs between the two genomes. This strategy can reduce the number of genes needing to be subjected subsequently to a specific and systematic functional analysis using 'knock-out' techniques, for instance. Comparing the genome of newly sequenced species to the genome of a well-known model organism in which most of the gene functions have been characterized through systematic biotechnological approaches is also a good way to identify gene function. Comparative genomics is therefore a powerful tool helping to gain greater insight into the genes present in a pathogen's genome. The ability to do this will increase with the amount of information available on different model organisms and the variety of these organisms. Obviously, the greater the number of completely sequenced genomes available, the more widespread this type of fast and economic *in silico* approach will become.

(c) *Discovering new drug and vaccine targets.* Comparative genomics can be a good strategy for identifying and discovering genes that might encode novel drug targets or protective antigens (Gutierrez, 2000; Carlton, 2003; McCarter, 2004). This can involve the use of comparative bioinformatics to identify single genes or families of genes which encode proteins whose function is known or which are already considered to be good drug targets in other organisms. In addition, the identification of genes involved in novel pathways or biochemical processes that are clearly essential for life but fundamentally different from the pathways found in the host can provide an important lead to potential new drug targets. The availability of genome sequences also makes it possible to develop the approach of reverse vaccinology, which uses genome comparison to predict new putative antigens.

Practically speaking, to identify new drug targets, BLAST research can be conducted against specific databases to find genes that are essential for the pathogen—i.e., which produce molecules essential to their life cycle—or genes already known to be good drug targets in related pathogen species. If the homologous gene is found, the queried genes may also be essential. Based on comparative genomics, the essential genes that are restricted to a group of species or a given species could represent novel targets for highly specific chemotherapy, whereas those that are preserved across many pathogens might be suitable targets for broad-spectrum antiparasitic agents. Such analyses must obviously be supplemented by systematic functional genomics to filter and ensure the validity as drug targets of the different candidate genes obtained through comparative genomics.

Comparative genomic methods have been successfully used to identify new drug targets in different types of pathogens. For instance, the genomic approach has been applied to tuberculosis, a major cause of transmissible morbidity and mortality, with notable success. Complete genome sequences are available for three members of the *Mycobacterium tuberculosis* complex and the related intracellular pathogen *M. leprae*. Many of the predictions generated *in silico* by comparative genomics have been validated through functional analysis, including studies of the transcriptome (Mostowy *et al*., 2004) and proteome (Betts *et al*., 2000), and have led to the identification of many genes essential for the life of the bacteria (Mushegian and Koonin, 1996). Knowledge of the latter genes defines potential targets for new and existing drugs. Their specificity has been assessed by comparative genomics with the host or other pathogens (Cole, 2002). Genomics has also advanced tuberculosis vaccine development by pinpointing potentially new antigenic proteins (Cockle *et al*., 2002).

In the genus *Plasmodium*, comparative genomics has also been applied to the search for new drug targets. One of the best examples concerns the identification of pathways in the apicoplast similar to those found in bacteria (Foth and McFadden, 2003). The bacterial nature of these pathways provided several new potential targets to which inhibitors had already been developed as antibiotics. Several experimental studies demonstrated *a posteriori* the validity of these candidate genes as good drug targets (Jomaa *et al*., 1999).

Several *Leishmania* genomes are or will soon be available on GeneDB: *L. major*, *L. infantum* and *L. braziliensis*. A great deal of interesting information will be provided by comparative genomics when the annotations are completely finished.

(d) *Understanding species evolution: what about selection?* As previously noted, traces of the evolutionary processes remain in the information of similarities and dissimilarities found between genomic sequences of different species. A number of useful evolutionary quantities can thus be analysed or estimated from between-species comparisons of genome-wide divergence patterns: the magnitude and variation of mutation rates within a genome, the magnitude of positive and negative (purifying) selection, the variation in selection across different lineages, chromosomes, gene families and individual genes as well as the number of genes participating in the speciation process or involved in adaptation to a new environment.

One of the best and most famous examples of how comparative genomics can give insight into the evolution of species is the analysis of how selection has shaped protein-coding genes in the genome.

The most direct method for showing the presence of positive selection is comparing and analysing the levels of divergence between sequences at two different types of sites within the DNA-coding region: the synonymous sites (also called silent sites because mutations there do not lead to a change in the protein sequence) and the non-synonymous sites (also called replacement sites because mutations at these sites lead to a change in the protein sequence) (Yang and Bielawski, 2000). Non-synonymous (N) and synonymous (S) sites undergo different selective forces in the genome. Whereas the former may experience positive and negative selection, the latter are generally assumed to be neutral and dependent on the mutation rate alone (but see Akashi *et al.*, 1998). The contrast between the differences observed between sequences at N and S sites has thus opened up ample opportunities for rigorous analyses of the effects of both positive and negative selection. In particular, the non-synonymous-to-synonymous substitution rate ratio (dN/dS) provides a sensitive measure of selective pressure on the protein. Values of $dN/dS<1$ indicate that a substantial proportion of amino acid changes must have been eliminated by purifying selection. Assuming that synonymous

substitutions are neutral, dN/dS > 1 implies, but is not a necessary condition for, adaptive or positive selection. However, changes in the dN/dS ratio, regardless of whether values are significantly > 1 within genes, among genes, among regions of the genome or among groups of species, can be indicative of changes in selective constraints and/or positive selection. A statistical framework for making inferences regarding dN and dS was developed by Goldman and Yang (1994). These methods (and new ones) are included in PAML software.

Overall genome analyses of selection acting on protein-coding genes have been applied to pathogenic species such as those of *Plasmodium* and *Trypanosoma*, for which multiple genomes of related species are available. In particular, it has been demonstrated in trypanosomatids that the genes of unknown function were those that displayed the highest rate of non-synonymous substitutions, suggesting that they were subject to positive selection or neutral evolution. Some of the genes undergoing positive selection probably included genes involved in single processes such as interaction with the host. For *Plasmodium* species, the analysis demonstrated that genes involved in host–parasite interactions or those which governed production of antigens recognized by the human immune system were evolving at the fastest rates (Carlton *et al.*, 2002).

5.2.2. *Other Tools: The Example of Single Nucleotide Polymorphisms*

Single nucleotide polymorphisms (SNPs) are single nucleotides that are polymorphic in the population. SNPs represent the most widespread type of sequence variation in genomes and are quite easily characterized and genotyped. As a consequence, they constitute interesting genetic markers that can be used for various purposes: revealing and analysing the evolutionary history of populations (Brumfield *et al.*, 2003), analysing the recombination rate in the genome, mapping genomes or conducting association studies, i.e. linking particular genotypes to particular phenotypes.

SNP discovery is the process by which a single nucleotide position in the genome is determined to be polymorphic in the population of interest (Marth *et al.*, 1999; Lindblad-Toh *et al.*, 2000). Generally,

SNPs have to be found through sequencing and comparison of segments of the genome from multiple individuals. Ideally the entire genome is sequenced in several individuals, so as to determine the number of SNPs overall in both coding and non-coding regions. For less ambitious and less costly projects, the number of genome segments that must be screened is largely dependent on the density of the SNPs present in each species. However, in many species, SNPs occur every 200–500 bp, suggesting that screening 100 segments of the genome is generally sufficient to obtain more than 50 SNPs.

For genotyping, the choice of method depends on many criteria and it is not our purpose to explain and develop all of them. Details of potential methods are given in the reviews by Kwok (2001) and Syvanen (2005). For instance, SNP genotyping can be performed using allele-specific methods, hybridization or primer extension (Kwok, 2001).

(a) *Recombination studies.* The nature and scale of recombination rate variation are largely unknown for most species. Recombination allows sites to evolve independently, and thus may act as a diversifying force, generating new variants of the parasite. SNPs can help to analyse and/or reveal patterns of recombination in the genome of pathogens (Mu *et al*., 2005). Different methods, parametric and non-parametric (see Awadalla, 2003 for more details), can be used to estimate the effective recombination rate within populations of pathogens. Such SNP-based approaches have been used in several species including *Plasmodium falciparum*. Mu *et al.* (2005) analysed the recombination rate of *P. falciparum* using SNPs distributed all over chromosome 3. This study revealed in particular that variation in effective recombination rates was high, both among populations and along the chromosome, with recombination 'hotspots' conserved among populations at chromosome ends. These markers might be used to infer the level of recombination in the genome of *Leishmania* species. This can be very informative since several recombination events have been demonstrated but their frequency in natural populations and their real impact on the population structure remain unknown.

(b) *Association studies and their limitations.* SNPs can also be used to genetically map DNA sequences that contribute to heritable

phenotypes (Pardi *et al*., 2005). While most SNPs are found outside genes and probably do not have an effect, those located in and near genes may contribute to the genetic basis of certain phenotypes, for example pathogenicity, virulence or resistance to certain drugs. The approach used to identify SNP alleles associated with particular phenotypes is conceptually quite straightforward. Two populations of individuals are screened, one with the phenotype of interest (e.g. resistant to a drug) and one without (e.g. susceptible to the same drug). The frequencies of the different alleles at each SNP in each sample population are then compared. If one allele is significantly more common in the first population than in the other, this suggests that one of the alleles confers the phenotype of interest or is linked to a site that directly confers the phenotype. Where an association is found, the SNP is therefore likely to be within or at least close to a gene that influences the phenotype. The ability of SNPs to provide information on that gene depends on the linkage disequilibrium existing between the SNP and the gene of interest, hence on the effective recombination rate in the genome and in the population being scrutinized. Unfortunately, SNPs are not useful or very informative in the case of association studies for asexual species or species displaying a 'clonal structure', as has been suggested for *Leishmania* and certain other pathogens (Wickstead *et al*., 2003), because linkage disequilibrium is expected to be high even for SNPs located far away from the gene of interest.

5.3. Towards an Integrated Approach

As mentioned above, the life cycle of *Leishmania* basically includes the vector, the parasite and the host, but the outcome of transmission—infection and disease—are dependent on the intrinsic characteristics of these three factors. Indeed, the epidemiology of leishmaniasis reflects the particular combination of interactions among these factors, but in many endemic areas the exact role of each factor and its relation to human infection are unknown. It is known, however, that all of them have an impact on the manifestation of the disease: (i) there is strong vector–parasite specificity, (ii) *Leishmania*

species are statistically associated with certain clinical forms and various factors have been described as associated with clinical diversity, and (iii) the risk of leishmaniasis is markedly increased by allelic variants at specific host genetic loci. This strongly suggests that it is necessary to study all these factors in a single-focus population in order to understand fully the epidemiology of leishmaniasis.

Concerning interactions with vectors, to complete their development in the sand fly, *Leishmania* have to overcome several adverse conditions (Killick-Kendrick, 1985; Sacks, 2001) such as digestive enzymes secreted by the insect (Borovsky and Schlein, 1987; Dillon and Lane, 1993; Ramalho-Ortigao *et al.*, 2003), midgut lectins (Wallbanks *et al.*, 1986; Volf *et al.*, 1994, 2002), excretion of bloodmeal remnants (Killick-Kendrick *et al.*, 1994; Pimenta *et al.*, 1994), and the sand fly's innate immune responses (Ramalho-Ortigao *et al.*, 2001; Boulanger *et al.*, 2004). The consequences of these interactions are termed vector competence. Some vectors have evolved as restricted vectors, such as *Phlebotomus (Phlebotomus) papatasi* and *P. (Paraphlebotomus) sergenti*, because they can be infected by only one species of *Leishmania* (*L. major* and *L. tropica*, respectively) (Pimenta *et al.*, 1994; Kamhawi *et al.*, 2000), whereas *Lutzomyia longipalpis* and *Phlebotomus argentipes* have evolved as permissive vectors, able to develop mature transmissible infections when infected with any of several *Leishmania* species (Pimenta *et al.*, 1994; Kamhawi *et al.*, 2000; Sadlova *et al.*, 2003; Rogers *et al.*, 2004). Numerous studies have shown that sand fly saliva enhances *Leishmania* infection in mammals and can modulate the immune response of the latter through the modulatory effect of molecules injected into the skin. These include salivary molecules and/or molecules of parasite origin, such as PSG (promastigote secretory gel) (reviewed by Gillespie *et al.*, 2000; Kamhawi *et al.*, 2000; Sacks, 2001). However, pre-exposure to sand fly saliva protected mice against infection with *L. major* (see Belkaid *et al.*, 1998; Kamhawi *et al.*, 2000). Sand fly saliva consists of a complex mixture of pharmacologically active compounds such as vasodilators, anticoagulants, platelet inhibitors and immunogenic proteins (Ribeiro, 1995; Charlab *et al.*, 1999; Kamhawi *et al.*, 2000; Valenzuela *et al.*, 2001; Valenzuela, 2002).

From numerous studies it is now emerging that host genetic factors also influence the outcome of human infection by *Leishmania*. Indeed, several epidemiological studies have shown that human infection by *Leishmania* parasites remains asymptomatic in most cases (Ho *et al.*, 1982; Badaro *et al.*, 1986; Zijlstra *et al.*, 1994; Bucheton *et al.*, 2003). Such asymptomatic subjects are able either to clear infection or to remain asymptomatic carriers for years (as shown by the development of overt leishmaniasis in immunosuppressed patients several years after their last visit to an endemic area). Other subjects, however, are unable to control parasite dissemination and/or multiplication and develop clinical symptoms of diverse severity. Several risk factors are associated with the development of leishmaniasis such as malnutrition, immunosuppression (acquired immunodeficiency syndrome, malignancy), pregnancy, age, immunological capacity and genetic factors. For example, the effect of malnutrition on persons infected with *L. donovani* infection is to alter the immune response and lead to increased parasite visceralization (Harrison *et al.*, 1986; Anstead *et al.*, 2001). The involvement of genetic host factors in resistance/susceptibility was first suggested by the fact that genetically distinct inbred strains of mice exhibited substantial differences in the outcome of experimental infection by a single strain of *Leishmania*. Overall studies in mice and in populations living in endemic areas suggest that the risk of clinical leishmaniasis (CL, MCL and VL) is markedly increased by allelic variants at specific genetic loci. During the early stages of *L. donovani* infection, control of the parasite is associated with a mutation in the transmembrane domain of the *Nramp1* gene (natural resistance-associated macrophage protein 1 gene or *Sl11a1*) (Bradley *et al.*, 1979; Vidal *et al.*, 1995). Alleles at the MHC locus have been associated with the late control of *L. donovani* infection in susceptible mouse strains (Blackwell *et al.*, 1980). Furthermore, in humans, some familial aggregations of clinical phenotypes, consistent with the existence of inherited factors in susceptibility to CL and VL, have been revealed by several epidemiological studies (Zijlstra *et al.*, 1994; Cabello *et al.*, 1995; Bucheton *et al.*, 2003). Furthermore, some studies indicated that the distribution of disease phenotypes (CL or VL) in extended pedigrees of persons living in endemic areas were statistically best explained by the

segregation of one or two major susceptibility loci (Shaw *et al.*, 1995; Alcais *et al.*, 1997; Peacock *et al.*, 2001). Together with the observation of profound ethnic differences in the ratio of asymptomatic to symptomatic infections (Ibrahim *et al.*, 1999; Bucheton *et al.*, 2003), these epidemiological observations strongly suggest that human susceptibility to cutaneous or visceral leishmaniasis is also mediated by host genetic factors. The integrated analysis of parasite genetics, parasite virulence factors, host immune responses, vector competence, host genetics, socioeconomic and environmental risk factors is now necessary for a better understanding of the interplay between these different factors and the risk of developing leishmaniasis. This approach could also provide information on the critical biological pathways involved in host resistance or susceptibility to leishmaniasis and therefore help to design new therapeutic or vaccine strategies.

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Human Waterborne Trematode and Protozoan Infections

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ABSTRACT

Waterborne trematode and protozoan infections inflict considerable morbidity on healthy, i.e., immunocompetent people, and may cause life-threatening diseases among immunocompromised and immunosuppressed populations. These infections are common, easily transmissible, and maintain a worldwide distribution, although waterborne trematode infections remain predominantly confined to the developing countries. Waterborne transmission of trematodes is enhanced by cultural practices of eating raw or inadequately cooked food, socioeconomical factors, and wide zoonotic and sylvatic reservoirs of these helminths. Waterborne protozoan infections remain common in both developed and developing countries (although better statistics exist for developed countries), and their transmission is facilitated via contacts with recreational and surface waters, or via consumption of contaminated drinking water. The transmissive stages of human protozoan parasites are small, shed in large numbers in feces of infected people or animals, resistant to environmental stressors while in the environment, and few are (e.g., *Cryptosporidium* oocysts) able to resist standard disinfection applied to drinking water.

1. INTRODUCTION

Current literature on human waterborne protozoan and helminthic infections is abundant and enormously scattered over various

epidemiological and parasitological journals, and is presented in diverse aspects and approaches. This review is concerned mainly with humans as hosts of waterborne protozoan and trematode infections, but other hosts that play an important zoonotic role are also covered in our review. The waterborne infections are mainly concerned with protozoans transferred as cysts, oocysts, or spores, and trematodes (exclusive of schistosomes) transferred as cercariae or metacercariae. Main coverage includes the following genera: *Cryptosporidium*, *Giardia*, *Cyclospora*, *Toxoplasma*, human-infective microsporidia, *Philophthalmus*, *Echinochasmus*, *Fasciola*, and *Fasciolopsis*. Information on the important topic of Travel Medicine and Risk Factors is included in one or more of the major sections in the text for most genera of organisms covered. Where such information is available, the modes of environmental contamination and transmission of some of these parasites is explained and the zoonotic reservoir characterized. The epidemiology of the diseases caused by some of these parasites is described for immunocompetent and immunocompromised human populations. The graphical presentation of the transmission cycles are utilized for certain parasites showing various epidemiological transmission sub-cycles. The article also presents the ways in which the environment is contaminated by some of these parasites. In this regard, we focus on agricultural runoff and wastewater discharges. The current statistics on waterborne infections, i.e., outbreaks and cases, are presented based on electronically available data collected by the Centers for Disease Control (CDC), Atlanta, GA, USA. The biological features of the life cycles of some of these parasites that facilitate environmental contamination, particularly waterborne transmission, are characterized. The article also covers the modern conventional molecular diagnostic techniques used for identification of some of these parasites in environmental samples, and in water, and for the assessment of their infectivity.

2. FASCIOLIDS

The purpose of this section is to review the salient information on the role of fasciolids in waterborne trematode infections. The backdrop of the review is the edited book by Dalton (1999) on all aspects of

Fasciola and fasciolosis. Although the book is concerned mainly with the cosmopolitan species *Fasciola hepatica*, it also documents some information on various aspects of the biology of the less-studied Asian liver fluke *Fasciola gigantica*. Dalton's edited book should be examined to appreciate the voluminous literature on fasciolids covered prior to about 1998, including all aspects of the biology of fasciolids. Generic characteristics and keys to the family Fasciolidae Ralliet, 1895 were provided by Jones (2005) in volume 2 of the *Keys to the Trematoda*. The major genera considered in our review are *Fasciola* Linnaeus, 1758 and *Fasciolopsis* Loos, 1899. Species of both genera are usually considered plantborne pathogens with humans and animals becoming infected following the ingestion of raw or improperly cooked vegetation tainted with metacercarial cysts. The topic of fascioliasis and other plantborne trematode zoonoses has been reviewed recently by Mas-Coma *et al.* (2005). Earlier reviews by Mas-Coma *et al.* (1999a, b) suggested that *F. hepatica* should also be considered as a waterborne trematode; recent information in Mas-Coma *et al.* (2005) also suggested that this is true for both *F. gigantica* and *Fasciolopsis buski*. Further information on this topic is considered in the section on fasciolids as waterborne trematodes.

A review of the literature using the ISI Web of Science indicates that most of the fasciolid literature is on *F. hepatica*. An ISI search in June 2006 covering the literature from 1998 to 2006 showed 1508 citations on *F. hepatica*, 235 on *F. gigantica*, and 23 on *F. buski*.

Whereas adults of *F. hepatica* and *F. gigantica* parasitize the liver and the biliary system of their human and animal hosts, *F. buski* is a gastrointestinal trematode. The topic of *F. buski* and fasciolopsiasis as a foodborne disease in humans has been reviewed by Graczyk *et al.* (2001a). Because the literature on fasciolopsiasis as a waterborne trematode intestinal infection is sparse, little further information on the topic is provided in this review. Although transmission of this digenean is generally associated with the ingestion of raw contaminated aquatic plants, Weng *et al.* (1989) noted that cercariae of this species may encyst on the water surface and that about 10% of all human infections may result from drinking tainted water.

Relative to human *F. hepatica*, this is a cosmopolitan species found in developed and under-developed countries. Human fascioliasis has

been an underestimated and under-explored disease but is now considered an emerging/reemerging disease (Mas-Coma *et al.*, 2005). Recent estimates have cited figures ranging from 2.4 to 17 million humans infected with fascioliasis, with most infections probably due to *F. hepatica* (Mas-Coma *et al.*, 1999a). It should be noted that *F. gigantica* has considerable impact on humans in under-developed countries and consequently the number of studies on this species is relatively sparse compared to those on *F. hepatica* (compare ISI WEB data as noted above). Undisputed proof of the distinctness of these two fasciolid species comes from the PCR-restriction fragment length polymorphism (RFLP) work of Marcilla *et al.* (2002). This is an important study for future work on the epidemiology of fascioliasis.

2.1. Waterborne Infections with *Fasciola*

Several studies (Mas-Coma *et al.*, 1999a; Esteban *et al.*, 2002) have implicated water as a source of infection with *Fasciola* metacercariae. Bargues *et al.* (1996), in their studies on the fascioliasis in the Bolivian Altiplano, noted that 13% of the experimentally obtained metacercariae were always floating. Mas-Coma *et al.* (2005) stated that water containing fasciolid metacercariae may contaminate food, kitchen utensils, and other objects, thus becoming a source of infection. Several indirect studies also show the importance of fascioliasis transmission through tainted water. Estaban *et al.* (2002, 2003) showed significant positive associations between fasciolid infection and infection with other waterborne parasites. For instance, the association of *Giardia intestinalis* with *Fasciola* in Peru (Esteban *et al.*, 2002) and of *Schistosoma mansoni* with *Fasciola* in Egypt (Esteban *et al.*, 2003) supported this idea. Hillyer and Apt (1997) noted that in many fasciolid hyperendemic areas of South America, humans do not eat watercress, and in the Asillo irrigation area of the Peruvian Altiplano, people do not eat freshwater plants. Esteban *et al.* (2002) cited additional evidences that infection of humans in the high areas of Peru occur mainly through drinking water, i.e., there is an absence of typical aquatic vegetation in the drainage channels inhabited by the fasciolid intermediate lymnacid snail hosts and the lack of potable

water systems inside dwellings, which required the residents to obtain water from irrigation canals and drainage channels.

Some studies have been designed to examine fasciolids as floating cysts. Thus, Cheriyof and Warne (1990) examined the distribution of the metacercariae of *F. gigantica* on some objects in containers in the laboratory. Cercariae of *F. gigantica* from *Lymnaea natalensis* were observed to encyst in varying degrees on different objects in the containers. However, some cysts were found unattached on the bottom of the containers or floating on the surface and bottom of the water. Vareille-Morel *et al.* (1993) provided data on the dispersion and fate of floating *F. hepatica* metacercariae. The authors concluded that the role of floating cysts is important in the transmission of fascioliasis to man.

3. THE GENUS *ECHINOCHASMUS*

The genus *Echinochasmus* contains a number of species that are transmitted to humans as foodborne trematode infections. Transmission is mainly by way of the ingestion of metacercarial cysts by humans with raw or improperly cooked freshwater fish. The genus has at least one species, *Echinochasmus liliputanus*, which is transmitted to humans as a waterborne infection. Humans ingesting cercariae of this species of echinostome may develop an intestinal trematodiasis referred to as echinochasmiasis. Fried (2001) reviewed the earlier literature till about the year 2000 on the descriptive, experimental, biochemical, and molecular studies of the various species in this genus. In the current review, emphasis is placed on significant studies on *E. liliputanus* as waterborne trematodes. We also review other significant literature on the genus *Echinochasmus* from 2000 to 2006.

3.1. Significant Literature on Species of *Echinochasmus* Exclusive of *E. liliputanus* from 2000 to 2006

Several studies have described species of *Echinochasmus* from animals of significance in wildlife biology and from humans. Thus, Borgsteede *et al.* (2000) described *E. beleocephalus* from passeriform birds, *Sitta europacea*, in the Netherlands and Rajkovic-Janje *et al.* (2002)

reported the species *E. pertoliatus* from the wild boar, *Sus scrofa*, in eastern Croatia. Dronen *et al.* (2003) reported on the species *E. donaldsoni* from the brown pelican, *Pelecanus occidentalis*, and the American white pelican, *Pelecanus erythrochynchus*, from Galveston Bay, TX, USA. Chai and Lee (2002), in their studies on foodborne intestinal trematodiases in the Republic of Korea, reported *E. japonicus* from humans. Although this echinostome is typically a parasite of birds in Korea, humans become infected by eating a variety of freshwater fish contaminated with metacercariae of this species.

Various studies on the biochemistry, systematics, and biology of echinochasmid species were done during 2000–2006. Thus, Cheng *et al.* (2000) analyzed the LDH isoenzymes and some proteins in two strains of *E. fujianeses* from China (the Guangdong strain versus the Fujian strain). Methods used in their study were isoenzyme electrophoresis, discontinuous PAGE, and thin-layer isoelectric equilibrium PAGE. The observed biochemical differences between the strains were minor, indicating that both strains are of the species *E. fujianeses*. Kostadinova (2005), in her revision of the family Echinostomatidae Looss, 1899, described the major characteristics of the genus *Echinochasmus* Dietz, 1909 and provided excellent figures (line drawings) of the species *E. coaxatus* Dietz, 1909. The Kostadinova (2005) key provides for separation of the genus *Echinochasmus* from related genera of Echinostomatidae. A distinction of the major species in the genus *Echinochasmus* was beyond the scope of the key. However, Kostadinova (2005) provided excellent morphological characterizations and considerable generalized life cycle information on the genus. Recently, Choi *et al.* (2006) provided morphological observations on the cercariae of *E. japonicus* and detailed methods used to maintain the life cycle of this species from the cercaria to the adult. Such information is valuable to workers who need to test the direct infectivity of cercariae via a water infection route by *per os* feeding of this larval stage to experimental definitive hosts.

3.2. *E. liliputanus* as a Waterborne Trematode

Numerous cases of human infection with *E. liliputanus* have been reported in China by Xiao and his collaborators from about 1990 to

the present (2006). The fact that this echinostome species can infect humans via the cercarial stage in contaminated water is of considerable interest to the medical community.

Xiao *et al.* (1992) reported the first case of natural infection of *E. liliputanus* in China. Because of the prevalence of echinochasmiasis in Anhui province, China, Wang *et al.* (1993) were able to determine the efficacy of praziquantel (often referred to as pyquiton in China) in the treatment of this echinostome in humans. Two hundred cases of *E. liliputanus* infection were divided into three groups and treated with 10, 5, or 2.5 mg of praziquantel per kg of body weight as a single dose. One month later, egg numbers were reduced by 100%, 98.7%, and 96.5%, respectively. The major symptoms of human echinochasmiasis, i.e., abdominal pain, distention of the abdomen, diarrhea, and anorexia, were alleviated. Xiao *et al.* (1994) carried out an epidemiological survey of *E. liliputanus* in Anhui province, China from 1991 to 1993. The overall infection rate was 13.7% with no significant difference in the infection rates between males and females. The infection rate was closely related to age with the highest rates occurring in relatively young persons. For instance, among all the infected people, the age group 3–30 had 78.3% of the infections. The study also determined a link between infection and drinking water. The infection rate in the major reservoir hosts (dogs and cats) was 62.2% in dogs and 39.8% in cats.

Xiao *et al.* (1995a) conducted epidemiological studies on *E. liliputanus* infections in intermediate hosts in Anhui province, China from 1991 to 1993. Seasonal variations in the infections in the intermediate hosts were recorded. The viviparid prosobranch snail, *Bellamya aeruginosa*, was reported as a new first intermediate host for this echinostome. Patent infections (those releasing cercariae) were reported from *B. aeruginosa* in late July, and peaked in September through November; they disappeared by early December. The overall infection prevalence of metacercariae in 20 species of freshwater fishes was 83.7% with the peak prevalence of infection in the fishes occurring between November and April. Xiao *et al.* (1995b) showed direct infection of *E. liliputanus* cercariae in nine dogs and two human volunteers. The subjects were infected *per os* with 1500–88 000 cercariae of this echinostome species. All the experimental subjects became infected showing

that humans and animals may become infected with this echinostome by drinking water containing the cercariae. In the two human subjects, eggs appeared in the stools on days 15 and 16 postinfection, respectively. In the dogs, the worm recovery rate was 4.0% and eggs first appeared in the feces at 14 days postinfection.

Xiao *et al.* (1995c) reported on epidemiological studies done in 1992 in Anhui province, China on the mode of human infection with *E. liliputanus*. The results showed that humans could become infected with this echinostome by drinking unboiled water containing cercariae or by eating raw fish containing metacercariae. Infection rates of *E. liliputanus* were 1.5% among the persons who did not drink unboiled water, but 20.1% for those who did. Only cercariae and not metacercariae were observed in the water of ponds used by these subjects. None of the people claimed to have eaten raw fish. Wang *et al.* (1998) conducted a longitudinal survey in a primary school in Anhui province, China to investigate the seasonal distribution, annual infection rate, and factors influencing infection by *E. liliputanus*. Infection with this echinostome in children reached a peak in autumn, particularly in September and October. The main reason for this seasonal distribution was that children drank untreated water from local ponds where *E. liliputanus* cercariae occurred. There was a significant correlation between *E. liliputanus* infection rate and the rate, frequency, and quantity of untreated water consumption. The annual infection rate of the echinostome in children was 31.9%.

3.3. Experimental Studies on *E. liliputanus*

Because of the importance of this echinostome as a waterborne infective agent, some basic experimental work has been done on this organism. Thus, Wu *et al.* (1997) used scanning electron microscopy (SEM) to examine the tegument of adults obtained from experimentally infected dogs. Most of the body surface is covered with spines except for the collar, acetabulum, and the posterior aspect of the body. There are three types of papillae on the tegument: two types of papillae are ciliate and the third type is aciliate. The acetabulum has 32 protruded aciliate papillae. Knowledge of the ultrastructure of the tegument of *E. liliputanus* may be of taxonomic significance in

distinguishing this medically important species of *Echinochasmus* from other closely related species.

Xiao *et al*. (2000) provided basic information on the cercariae of *E. liliputanus* obtained from infected *B. aeruginosa* (Viviparidae) snails. Daughter rediae of this echinostome species were able to produce enormous numbers of cercariae. For instance, in October with a mean temperature of 23°C, average cercarial release was 209 200 cercariae daily per snail. Under natural conditions, the release of cercariae from snails showed a typical diurnal pattern with cercariae being shed mainly between 08:00 and 16:00 h with a significant peak between 10:00 and 12:00 h and no cercariae were released at night. The main factors affecting the emergence of cercariae were temperature and light. Cercariae were phototactic and the newly shed cercariae tended to remain at the water surface. Survival and infectivity data of these cercariae at different temperatures were also reported. As in other studies on digeneans, cercarial longevity exceeded the period of infectivity at a given temperature. Xiao *et al*. (2006) examined the *in vivo* and *in vitro* encystment of cercariae *of E. liliputanus* and also reported on the biological activity of the metacercariae. *In vivo* encystment occurred in the gills of goldfish, a second intermediate host for this echinostome. The cercariae also encysted in various dilutions of Locke's solution, NaCl solutions, artificial gastric juice, and human gastric juice. *In vitro* formed cysts were capable of excysting when treated with a bile salt medium. Cysts formed *in vitro* and *in vivo* were both capable of infecting experimental rabbit hosts. The authors concluded that the finding of *E. liliputanus* cercariae encysting *in vitro*, especially in human gastric juice, might be helpful in elucidating mechanisms of the definitive host that allow for direct infection by the cercariae.

4. PHILOPHTHALMIDS (EYE FLUKES)

4.1. Introduction

Philophthalmid eye flukes may be considered waterborne trematodes (WBT) transmitted to the human eye by direct contact with cercariae

or metacercariae of a species of *Philophthalmus* in fresh or salt water. Probably, the usual mode of transmission of philophthalmid eye flukes to birds is by the oral route. Birds presumably ingest encysted metacercariae contained on the surfaces of objects, i.e., the shells of mollusks or crustaceans and the encysted metacercariae excyst in the oral cavity, aided by the warmth in the mouth; excysted metacercariae then migrate to the eye via the median slit and nasolacrimal canals. There is the possibility that some humans with a defective dorsal pallet can be infected by transmission of larval to stages via an opening in the dorsal pallet that connects to the nasal–lacrinal canals; the eye fluke larvae would then migrate to the orbit, probably the conjunctiva, where maturation from larva to adult would occur. Definitive evidence for this latter mode of transmission in humans is lacking.

This review examines significant studies on the biology of philophthalmids with coverage mainly from 1995 to 2005. The review on the taxonomy and biology of philophthalmids by Nollen and Kanev (1995) serves as background for our work. Significant articles earlier than 1995, but not cited in Nollen and Kanev (1995), are also included herein. Our coverage is in reverse chronological order and considers studies from about 1993 to 2005 on the biology, transmission, life cycles, epidemiology, and experimental studies of eye flukes in the genus *Philophthalmus*. Emphasis in the review is on human philophthalmosis, but we also consider studies on eye fluke diseases in non-human mammals and birds. Molecular biology studies on philophthalmid eye flukes are not available and therefore there is no coverage of this topic.

4.2. Biological Studies

Mukaratirwa *et al*. (2005) described a field outbreak of the oriental eye fluke, *Philophthalmus gralli*, in 17 commercially reared ostriches, *Struthio camelus*, in Zimbabwe. The ostriches had swollen eyes, severe conjunctivitis, and constant tears with a purulent exudate. Some birds were almost blind and showed severe loss of body condition. The eye flukes, attached to the nictitating membranes and conjunctival sacs of both eyes, were identified as *P. gralli*. The freshwater prosobranch snail, *Melanoides tuberculata*, was identified as the snail intermediate host based on natural and experimental infections. This was the first

report of *P. gralli* in birds in Zimbabwe and Africa and extends the known geographical range of this species.

Pinto *et al.* (2005) described the pathology and natural infection of *P. lachrymosus* in a non-human mammalian host. There are no reports of this eye fluke in humans, and information on any philophthalmid in non-human mammalian hosts is sparse. This report, concerned with philophthalmosis in two naturally infected capybaras, *Hydrochaeus hydrochaeris*, in Brazil, described the clinical signs, and gross and microscopic lesions in the eyes of these hosts; the study also provided new morphometric data on adults of *P. lachrymosus*.

Dailey *et al.* (2005) described a new philophthalmid, *Philophthalmus zalophi*, from the eyes of pinnepids, *Zalophus wollebaeki*, in the Galapagos Islands, Ecuador. This marine species differed from the four other described marine species (*P. andersoni*, *P. hegeneri*, *P. burrilli*, and *P. larsoni*) based on the mammalian host, large body size, lack of tegumental spines, and other morphometric features.

Abdul-Salam *et al.* (2004) described the cercariae of *P. hegeneri* from Kuwait Bay (Kuwait) in the marine snail *Cerithium scabridum*. Infected snails released megalurous cecariae, which produced the characteristic flask-shaped cysts described by Penner and Fried (1963) for *P. hegeneri*. Adult philophthalmids were recovered from the orbit of domestic ducks experimentally infected with excysted metacercariae. The study of Abdul-Salam *et al.* (2004) extended the range of *P. hegeneri* to Kuwait. Previous reports of this marine eye fluke by Penner and Fried (1963) were based on natural and experimental infections of various avian hosts in the Gulf of Mexico (FL, USA); the snail vector for *P. hegeneri* was from naturally infected marine snails, *Batillaria minima*, also from the Gulf of Mexico.

Urabe (2005) described a megalurous cercaria in the genus *Philophthalmus* from the freshwater snail, *Semisulcospira libertine*, in Kyushi, Japan. This was the first record of a philophthalmid cercaria from East Asia and from snails in the genus *Semisulcopira.*

Rathinam *et al.* (2002) described a presumed trematode-induced granulomatous anterior uveitis as a cause of intraocular inflammation in children from South India. The affected children had a history of bathing and swimming in local ponds and rivers. It was presumed, but not proven, that the infection was caused by a waterborne, infectious agent, possibly a philophthalmid.

Lamothe-Argumedo *et al.* (2003) reported the first case in Mexico of conjunctivitis in the human eye caused by the avian eye fluke, *Philophthalmus lacrymosus*. The patient, a 31-year-old male, visited an ophthalmologist in Sinaloa, Mexico because of a foreign body sensation in his left eye for 2 months. An eye fluke was removed surgically under local anesthesia from the connective tissue of the bulbar conjunctiva and the fluke was identified by morphological means as *P. lacrymosus*. Diaz *et al.* (2002) completed the life cycle of *P. gralli* in experimentally infected domestic chicks from larval stages found in *M. tuberculata* snails from freshwater streams in Sucre State, Venezuela. Their report provided a new geographical record for this species.

Dimitrov *et al.* (2000) described argentophilic structures in miracidia and cercariae of *Philophthalmus distomatosa* n. comb. The epidermal plate arrangement of most miracidia conformed to the formula 6:8:4:2 = 20. The distribution of tegumentary papillae on the cephalic and periaceablular body regions and the tail of the cercariae were given. The authors discussed the potential use of both miracidial and cercarial papillae patterns in identifying valid species of *Philophthalmus*.

Radev *et al.* (2000) studied the biology and life cycle of an eye fluke from larval stages in the freshwater snail *M. tuberculata* in Israel based on comparisons of the life cycle stage of the Israeli species with numerous philophthalmids from many parts of the world; the authors described the species from Israel as *P. distomatosa* n. comb.

Radev *et al.* (1999) identified *Philophthalmus lucipetus* from larval stages of a philophthalmid from *Melanopsis praemorsa* snails collected in Israel. The work included extensive studies on natural and lab-reared infections with larval and adult stages of this eye fluke.

Lang *et al.* (1993) reported the first instance of human philophthalmosis in Israel, based on conjunctivitis caused by a *Philophthalmus palperbrarum* adult in a 13-year-old Israeli girl. A single mature worm was detected on the palpebral conjunctiva of the upper eyelid of the right eye. Removal of the worm resulted in a cessation of the ocular symptoms.

Munizpereira and Amato (1993) reported the occurrence of *P. gralli* from the white-cheeked pintail duck, *Anas bahamensis*, and the Brazilian duck, *Amazonetta grasilieassis*, from lagoons in Marica County, Rio-de Janero, Brazil. This was the first record of a species of

eye fluke in the Neotropical region and both species of ducks were new hosts for *P. gralli*.

4.3. Experimental Studies

As reviewed by Nollen and Kanev (1995) philophthalmid eye flukes have provided useful models for various experimental studies on digeneans including work on chemoattraction of larval and adult stages, wound healing of adults, *in vitro* and *in vivo* cultivation of larval and adult stages and studies on the reproductive biology within and between species of philophthalmids. For detailed coverage of these topics, the reader is referred to Nollen and Kanev (1995). Two additional experimental studies after the review are listed below.

Radev *et al*. (1998a, b) used light microscopy to study the "so-called" body spines of adults of *P. hegeneri* and *P. lucipetus* obtained experimentally from the eyes of domestic chicks. The authors described four types of body spines based on light microscopy, but were cautioned that more detailed descriptions of these species by electron microscopy were needed. In a follow-up study using *P. lucipetus*, Radev *et al*. (1998a) used light and electron microscopy to study the body spines of *P. lucipetus* during maturation of this fluke in the eye cavity of bird hosts. Light microscopy showed long and thin spine-like structures clearly visible in young worms (up to 10 days olds), but absent in most of the older worms. Observations with electron microscopy showed that what were thought to be tegumentary spines were in fact papilla-like structures. It was concluded that the so-called body spines of *P. lucipetus* are different from the true tegumentary spines of echinostomatid-like digeneans that live in the intestine. The question of tegumentary papillae versus tegumentary spines in most species of philophthalmids needs more work.

5. HUMAN WATERBORNE PROTOZOAN PARASITES

Cryptosporidium, Giardia, Cyclospora, Toxoplasma, and human-infective microsporidia (e.g., *Encephalitozoon intestinalis, Encephalitozoon hellem, Encephalitozoon cuniculi*, and *Enterocytozoon bieneusi*)

are human enteric parasites in which transmission is associated with water (Wolfe, 1992; Ortega *et al.*, 1993; Graczyk *et al.*, 1997a; Lindsay *et al.*, 2001; Weiss, 2001). *Cryptosporidium, Giardia, Cyclospora, and Toxoplasma* are protozoan enteropathogens, and recent genetic information indicates that microsporidia are closely related to fungi (Weiss, 2001). All aforementioned parasites inflict considerable morbidity in immunocompetent people. Medically, the most important is *Cryptosporidium* as it significantly contributes to the mortality of people with impaired immune systems due to the lack of effective prophylaxis or therapy (Blagburn and Soave, 1997). Mortality rates due to *Cryptosporidium* among these individuals vary from 52% to 68% (Rose, 1997). The infectious dose of *Cryptosporidium* for immunosuppressed people has not been established, but it is believed that the disease can be caused by a single parasite cell (Rose, 1997). Although *Giardia lamblia* (syn. *G. intestinalis, G. duodenalis*) and *Cyclospora cayetanensis* cause serious diarrheal illness in adults and children worldwide (Pozio, 2003), the infections usually respond well to pharmacological treatment (Wolfe, 1992; Ortega *et al.*, 1993; Mansfield and Gajadhar, 2004). *Toxoplasma gondii* infections are life-threatening to persons with severely weakened immune systems, such as individuals with HIV/AIDS, those taking certain types of chemotherapy, and those who have recently received an organ transplant (Arkush *et al.*, 2003; Bonfioli and Orefice, 2005). *Toxoplasma* infections are also life-threatening to infants born to mothers who became infected for the first time during or just before pregnancy. Several pharmacological options are available for treatment of toxoplasmosis (Bonfioli and Orefice, 2005). Human-infective microsporidia cause serious illness in immunodeficient patients; however, they also respond well to pharmacological treatment (Weiss, 2001). *Cryptosporidium parvum, G. lamblia, T. gondii*, and human-infective microsporidia are anthropozoonotic pathogens (Wolfe, 1992; Graczyk *et al.*, 1997a; Lindsay *et al.*, 2001), and no zoonotic reservoir has been revealed for *C. cayetanensis* despite considerable efforts worldwide (Ortega *et al.*, 1993; Mansfield and Gajadhar, 2004). Clinical infections of *C. parvum* are mainly confined to calves, which can shed up to 10^6 oocysts per g of their feces, and exceed 10^9 oocysts in daily output (Anderson, 1981). As many as 10^6 *C. parvum* oocysts can be found in human diarrhetic feces (Rose, 1997). All aforementioned

parasites produce a robust, long-lasting, and environmentally resistant transmissive infectious stage, the oocyst (i.e., *Cryptosporidium, Cyclospora, and Toxoplasma*), the cyst (i.e., *Giardia*), and the spore (i.e., microsporidia), which can be effectively and efficiently transmitted via water (Wolfe, 1992; Ortega *et al*., 1993; Graczyk *et al*., 1997a; Lindsay *et al*., 2001). Therefore, transmissive stages of these parasites are ubiquitous in the environment (Wolfe, 1992; Rose *et al*., 1997; Kucerova-Pospisilova *et al*., 1999) and pollute surface waters via point sources of contamination such as wastewater discharges, leaky septic tanks, urban runoff, recreational activities, and agricultural runoff predominantly from livestock operations (Graczyk *et al*., 2000a, b).

6. THE ORGANISMS

6.1. *Cryptosporidium*

Parasites of the genus *Cryptosporidium* are cyst-forming protozoons, which have a monogenous life cycle and inhabit epithelial cells of the gastrointestinal or respiratory tracts (Fayer *et al*., 1997). Of approximately11 valid species infecting all vertebrate groups, two species, *C. parvum* and *C. hominis*, represent a global public health problem (Morgan-Ryan *et al*., 2002; Thompson *et al*., 2005). The only exogenous stage, the oocyst, is long-lived and resistant to standard water disinfection (Graczyk *et al*., 1997a). As a result, *Cryptosporidium* has caused massive epidemics and has become recognized as the most important water biological contaminant in the USA (Rose *et al*., 2002; Gupta and Hass, 2004; Eisenberg *et al*., 2005). Waterborne transmission is facilitated by the small size of the oocyst (3.5–5.0 μm), sub-optimal processing at water-treatment facilities, chlorine resistance, and long-lasting infectivity of the oocysts in the environment (Graczyk *et al*., 1997a). Approximately 1 week after ingestion of oocysts, severe chronic or self-limiting diarrheal disease may result depending on a person's immune status (Fayer *et al*., 1997). As few as 10 oocysts initiated infection in a dose–response study involving immunocompetent humans (Okhuysen *et al*., 1999, 2004). Over one

billion oocysts can be excreted daily in diarrheal stools (up to 30 bowel motions, totaling 3 L/day) by an immunodeficient person (Blagburn and Soave, 1997). Persons at greatest risk are young children and immunocompromised and immunosuppressed individuals (Graczyk *et al.*, 1997a). The pathogen significantly contributes to mortality of AIDS patients; extraintestinal infections in the pancreas, gallbladder, bile ducts, and even the lungs has been observed in such patients (Fayer *et al.*, 1997). The oocysts can remain viable when exposed to temperatures as low as −22 °C, remain infectious for up to 12 months in an aqueous suspension at 4 °C, resist the lethal effects of chlorine and chloramine used in drinking water disinfection, and can survive contact with commonly used flocculents, e.g., alum, ferric sulfate, and lime (Rose *et al.*, 1997). The oocysts remain viable after an 18 h exposure at 4 °C to 3% sodium hypochlorite (Rose *et al.*, 1997). In immunocompetent (healthy) people, the ID_{50} is 132 oocysts (Chappell *et al.*, 1999). Symptoms of cryptosporidiosis include profuse, watery diarrhea, abdominal cramping, nausea, vomiting, anorexia, low-grade fever, and headache. In young malnourished children, *Cryptosporidium* infections can last longer than 3 weeks (Okhuysen *et al.*, 1999, 2004).

Cryptosporidium oocysts are continuously (as distinct from intermittently) prevalent in surface waters (Rose *et al.*, 1997) in which the prevalence of positive samples varies from 6% to 100% with oocyst concentrations of 0.003 to 5800 oocyst/L, respectively (Rose *et al.*, 1997). Adverse weather conditions such as heavy rains, snow melts, and floods wash oocysts from land areas into surface waters, elevate turbidity, cause sewage overflow, and increase urban and agricultural runoff resulting in water contamination (Graczyk *et al.*, 2000a, b). Although molecular techniques have demonstrated differences among *Cryptosporidium* species and genotypes, no methods to speciate waterborne oocysts at a water-treatment facility have been adopted (Graczyk *et al.*, 1997a). Assessment of infectivity of oocysts recovered from treated drinking water would be invaluable in providing accurate information facilitating communication of health officials with waterplant managers (Arnone and Walling, 2006).

6.1.1. Historical Prospective on Waterborne Cryptosporidiosis

Cryptosporidium was known to parasitologists long before human cryptosporidiosis emerged as a global problem. The zoonotic potential of *C. parvum* raised early concerns that large concentrations of domestic animals, particularly dairy farms and cattle grazing lands and pastures, were possible sources for waterborne oocyst (Lisle and Rose, 1995). Concern of environmental contamination by cattle feces increased when cider from fallen apples collected in a pasture caused outbreak of cryptosporidiosis (Fayer *et al*., 1997). Agricultural, sewage waste and discharges, and urban runoff have become recognized as potential sources of water contamination (Lisle and Rose, 1995). Consequently, current watershed protection programs include elimination of human activity from the area of water catchment (Lisle and Rose, 1995). Surprisingly, waters from the protected watersheds have been found to be contaminated at similar levels to waters from unprotected or industrially impacted areas (Rose *et al*., 1997), raising the concern that environmental factors contributing to the contamination may not be fully recognized (Lisle and Rose, 1995). Wildlife, particularly large game-animals, was postulated as a potential factor contributing to water contamination (Hansen and Ongerth, 1991). Currently, mammalian wildlife is recognized as a significant source of waterborne *Cryptosporidium* oocysts in a watershed (Macpherson, 2005).

6.1.2. Cryptosporidium *in Recreational, Drinking, and Surface Waters*

Cryptosporidium is the most frequent etiologic agent identified in recreational waters in the United States (Craun *et al*., 2005). Outbreaks caused by *Cryptosporidium* are primarily associated with treated waters in swimming and wading pools, and the important sources of contamination for both treated and untreated recreational waters are the bathers themselves (Craun *et al*., 2005). Contamination from sewage discharges and wild or domestic animals are important contamination sources for untreated waters (Craun *et al*., 2005). Factors contributing to swimming pool-related outbreaks include inadequate attention to maintenance, operation, disinfection, and filtration (Craun *et al*., 2005).

Most of the outbreaks of *Cryptosporidium* in drinking water were associated with semi-public water systems, followed by public and private water systems (Schuster *et al*., 2005). Severe weather conditions, close proximity to animal populations, treatment system malfunctions, and poor maintenance and treatment practices were associated with reported *Cryptosporidium* outbreaks in drinking water (Schuster *et al*., 2005).

Monitoring of the water samples from 14 states of the USA and one Canadian province showed that *Cryptosporidium* oocysts were found in 87% of the 85 raw surface water samples with the geometric mean of 2.70 oocysts/L (Rose *et al*., 1997). All of the river water samples contained *Cryptosporidium* oocysts (Rose *et al*., 1997). In 97% of 85 samples, either *Giardia* spp. cysts or *Cryptosporidium* oocysts were found with *Cryptosporidium* being about 1.5 times more numerous than *Giardia* spp. (Rose *et al*., 1997). Testing of river water in Washington State and California (USA) showed 100% positivity for *Cryptosporidium* oocysts; the oocyst concentrations varied from two to 112 oocysts/L with a mean of 25.1 oocysts/L (Rose *et al*., 1997). The other study that examined two rivers (Snoqualmie and Cedar) in Washington State showed that 34 of the 35 samples (97%) were positive for *Cryptosporidium* with oocysts concentrations ranging from 0.15 to 63.5 oocysts/L (Pan and Graczyk, 1997). Although *Cryptosporidium* oocysts were present in the Cedar river (considered as pristine), the oocyst concentrations were nearly 10 times lower than those in the Snoqualmie River (Pan and Graczyk, 1997). The highest concentrations were found in samples taken farthest downstream on the Snoqualmie River, below numerous dairy farms (Pan and Graczyk, 1997). *Cryptosporidium* oocysts were found consistently over the 3-month study period, leading to the conclusion that *Cryptosporidium* does not appear intermittently, but rather is present continuously (Graczyk *et al*., 1997a), and that seasonal factors such as runoff of land drainage, affect oocyst concentrations (Pan and Graczyk, 1997). In other studies, 77% of 107 samples taken from surface waters in Arizona, California, Colorado, Oregon, Texas, and Utah (USA) contained *Cryptosporidium* oocysts with the mean concentration within the limits of 0.91–0.94 oocysts/L (Rose *et al*., 1997). Higher oocyst concentrations were found in waters receiving sewage effluents

(Pan and Graczyk, 1997; Rose *et al.*, 1997). In studies conducted in 17 states of the USA, 55% of 257 water samples contained *Cryptosporidium* oocysts with an average concentration of 0.43 oocysts/L (Pan and Graczyk, 1997; Rose *et al.*, 1997). Higher concentrations of oocysts were found in polluted waters; the oocyst concentrations were 11 times greater in polluted lakes as opposed to pristine lakes (Pan and Graczyk, 1997; Rose *et al.*, 1997). Polluted rivers contained 2.2 times greater concentrations of *Cryptosporidium* oocysts over their pristine counterparts. Although the differences between pristine and polluted lakes was greater than the difference between pristine and polluted rivers, rivers demonstrated higher peaks of oocyst concentrations (Pan and Graczyk, 1997; Rose *et al.*, 1997). Polluted rivers contained approximately four times greater oocyst concentrations when compared to polluted lake waters (Pan and Graczyk, 1997; Rose *et al.*, 1997). In addition, concentrations of *Cryptosporidium* oocysts were compared with concentrations of *Giardia* spp. cysts in the same samples. On average, *Cryptosporidium* oocyst concentrations were 10–100-fold greater than concentration of *Giardia* spp. cysts (Pan and Graczyk, 1997; Rose *et al.*, 1997). Although *Cryptosporidium* oocysts were found in greater numbers than *Giardia* spp. cysts, their levels were found to be significantly correlated (Pan and Graczyk, 1997; Rose *et al.*, 1997). In the study conducted in Canada, 4.5% of 1173 water samples collected from 72 municipalities across the country contained *Cryptosporidium* oocysts (Pan and Graczyk, 1997; Rose *et al.*, 1997). In Yukon (Canada), 5% of 63 samples were positive for *Cryptosporidium* oocysts (Pan and Graczyk, 1997).

The 1993 outbreak of cryptosporidiosis in Milwaukee (WI, USA) affected over 403 000 people out of a population of approximately 800 000 (Gupta and Hass, 2004; Eisenberg *et al.*, 2005). At least 100 deaths are attributed to the outbreak, most of whom were HIV-positive (Gupta and Hass, 2004; Eisenberg *et al.*, 2005). The source of the outbreak was traced to one of two water treatment plants that drew its water from Lake Michigan at a point 42 ft [13 m] below the water's surface. Suggested sources of oocyst contamination of the raw water supply included a wastewater discharge (Gupta and Hass, 2004; Eisenberg *et al.*, 2005).

6.2. *Giardia* spp.

Giardia spp. are flagellated protozoan parasites of vertebrates; their infectious stage, the cyst, is transmitted via the fecal–oral route and frequently via water (Wolfe, 1992). The genus *Giardia* has been divided into three morphological types: *G. duodenalis* (= *lamblia*, *intestinalis*), *G. muris*, and *G. agilis* (Thompson *et al*., 1993). *Giardia duodenalis* and *G. muris* have been reported from mammals and birds (Thompson *et al*., 1993). In the wild, a wide variety of aquatic and semi-aquatic mammals can be a source of waterborne *Giardia* cysts (Erlandsen and Bemrick, 1988). Giardiasis is one of the most common protozoan intestinal zoonotic diseases. The pathogen is the most frequently identified etiologic agent of waterborne and foodborne outbreaks of intestinal illnesses worldwide (Wolfe, 1992). Over 44% of the outbreaks in which *Giardia* was identified as an etiologic agent were foodborne, versus 30% and 26% drinking and recreational water-related epidemics, respectively (Graczyk *et al*., 1999b; Mead *et al*., 1999). The zoonotic reservoirs for waterborne cysts include aquatic and semi-aquatic mammals, e.g., beavers and muskrats, small rodents, and wild or domestic mammals (Monzingo and Hibler, 1987). Contamination of surface waters is enhanced by adverse weather conditions, which carry cysts from fields containing feces of livestock and wildlife, and cause sewage and waste-related water contamination (Graczyk *et al*., 1999b). Human infections manifested by a transient or persistent acute steatorrhea, or intermittent acute diarrhea and abdominal cramps, malabsorption, nausea, abdominal distention, malodorous flatulence, and occasional vomiting are associated with weight loss (Wolfe, 1992). *Giardia* infections can be generated by as few as 10 waterborne cysts (Wolfe, 1992). Giardiasis has a median prepatent period of 14 days, the median incubation time of 8 days, and infections are usually self-limiting. Treatment includes quinacrine, metronidazole, tinidazole, or furazolidone (Wolfe, 1992).

Giardia cysts can remain viable in surface water for approximately 2 months (deRegnier *et al*., 1998). In the water, these cysts gravitate to the bottom of the reservoir and are more easily found in the sediment than in the water (deRegnier *et al*., 1998). Also, sedimentation is

the most efficient procedure for the removal of *Giardia* cysts during drinking water treatment (Rose *et al.*, 1997). A long-term (over 14 months) ecological investigation demonstrated that *Giardia* sp. cysts originating from beaver and muskrat colonies settled rapidly to the bottom of slow-moving water reservoirs and contaminated the sediment (Monzingo and Hibler, 1987).

Studies on giardiasis in a variety of birds suggest they may be zoonotic reservoirs (Upcroft *et al.*, 1997); however, these indications have not been substantiated. As reviewed by Hopkins *et al.* (1997), numerous studies demonstrated genetic and antigenic similarities among *Giardia* cyst isolates from humans and other mammalian hosts. Identification of the genotype of *Giardia* directly from cysts appears to be the most reliable means to demonstrate zoonotic transmission (Hopkins *et al.*, 1997; Issac-Renton *et al.*, 1997). Genotyping characterization of *G. duodenalis* from cyst samples originating from a human waterborne outbreak was identical to the genotype of cysts derived from beavers living in the same epidemic locality (Issac-Renton *et al.*, 1997). For studying the epidemiology of giardiasis, a PCR-based method for genotyping *Giardia* isolates directly from cysts appears to be most successful to differentiate genotypes of the parasites (Hopkins *et al.*, 1997). This molecular identification does not require cultivation to increase the number of organisms and can be applied directly to the cysts recovered from environmental sources (Hopkins *et al.*, 1997). Current data on *Giardia* do not support the traditional view that numerous, highly host-specific *Giardia* species exist (Hopkins *et al.*, 1997; Issac-Renton *et al.*, 1997), but rather indicate the existence of a limited number of species. Of these, *G. duodenalis* exhibits considerable intraspecific variability with a number of genotypes, some of which are not rigidly host-specific (Hopkins *et al.*, 1997; Issac-Renton *et al.*, 1997).

6.2.1. Giardia *spp. in Recreational, Drinking, and Surface Waters*

Giardia is a one of the most frequent biological contaminant reported from recreational and drinking waters (Craun *et al.*, 2005; Schuster *et al.*, 2005). Close proximity to domestic livestock populations and inadequate attention to maintenance, operation, disinfection, and

filtration were main factors contributing to contamination of water in swimming pools (Craun *et al.*, 2005). Deficiencies related to drinking water productions were predominant reasons for presence of *Giardia* cysts in finished drinking water (Schuster *et al.*, 2005).

Giardia is considered to be a problem in waters that received sewage, agricultural or industrial runoff (Rose *et al.*, 1997). Higher cyst concentrations are found in human-impacted surface waters, consistent levels of cysts are also being found in waters considered as pristine (Rose *et al.*, 1997). In the study that examined 66 surface water sources for treatment plants in 14 states of the USA and one Canadian province, *Giardia* spp. cysts were found in 81.2% of the samples (Pan and Graczyk, 1997; Rose *et al.*, 1997). The cyst concentration ranged from 0.04 to 66 cysts/L, with a geometric mean of 2.77 cysts/L (Pan and Graczyk, 1997; Rose *et al.*, 1997). While these numbers may seem high, examination of the cysts for a possible viable morphology showed that only 12.8% resembled viable pathogen types (Pan and Graczyk, 1997; Rose *et al.*, 1997). This study also concluded that higher cyst concentrations were found in waters that received industrial or sewage effluents (Pan and Graczyk, 1997; Rose *et al.*, 1997). In another study of water samples from 17 states of the USA, *Giardia* spp. cysts were found in only 16% of the samples (Pan and Graczyk, 1997; Rose *et al.*, 1997). The presence of *Giardia* spp. cysts was compared in two rivers in Washington state (USA) (Pan and Graczyk, 1997). One of the river, the Hoh River, is heavily visited by tourists and contains many recreational facilities. The other, the Queets River, sees 10 times less human usage. Concentration of 0.15 cysts/L was found in Hoh River, while concentration of only 0.05 cysts/L was found for the Queets River (Pan and Graczyk, 1997). On the basis of these data, it was concluded that concentrations of *Giardia* spp. cysts of 0.05 cysts/L can be expected in relatively pristine rivers (Pan and Graczyk, 1997). Another study examined three pristine rivers in Washington state (USA) and found *Giardia* spp. cysts in 43% of the 222 samples; cyst concentration ranged from 0.1 to 5.2 cysts/L. The conclusion reached by the authors stated that *Giardia* spp. cysts are continuously present in pristine waters at low concentrations (Pan and Graczyk, 1997). In another study that examined remote pristine surface water sources in the Yukon (Canada), *Giardia*

spp. cysts were found in 32% of 22 samples (Pan and Graczyk, 1997). In this study, animal scats in the watersheds of these water sources were also examined and 21% of them contained *Giardia* spp. cysts (Pan and Graczyk, 1997). The animals that were harboring the cysts included beavers, coyotes, sheep, grizzly bears, muskrats, and wolves (Pan and Graczyk, 1997). Another study conducted in Canada sampled water from 72 municipalities across the country (Pan and Graczyk, 1997). *Giardia* spp. cysts were found in 21% of the raw water samples (Pan and Graczyk, 1997). Concentrations tended to be low, with most samples containing fewer than 2 cysts/100 L (Pan and Graczyk, 1997). High *Giardia* spp. cyst concentrations were found during an outbreak in Ontario (Canada) (230 cysts/100 L). Although *Giardia* spp. cysts were found in samples collected throughout the year, the incidence and concentration tended to be higher during the late winter–early spring and fall (Pan and Graczyk, 1997). The study that examined the prevalence of *Giardia* spp. cysts in two watersheds in British Columbia (Canada) showed that the pathogen was present in 100% of the water samples (Pan and Graczyk, 1997). These two watersheds serviced communities that are active in cattle ranching. Cyst concentrations ranged from 0.006 to 22.15 cysts/L (Pan and Graczyk, 1997; Rose *et al*., 1997). The concentrations of *Giardia* spp. cysts were also found to be higher downstream of cattle ranches, with a peak coinciding with calving activity, implicating cattle ranching as a main factor for water contamination (Pan and Graczyk, 1997; Rose *et al*., 1997). *Giardia* spp. cysts are ubiquitous in surface waters, both pristine and those impacted by human activity. While water sources that are industrially or agriculturally impacted show higher concentrations of *Giardia* spp. cysts, pristine water still consistently demonstrated low cyst concentration.

6.3. *Cyclospora*

C. cayetanensis is an intestinal coccidian protozoon that causes prolonged diarrheal illness in adults and children worldwide (Ortega *et al*., 1993; Madico *et al*., 1997). *Cyclospora* infects the small intestine and usually causes watery diarrhea, with frequent, sometimes explosive,

bowel movements (Mansfield and Gajadhar, 2004). Other symptoms can include loss of appetite, substantial loss of weight, bloating, increased gas, stomach cramps, nausea, vomiting, muscle aches, low-grade fever, and fatigue (Mansfield and Gajadhar, 2004). However, some people who are infected with *Cyclospora* do not have any symptoms, and can spread this pathogen to other persons (Mansfield and Gajadhar, 2004). People of all ages are at risk for infection. In the past, *Cyclospora* infection was usually found in people who lived or traveled in developing countries. However, nowadays the infection is found worldwide. The recommended treatment for infection with *Cyclospora* is a combination of two antibiotics, trimethoprim–sulfamethoxazole, also known as Bactrim, Septra, or Cotrim (Madico *et al*., 1997; Mansfield and Gajadhar, 2004). People who have diarrhea should rest and drink plenty of fluids. Avoiding water or food that may be contaminated with stool may help prevent *Cyclospora* infection. People who have previously been infected with *Cyclospora* can become infected again.

Waterborne transmission of *C. cayetanensis* is believed to be the main mode of transmission (Ortega *et al*., 1993; Graczyk *et al*., 1998); however, with the exception of one confirmed drinking water outbreak, it remains epidemiologically unproven (Graczyk *et al*., 1998; Mansfield and Gajadhar, 2004). The outbreaks of cyclosporiasis epidemiologically linked to contamination of berries or vegetables have been consequently classified as foodborne, although contamination of field watering systems with the oocysts was suggested (Mansfield and Gajadhar, 2004). Epidemiological circumstances presented in the cases, outbreaks, or non-outbreak settings of *C. cayetanensis* infections, together with the prolonged pathogen sporulation period, strongly indicate waterborne transmission of the oocysts (Mansfield and Gajadhar, 2004).

6.4. *Toxoplasma gondii*

T. gondii causes a disease known as toxoplasmosis. While the parasite is found throughout the world, more than 60 million people in the United States may be infected with this parasite (Dawson, 2005). Of

those who are infected, very few have symptoms because a healthy person's immune system usually keeps the parasite from causing illness (Dawson, 2005). However, *Toxoplasma* infection could cause serious health problems for pregnant women and individuals who have compromised immune systems. Infections are acquired via ingestion of oocysts originating from cat feces (Lindsay *et al.*, 2001). This might happen accidentally when the hands touch the mouth after gardening, after cleaning a cat's litter box, or after touching anything that has come into contact with cat feces. Toxoplasmosis can also be acquired via eating contaminated raw or partly cooked meat, especially pork, lamb, or venison, or drinking water contaminated with the oocysts (Arkush *et al.*, 2003; Sroka *et al.*, 2006).

Most people who become infected with *Toxoplasma* are not aware of it (Dawson, 2005). Some people who have toxoplasmosis may feel as if they have the ''flu'' with swollen lymph glands or muscle aches and pains that last for a month or more. Severe toxoplasmosis, causing damage to the brain, eyes, or other organs, can develop from an acute *Toxoplasma* infection or one that had occurred earlier in life and is now reactivated. Severe cases are more likely in individuals who have weak immune systems, though occasionally, even persons with healthy immune systems may experience eye damage from toxoplasmosis (Dawson, 2005). Most infants who are infected while still in the womb have no symptoms at birth, but they may develop symptoms later in life. A small percentage of infected newborns have serious eye or brain damage at birth (Dawson, 2005; Bonfioli and Orefice, 2005).

6.5. Microsporidia

Microsporidia are obligate intracellular parasites that are emerging opportunistic pathogens among both immunocompromised and immunocompetent people (Weiss, 2001). Microsporidia are on the Contaminant Candidate List of the U.S. Environmental Protection Agency because their transmission routes are unknown, spore identification, removal, or inactivation in drinking water is technologically challenging (Nwachcuku and Gerba, 2004), and human

infections are difficult to treat (Weiss, 2001). Considerable evidence gathered to date indicates involvement of water in the epidemiology of human microsporidiosis (Slodkowicz-Kowalska *et al*., 2006); however, this epidemiological link has not been conclusively substantiated. Identification of microsporidian spores of species known to infect humans (i.e., *Encephalitozoon intestinalis*, *E. hellem*, *E. cuniculi*, and *Enterocytozoon bieneusi*) represents a challenge because microsporidia can infect a variety of non-human hosts, and spore morphology is insufficient for species identification (Weiss, 2001).

Human microsporidiosis is a serious disease, which currently occurs more frequently than in the past in immunocompetent and immunosuppressed people with the most common species identified as *E. intestinalis*, *E. cuniculi*, *E. hellem*, and *E. bieneusi* (Weiss, 2001). Besides the frequent intestinal infections, extraintestinal infections (e.g., urinary, respiratory, disseminated systemic infections, sinusitis, otitis, keratoconjunctivitis) are common (Kotler and Orenstein, 1999; Weiss, 2001). Despite the advances in molecular technology, most epidemiological aspects of human microsporidiosis, particularly transmission cycles, remain unresolved (Weiss, 2001). Zoonotic (i.e., understood as a result of direct-contact exposure) and foodborne and waterborne transmissions are generally postulated to be predominant (Deplazes *et al*., 1996; Breitenmoser *et al*., 1999; Rinder *et al*., 2000; Weiss, 2001; Graczyk *et al*., 2002; Slodkowicz-Kowalska *et al*., 2006).

7. DETECTION METHODS FOR HUMAN WATERBORNE PROTOZOAN PARASITES

7.1. The American Society for Testing and Materials (ASTM) Method

The ASTM method was first described as a protocol for the detection of *Giardia* spp. cysts, but it was later adapted for use with both *Giardia* spp. cysts and *Cryptosporidium* oocysts (Graczyk *et al*., 1997b, c). The method involves three stages: sampling, concentration and purification, and identification and enumeration. The sampling

utilizes polypropylene yarn-wound cartridge filters with a 1.0 μm nominal pore size and sample volumes range from 100 to 400 l (Graczyk *et al.*, 1997b, c). Further processing involves cutting the filter fibers, removing the fibers from the cartridge core, and eluting the entrapped particles in 3–4 L of eluting solution. The fluid is concentrated by centrifugation, the pellet resuspended in an equal volume of 10% formalin (Graczyk *et al.*, 1997b, c), and the cysts and oocysts separated from debris by centrifugation, and detected by immunofluorescent antibody (IFA).

7.2. The Alternate Method (AM)

The AM utilizes a 40 l water sample passed through a 2.0 μm pore polycarbonate membrane filter of 293 mm diameter. After the sample is passed under vacuum, the filter is rinsed with a 0.01% Tween 80 solution and scraped with a small rubber squeegee to remove the cysts and oocysts. The washing solution containing the cysts and oocysts is centrifuged and decanted to 10% of its original volume (Graczyk *et al.*, 1997b, c). The pellet is resuspended and processed by Percoll gradients; *Giardia* spp. cysts and *Cryptosporidium* oocysts are then detected by IFA.

7.3. Information Collection Rule (ICR) Method

Sampling for this method involves concentrating large volume samples using nominal 1 μm pore size cartridge filters. Laboratory processing includes eluting the particulates from the filter and concentrating the eluant suspension by centrifugation. A Percoll–sucrose flotation procedure is used to separate the biological and non-biological particulates and a portion of the floated material is stained with IFA to enhance the detection of *Giardia* and *Cryptosporidium* under fluorescent microscopy. Numerous methodological shortcomings of this procedure have been identified, including the use of a nominal 1 μm filter; losses associated with the elution, concentration, and purification steps; the subjective nature of microscopic examination; and the lack of viability and infectivity data it yields (Rose *et al.*, 1997).

7.4. The Cellulose Acetate Membrane (CAM)-Filter Dissolution Method

This method is unique in that the filter washing steps in the AM and ASTM method are replaced by the dissolution of the entire membrane filter (Graczyk *et al.*, 1997b, c). This prevents any losses during the washing steps (Graczyk *et al.*, 1997b, c). The method is optimized for the detection of *Giardia* cysts and *Cryptosporidium* oocysts in drinking water. The sampling step of this method begins with the filtration of up to 300 l sample through a 1.2 μm pore cellulose acetate membrane filter (Graczyk *et al.*, 1997b, c). The filter is dissolved in acetone and centrifuged. The pellet is subsequently washed with 95% ethanol, 75% ethanol, and water (Graczyk *et al.*, 1997b, c). The rest of the protocol follows the ASTM method (Graczyk *et al.*, 1997b, c). The use of this method has shown recovery rates as high as 70.5% and 77.8% for *Cryptosporidium* oocysts, which is a vast improvement over the previous methods (Graczyk *et al.*, 1997b, c). In addition, *C. parvum* oocysts retain their infectivity after isolation with this method, which allows viability assays to be completed on the recovered oocysts (Graczyk *et al.*, 1997b, c). This is important for the monitoring of *Cryptosporidium* in finished drinking water, where oocysts may still be present, but in an inactivated, non-viable form that poses no public health risk (Graczyk *et al.*, 1997b, c).

7.5. Method 1622/1623

Method 1622/1623 for the detection and enumeration of *Cryptosporidium* and *Giardia* uses filtration, immunomagnetic separation (IMS), and IFA microscopy (Arnone and Walling, 2006). Organisms may be confirmed using 4′,6-diamidoino-2-phenylindole (DAPI) vital dye staining and differential interference contrast (DIC) microscopy. Method 1622/1623 sampling involves collecting a 10 L volume of untreated water in a carboy in the field and shipment of the sample to a qualified laboratory for concentration and analysis (Arnone and Walling, 2006). It should also be noted that new techniques are still needed to answer questions researchers have about *Cryptosporidium* in water. For example, even Method 1622/1623 will not identify the

species of *Cryptosporidium* or the host origin, nor do they determine viability or infectivity of the oocysts (Arnone and Walling, 2006).

8. WATER TREATMENT

Giardia spp. cysts and *Cryptosporidium* oocysts have been detected in finished drinking water indicating that these biological contaminants of source waters can pass through water treatment processes (Rose *et al.*, 1997). Addressing the public health risks of waterborne giardiasis and cryptosporidiosis, the U.S. Environmental Protection Agency (EPA) instituted the Surface Water Treatment Rule (SWTR) and the Information Collection Rule (Nwachcuku and Gerba, 2004). These rules require filtration and disinfection of all surface water supplies with a goal to reduce the annual risk of acquiring a waterborne infections to less than 10^{-4} (under the assumption that the average person consumes 2 L of water per day) (Rose *et al.*, 1997). Calculation of the acceptable concentration of *Cryptosporidium* oocysts in drinking water that would not exceed the annual risk level showed a maximum concentration of 3.27×10^{-3} oocysts/100 L (Rose *et al.*, 1997).

Extensive monitoring of drinking water from 66 surface water treatment facilities in 14 states of the USA and one Canadian province showed that of the 83 samples, 17% contained *Giardia* spp. cysts and 27% contained *Cryptosporidium* oocysts (Rose *et al.*, 1997). All these water treatment plants employed filtration systems in addition to disinfection. These findings suggest that current water treatment practices may not reduce the load of *Giardia* spp. cysts and *Cryptosporidium* oocysts enough to achieve the 10^{-4} annual risk level (Rose *et al.*, 1997). The different types of filtration produced different levels of cyst and oocyst removal. It was demonstrated that rapid sand filters and granular activated carbon filters were more likely to have effluent samples positive for cysts and oocysts than dual media or mixed media filters (Rose *et al.*, 1997). Other studies have demonstrated that slow sand filtration may be more effective in removing *Giardia* cysts. The U.S. EPA attributes a 3 $\log_{10}$ reduction in *Giardia* cysts to conventional filtration methods. However, even if a 3 $\log_{10}$ cyst/oocyst removal is achieved, the levels of cysts and oocysts in the

raw water supply may still overcome the filtration if their concentrations are high (Rose *et al*., 1997). Common disinfectants used in water treatment are not entirely effective at inactivating *Giardia* spp. cysts and *Cryptosporidium* oocysts. Numerous studies have shown, however, that ozone may prove to be a much more effective disinfectant (Rose *et al*., 1997). Although ozone is not widely used due to the expense and expertise needed to properly set up and run such a disinfection plan, it should be considered as a viable and perhaps necessary addition to current water treatment plants.

9. ASSOCIATIONS OF HUMAN WATERBORNE PARASITES WITH MOLLUSCAN SHELLFISH

Molluscan shellfish are suspension- or sediment-feeding organisms, which filter unicellular algae, bacteria, other microorganisms, and detrital particles of approximately 1–30 μm size range (McMahon, 1991; Kennedy *et al*., 1996). Bivalves have an important role in aquatic habitats; by filtering suspended particles they clarify the water and generally improve water quality (McMahon, 1991). The diameter of transmissive stages of *Cryptosporidium*, *Cyclospora*, and *Toxoplasma* does not exceed 6 and 10 μm, respectively, and *Giardia* cysts are oval and no longer than 15 μm (Wolfe, 1992; Ortega *et al*., 1993; Graczyk *et al*., 1997a; Lindsay *et al*., 2001). Microsporidian spores range from 1.5 to 4 μm (Graczyk *et al*., 2004). Thus, cystic stages of these parasites fall within the range of particles filtered by bivalve mollusks. Multiple in vitro and in vivo experimental studies demonstrated that aforementioned parasites can be efficiently recovered from water, then retained and concentrated in shellfish (Munoz, 1999; Graczyk, 2003; Graczyk *et al*., 2005).

Historically, *C. parvum* oocysts of waterborne origin were first identified in the tissue of blue mussels in Ireland (Chalmers *et al*., 1997), initiating worldwide investigation of this pathogen in molluscan shellfish (Graczyk, 2003; Graczyk *et al*., 2005). Since then, multiple studies demonstrated that these filter-feeding organisms can harbor environmentally derived protozoan parasites as a result of concentrating the recovered particles (Graczyk, 2003).

A recent and interesting epidemiological discovery is the identification, for the first time, of human-infectious microsporidia spores, i.e., *E. intestinalis* and *E. bieneusi* in molluscan shellfish, zebra mussels (*Dreissena polymorpha*) (Graczyk *et al.*, 2004). Microsporidia infects a variety of vertebrate and invertebrate hosts, and approximately 14 species have been reported to infect people (Weiss, 2001). Of these *E. intestinalis* and *E. bieneusi* have been reported to be zoonotic and to infect domestic animals and livestock (Graczyk *et al.*, 2002; Slodkowicz-Kowalska *et al.*, 2006). Although the actual transmission route of this specific spore species is not known, it is quite possible that infectious spores of human or animal origin passed to the aquatic environments via feces or urine (Bryan and Schwartz, 1999). Spores of microsporidia have been detected in a variety of surface waters (Avery and Undeen, 1987), and water as a source of human infections has been concluded from epidemiological data (Cotte *et al.*, 1999). Spores of *E. intestinalis* and *E. bieneusi* have been detected previously in surface waters (Sparfel *et al.*, 1997).

Cryptosporidium oocysts have also been identified in feral bivalves, supporting the concept that estuarine shellfish can be used in the sanitary assessment of water quality as biological indicators for contamination of water and sediment (Graczyk *et al.*, 2003). Zebra mussels and *Corbicula* clams very efficiently concentrate *C. parvum* and *G. lamblia* in relation to low ambient concentrations (Graczyk *et al.*, 2003). In addition, zebra mussels are also able to recover spores of human-infective species of microsporidia such as *E. intestinalis* and *E. bieneusi* (Graczyk *et al.*, 2004). Bivalves such as zebra mussels or *Corbicula* clams are convenient for such purposes because they form dense populations and clusters that facilitate the collection of large samples, do not have economic value, have a relatively small size, and are easily collected throughout the year (McMahon, 1991; Graczyk *et al.*, 2004).

9.1. Quantitative Estimation of Removal of Human Waterborne Parasites by Molluscan Shellfish

Zebra mussels collected from the St. Lawrence River, Canada, near a wastewater discharge site contained on average approximately 440

C. parvum oocysts/mussel (Graczyk *et al*., 2001b). Knowing the *C. parvum* retention rate as 4.9×10^2 oocysts/mussel/24 h (Frischer *et al*., 1999) and *D. polymorpha* densities of approximately 30 000 specimens/m^2 for adult (>1-year-old) mussels (McMahon, 1991), it has been calculated that during 24 h approximately 1.3×10^7 waterborne *C. parvum* oocysts can be removed by each square meter of mussel bed in the St. Lawrence River (Graczyk *et al*., 2001b).

The concentration of *C. parvum* observed in zebra mussels from the Shannon River, Ireland (Graczyk *et al*., 2004), was much lower than that reported from the St. Lawrence River (Graczyk *et al*., 2001b). However, in the St. Lawrence River, mussels originated from sites impacted by wastewater discharge, and in the Shannon River, no apparent sources of water contamination have been identified near any of the sites. Considering the natural densities of zebra mussels (McMahon, 1991), and the fact that on average approximately eight parasites/mussel have been identified in the Shannon River study (Graczyk *et al*., 2004), at least 2.4×10^5 pathogens/24 h can be potentially removed per each square meter of zebra mussel bed in the Shannon River.

9.2. A Public Health Threat from Shellfish Contaminated with *Cryptosporidium*

Prior to 1992, the association between contamination derived from animal fecal wastes and the occurrence of shellfish-vectored illnesses was inconclusive (Stelma and McCabe, 1992). In 1994, enterohemorrhagic *Escherichia coli* 0157 became a major concern (Rippey, 1994). This bacterium has not been associated with shellfish; however, its frequent occurrence in cattle indicated potential public health problems with shellfish harvested from waters affected by runoff from cattle farms (Rippey, 1994).

Beginning in 1998, multiple studies worldwide indicated that molluscan shellfish intended for human consumption can be contaminated with *Cryptosporidium*. So far there has been no reported outbreak (or case) of foodborne cryptosporidiosis linked to

consumption of raw oysters in the US. However, (A) over 40% of all foodborne infections linked to oyster consumption are in the category of an unknown etiologic agent (Anonymous, 1996); (B) 20% of the general US population are vulnerable to *C. parvum* infection (Gerba *et al.*, 1996); (C) epidemiology of enteric infections, i.e., cryptosporidiosis, indicates an association with consumption of raw shellfish (Graczyk and Schwab, 2000); and (D) it is believed that in the United States and Canada the true incidence of shellfish-vectored gastroenteritis is underestimated as much as 20-fold (Hauschild and Bryan, 1980). Since there is no mandatory federal requirement for reporting of gastroenteritis of an unspecified nature, physicians and state health departments are not forwarding case reports to federal authorities (Rippey, 1994; Wallace *et al.*, 1999). In intensive seafood production regions such as northwest Gallicia, Spain, where molluscan shellfish production is the most important industry, cases of self-limiting diarrhea associated with consumption of raw oysters and clams are often reported (Freire-Santos *et al.*, 2000, 2001).

9.2.1. Why are the Shellfish-Caused Illness not Declining?

There are several reasons why shellfish-vectored outbreaks and cases of gastroenteritis due to waterborne protozoan parasites are not projected to decline.

A. The fecal coliform count, which is the main standard indicator for waterborne fecal contamination, is not reliable in determining the quality of water at shellfish harvesting sites (Rippey, 1994; Anonymous, 1996; Graczyk *et al.*, 2007). The transmissive stages of human protozoan parasites can persist in aquatic environments for greater lengths of time as compared to enteric and indicator bacteria (Richards, 1998; Graczyk and Schwab, 2000). Thus, waters considered to be "safe" based on the fecal coliform standards may, in fact, be contaminated by human enteric parasites (Rippey, 1994; Anonymous, 1996; Wallace *et al.*, 1999; Graczyk and Schwab, 2000; Graczyk *et al.*, 2007).
B. Animal operations such as individual farms or concentrated animal feeding operations (CAFO) located near shores and river

banks can generate enormous surface runoff particularly under adverse weather conditions and can cause water pollution (Todd *et al*., 1992; Freire-Santos *et al*., 2000; Gomez-Bautista *et al*., 2000; Gomez-Couso *et al*., 2003, 2004).

C. Deficiencies at the sewage treatment plants such as volume limitations related to designed capacity of a plant under adverse weather conditions, i.e., heavy rainfall, allow the discharge of large amounts of unprocessed waste waters. In addition, the periodic breakdown in particle removal, or inadequate disinfection can deliver human enterapathogens into surface waters including shellfish harvested waters (Rippey, 1994).

D. Transmissive stages of human enteric parasites are resistant to environmental degradation (i.e., heat, sunlight, temperature fluctuations, etc.) and may even remain infectious after exposure to chemical water treatment processes such as chlorination (Graczyk and Schwab, 2000). These pathogens can still be infectious even after the oyster meat has been processed (Tamburini and Pozio, 1999; Graczyk and Schwab, 2000) and are also very poorly, i.e., slowly, depurated (removed) from molluscan shellfish tissue (Graczyk and Schwab, 2000; Freire-Santos *et al*., 2002).

E. Increased fecal pollution determined on the fecal coliform counts has decreased the total area of coastal habitats approved for harvesting of molluscan shellfish, particularly oysters, for human consumption (Rippey, 1994). Thus, large and very productive areas have been closed, resulting in illegal harvesting of oysters from unapproved or closed, but profitable waters (Rippey, 1994). Such criminal activity unavoidably affects public health when contaminated shellfish enter the market (Rippey, 1994).

F. Improper postharvest handling and transportation of molluscan shellfish, i.e., temperature abuse, affect oysters directed for consumption in a raw form (Rippey, 1994). Holding of oysters at temperatures greater than 4 °C in transit or in the market place can contribute to multiplication of bacterial enteropathogens (Rippey, 1994).

G. Many shellfish-related outbreaks have more than one contributing factor (Altekruse *et al*., 1999; Wallace *et al*., 1999). For example, contaminated ingredients added to raw or lightly

cooked mollusks have been also reported as contributing factors for foodborne infections factor (Wallace *et al.*, 1999).

H. Development of new molecular techniques that can be applied to a wide variety of food items have dramatically increased the sensitivity and specificity of detection of human enteric parasites in the tissue of molluscan shellfish (Graczyk and Schwab, 2000; Graczyk *et al.*, 2007).

10. CONCLUSIONS

A. The role of certain species of fasciolids, echinostomids, and philophthalmids as waterborne trematode parasites is considered in this review. It is apparent from the review that species of *Fasciola*, *Philophthalmus*, and *Echinohasmus* must be considered as waterborne trematode parasites of humans. With the possible exception of philophthalmids, a prime mode of transmission of some fasciolid and echinochasmid species is by human ingestion of encysted metacercariae (cysts) in contaminated water. Echinochasmids are also transmitted to humans by the ingestion of free cercariae in tainted water. For species of *Philophthalmus*, most transmission occurs in water by direct infection of the human eye by cercariae or metacercariae. Transmission of fasciolids and echinostomids by the ingestion of plant and animal material tainted with metacercarial cysts (foodborne trematodiasis) is considered reasonably common knowledge in the biomedical community. This review provides the scientific community with new information on the possibility of certain trematodes transmitted to humans as waterborne infections.

B. The emergence and explosive spread of HIV around the world, together with the consequences of AIDS, greatly enhance the life-threatening manifestation of cryptosporidiosis and other water-mediated diseases as a global health problem (Graczyk *et al.*, 1997a). Prevention of waterborne infections by blocking transmission via water to which exposure cannot be avoided, constitutes a challenge that humans have to face in the developing and developed world (Graczyk *et al.*, 1997a). The importance

of ensuring the high quality of the water supply is greater now considering the growing population of immunocompromised and immunosuppressed people worldwide, and the fact that waterborne infections are predominantly related to poverty and low sanitation (Pozio, 2003).

C. *Giardia* spp. cysts and *Cryptosporidium* oocysts have been detected in finished drinking water in developed countries, indicating that these biological contaminants can pass through water treatment processes. The operational practices necessary to reduce the risk of waterborne infections to acceptable levels are not well understood. Also, current standards are obviously not stringent enough, since some waterborne outbreaks occurred while the treatment plants were operating within regulations. A systemic and coordinated national surveillance system for comparison purposes, trend identification, and policy development is needed so that future waterborne outbreaks can be avoided (Schuster *et al*., 2005).

D. Analysis of drinking water-related outbreaks of *Cryptosporidium* when coupled with genotyping data provides important epidemiologic information on the public health significance of zoonotic versus anthroponotic transmission of this pathogen (Hunter and Thompson, 2005). Such studies supported the hypothesis that *C. hominis* is spread among humans and a major reservoir for *C. parvum* are domestic animals, predominantly cattle, and that the contact with infected cattle and indirect transmission through drinking water represents the major transmission routes for *C. parvum* (Hunter and Thompson, 2005). The situation is less clear for *Giardia*; however, the evidence gathered to date does not support the zoonotic transmission as a major risk for human infection (Hunter and Thompson, 2005). Although neither all waterborne outbreaks of *Cryptosporidium* and *Giardia* are recognized nor reported, the national surveillance of these outbreaks has helped identify important sources of contamination of surface, recreational, and drinking waters (Craun *et al*., 2005).

E. Human waterborne protozoan parasites, particularly *Cryptosporidium*, represent a serious public health threat due to

waterborne transmission (Graczyk *et al.*, 1997a; Craun *et al.*, 2005; Schuster *et al.*, 2005). However, the extent to which water consumers are at risk cannot be precisely determined until more sensitive and reliable methods for the recovery and detection of these pathogens are developed (Graczyk *et al.*, 2007). The history of waterborne outbreaks of *Giardia* spp. and *C. parvum* infections related to drinking water does demonstrate that the risk is considerably high. By increasing knowledge in key areas of *Cryptosporidium* research such as etiology, transmission, and host interactions, the number of waterborne outbreaks and cases is expected to be reduced (Sunnotel *et al.*, 2006).

F. Economically important shellfish, such as oysters, which are harvested commercially and preferentially consumed raw can be of public health importance if contaminated with human waterborne parasites (Graczyk, 2003; Graczyk *et al.*, 2005, 2007). Shellfish which do not have an apparent economic value may serve as indicators in monitoring aquatic environments for pollution with human waterborne pathogens such as *Cryptosporidium*, *Giardia*, *Cyclospora*, *Toxoplasma*, and human-infective microsporidia (Lindsay *et al.*, 2001; Arkush *et al.*, 2003; Graczyk *et al.*, 2004, 2005). Foodborne illnesses following consumption of molluscan shellfish continue to occur despite that (1) testing of waters for fecal coliforms from which oysters are harvested for human consumption demonstrate that the water quality met the criteria of the National Shellfish Sanitation Program (NSSP) (Rippey, 1994; Anonymous, 1996). (2) Oysters harvested from the NSSP-approved waters are considered as ‘‘safe’’ with regards to fecal pollution (Rippey, 1994; Anonymous, 1996). (3) Sanitation procedures at oyster-harvesting facilities met standards set by the state authorities (Rippey, 1994; Anonymous, 1996). (4) In most instances, neither confirmed evidences of improper handling or processing of outbreak-implicated oysters nor the environmental source(s) of pollution were detected (Rippey, 1994; Anonymous, 1996). These facts indicate that the monitoring of water for fecal coliform at molluscan shellfish-harvesting sites may not be sufficient indicating the presence of human waterborne parasites such as *Cryptosporidium*, *Cyclospora*, *Giardia*, *Toxoplasma*, and

human-infectious microsporidia (Rippey, 1994; Wallace *et al.*, 1999; Lindsay *et al.*, 2001; Arkhus *et al.*, 2003; Graczyk *et al.*, 2005, 2007).

G. Estuarine molluscan shellfish, both commercial and feral, can be used for the sanitary assessment of water quality as biological indicators for water and sediment contamination with *Cryptosporidium*, *Giardia*, *Toxoplasma*, and *Cyclospora* (Graczyk *et al.*, 1999a; Lindsay *et al.*, 2001; Xiao *et al.*, 2001; Arkush *et al.*, 2003). Reducing the number of foodborne diseases due to bivalve mollusks will require the coordinated efforts of different agencies involved in water quality assessment, shellfish harvesting and processing, disease surveillance, and consumer education (Rippey, 1994; Anonymous, 1996; Wallace *et al.*, 1999). It may be useful to reduce or eliminate economic incentives for illegal harvesting of shellfish from unapproved or prohibited waters that results in contaminated shellfish reaching the market place (Rippey, 1994). Continued surveillance for outbreaks and cases of gastroenteritis associated with consumption of raw shellfish are needed to assess the efficacy of the NSSP in preventing human illnesses (Rippey, 1994; Wallace *et al.*, 1999; Graczyk and Schwab, 2000). Public health officials should consider consumption of raw shellfish as a possible source of infection during the evaluation of a gastroenteritis outbreak (Rippey, 1994; Wallace *et al.*, 1999; Graczyk and Schwab, 2000).

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The Biology of Gyrodactylid Monogeneans: The "Russian-Doll Killers"

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ABSTRACT

This article reviews the history of gyrodactylid research focussing on the unique anatomy, behaviour, ecology and evolution of the viviparous forms while identifying gaps in our knowledge and directions for future research. We provide the first summary of research on the oviparous

gyrodactylids from South American catfish, and highlight the plesiomorphic characters shared by gyrodactylids and other primitive monogeneans. Of these, the most important are the crawling, unciliated larva and the spike sensilla of the cephalic lobes. These characters allow gyrodactylids to transfer between hosts at any stage of the life cycle, without a specific transmission stage. We emphasise the importance of progenesis in shaping the evolution of the viviparous genera and discuss the relative extent of progenesis in the different genera. The validity of the familial classification is discussed and we conclude that the most significant division within the family is between the oviparous and the viviparous genera. The older divisions into Isancistrinae and Polyclithrinae should be allowed to lapse. We discuss approaches to the taxonomy of gyrodactylids, and we emphasise the importance of adequate morphological and molecular data in new descriptions. Host specificity patterns in gyrodactylids are discussed extensively and we note the importance of host shifts, revealed by molecular data, in the evolution of gyrodactylids. To date, the most closely related gyrodactylids have not been found on closely related hosts, demonstrating the importance of host shifts in their evolution. The most closely related species pair is that of *G. salaris* and *G. thymalli*, and we provide an account of the patterns of evolution taking place in different mitochondrial clades of this species complex. The host specificity of these clades is reviewed, demonstrating that, although each clade has its preferred host, there is a range of specificity to different salmonids, providing opportunities for complex patterns of survival and interbreeding in Scandinavia. At the same time, we identify trends in systematics and phylogeny relevant to the *G. salaris* epidemics on Atlantic salmon in Norway, which can be applied more generally to parasite epidemiology and evolution. Although much of gyrodactylid research in the last 30 years has been directed towards salmonid parasites, there is great potential in using other experimental systems, such as the gyrodactylids of poeciliids and sticklebacks. We also highlight the role of glacial lakes and modified river systems during the ice ages in gyrodactylid speciation, and suggest that salmon infecting clades of *G. salaris* first arose from *G. thymalli* in such lakes, but failed to spread fully across Scandinavia before further dispersal was ended by rising sea levels. This dispersal has been continued by human activity, leading to the appearance of *G. salaris* as a pathogen in Norway. We

review the history and current status of the epidemic, and current strategies for elimination of the parasite from Norway. Finally, we consider opportunities for further spread of the parasite within and beyond Europe.

1. INTRODUCTION

Monogeneans of the genus *Gyrodactylus* have been known for almost 180 years for their retention of fully grown daughters *in utero* until they themselves contain developing embryos (Figure 1). This ''Russian-doll'' reproduction, dubbed hyperviviparity by Cohen (1977), is extremely rare in the Animal Kingdom and was the focus for intensive study by late 19th century microscopists. We recently reviewed gyrodactylid reproduction (Cable and Harris, 2002) and specificity (Bakke *et al.*, 2002) but there are now compelling reasons to reevaluate their ecology. In the first place, although the viviparous gyrodactylids have attracted most attention, we are becoming increasingly aware of the diversity of oviparous gyrodactylids from South American catfish (Kritsky *et al.*, 2007) and a review of their relationship to the viviparous genera is timely. Furthermore, the viviparous gyrodactylids are increasingly reported as pathogens of

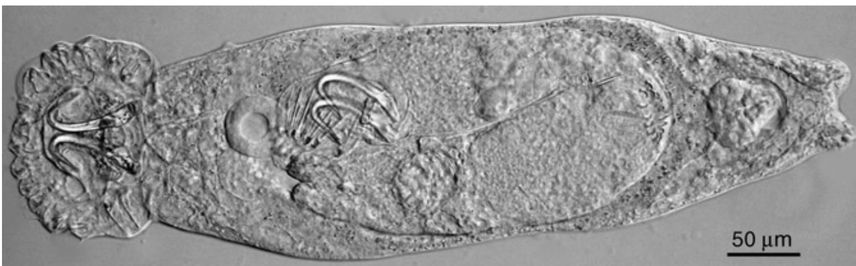


Figure 1 Light micrograph (interference contrast) of a living gravid *Gyrodactylus salaris* with two daughters *in utero* like ''a Russian-doll''. The lack of penis and spermatozoa in the seminal receptacle together with the near term F1 embryo *in utero* indicates that the parasite has not yet given birth for the first time. (G. Robertsen, unpublished.) See coloured version on the front cover.

farmed fish (Lile *et al.*, 1993; Woo, 1995; Mo and Lile, 1998; Jalali *et al.*, 2005; You *et al.*, 2006) and the notoriety of the *Gyrodactylus salaris* epidemic has stimulated research to such an extent that gyrodactylids are now the best studied of all monogeneans. Much research in Europe has focussed on three economically relevant species: *G. salaris* on Atlantic salmon (*Salmo salar*) and its non-pathogenic sibling *G. thymalli* on grayling (*Thymallus thymallus*), and *G. derjavini* on brown trout (*Salmo trutta*). Recent studies suggest that in the former case we are observing the evolution of a fish pathogen complex in real time. However, several other gyrodactylids have also been studied extensively, including those infecting guppies, gobies and sticklebacks, and represent ideal model systems for studying evolutionary and ecological processes in this group, and in parasitic organisms in general.

1.1. Early History

Gyrodactylus was first described from bream (*Abramis brama*) by von Nordmann (1832). Although he observed the embryo *in utero*, he failed to appreciate its significance, and the prominent embryonic hamuli (Figure 1) were interpreted as ‘‘stomach hooks’’. Viviparity was first recognised by von Sieboldt (1849) and the value of gyrodactylids for studies on reproduction was established. Gyrodactylids were particularly useful to early microscopists as flatworms without an impervious egg shell. Flatworms attracted much attention at the end of the 19th century because of their apparently basal position close to Haeckel's (1874) hypothetical gastraea ancestor of the Metazoa and their potential for regeneration. The enclosure of several embryos inside each other also represented an attractive model for the study of germ cell lineages, a paradigm which was just becoming established at this time. *Gyrodactylus* was therefore a popular choice for early studies on chromosome and embryonic cell behaviour (e.g. Wagener, 1860; Kathariner, 1893, 1899, 1904; Gille, 1914), remaining important until shortly before the First World War.

1.2. Taxonomic and Faunistic Research

Gyrodactylids soon became well known as pathogens in fish farms (e.g. Atkins, 1901; Embody, 1924; Guberlet *et al.*, 1927) and wild fish populations (e.g. Williams, 1964). Fish health textbooks describe "fluke" infections, often without differentiating the distinct epidemiology of viviparous gyrodactylids from that of oviparous dactylogyrids and ancyrocephalids. The gyrodactylids were known to be species-rich, and by 1970 some 200 species had been described, mostly from North America and Eurasia (Malmberg, 1970). There was considerable scepticism over the validity of many taxa, and gyrodactylids were largely ignored by evolutionary ecologists because of their complex taxonomy. They were perceived as difficult to work with, which undoubtedly has held back research into their ecology, and by the 1980s only a handful of researchers worldwide retained an interest in the group.

1.3. The *Gyrodactylus salaris* Epidemic

During the mid-1970s, Norwegian researchers began to report a highly pathogenic epidemic disease amongst both farmed and wild salmon populations, which they ascribed to *Gyrodactylus salaris* (see Tanum, 1983). The first international report of the epidemic (Johnsen, 1978) followed internal reports in Norway (Bergsjö and Vassvik, 1977). Research was at first slow, but over the next 20 years the disease was observed in many rivers within Norway (Heggberget and Johnsen, 1982; Johnsen and Jensen, 1986, 1991; Mo, 1994). The generally accepted conclusion is that the parasite was introduced from the Baltic region into East Atlantic stocks of salmon which lacked endogenous resistance to the parasite (but see Halvorsen and Hartvigsen, 1989; and Section 10 below). Subsequently, *G. salaris* has spread to cause epidemic disease in the Russian River Keret (Kola Peninsula) and high infection levels are also recorded in the River Högvadsån on the south west Coast of Sweden. Much effort is devoted to prevent the pathogen spreading to Scotland, Ireland and

other areas with large natural populations of Atlantic salmon. *G. salaris* now represents the most significant threat to the continued existence of large wild populations of East Atlantic stocks of Atlantic salmon, and endangers the reintroduction of salmon into rivers of the North Sea basin. It represents a major drain on resources for the EU and the potential for translocation with salmonid stocks to other parts of the world cannot be ignored. This pathogen has been a research driver which has led to huge improvements in our understanding of gyrodactylid biology. In addition, due to their species richness, ubiquity, economic importance in aquaculture and potential conservation threat, they are the most intensively studied group of monogeneans. From a research backwater, gyrodactylid research is now a growth area within parasitology and wildlife disease ecology.

1.4. Genus, Species Flock or Host Races?

One of the most interesting questions regarding *Gyrodactylus* today concerns the species concept; what is the relationship between the operational taxonomic units (OTU's) that we currently regard as valid species? *Gyrodactylus* is hugely species-rich but lacks obvious morphological diversity. Over 400 species have been described (Harris *et al.*, 2004) but from only ~200 predominantly teleost hosts (Bakke *et al.*, 2002). Extrapolation to the ~24 000 teleost species would suggest around 20 000 gyrodactylid species. The existence of such a mega-diverse genus has wider significance. There is no evidence that the rate of discovery of new gyrodactylids is slowing for any reason other than lack of research, and where close attention has been paid to specific host groups, species descriptions have multiplied (on poeciliid fishes, see Harris and Cable, 2000; Cable *et al.*, 2005; on gobiids see Longshaw *et al.*, 2003; Huyse and Malmberg, 2004; Huyse *et al.*, 2004a). The rate of discovery of new gyrodactylid taxa is set to risen again, as the availability of molecular markers reveals previously undetected species and strains (Lautraite *et al.*, 1999; Ziętara and Lumme, 2002; Hansen *et al.*, 2003).

1.5. *Gyrodactylus*: The Drosophilids of the Parasitic World

The discovery of *G. salaris* as a devastating pathogen marked a turning point in gyrodactylid research and by stimulating interest in molecular markers, has reinvigorated gyrodactylid taxonomy and evolutionary biology. Molecular probes (Cunningham *et al*., 1995a, b; Cunningham, 1997) were initially developed to provide objective and reliable means for identification of *Gyrodactylus* parasitising salmonid fish by non-specialists, and from this the modern field has developed. One of the most exciting observations has been the ability to record evolution in action by an actively adapting species, *G. salaris.* The boundaries between *G. salaris* on Atlantic salmon and on Arctic charr (Olstad *et al*., 2005, 2007; Robertsen *et al*., 2007a), and *G. thymalli* on grayling (Hansen *et al*., 2003; Meinilä *et al*., 2004) are not distinct, suggesting that we are watching the evolution of a gyrodactylid which has switched hosts and is developing new patterns of host specificity. This has stimulated interest in other gyrodactylids, for example those infecting guppies (Cable and van Oosterhout, 2007; King and Cable, 2007; van Oosterhout *et al.*, 2003, in press a, b) and gobies (Huyse and Volckaert, 2002; Huyse *et al*., 2003, 2004a, b), and has shown that the patterns of evolution of these organisms are subtle and informative about general evolutionary processes in parasites. It may not be too fanciful to consider the genus *Gyrodactylus* the *Drosophila* of the parasite world. Our strains of *G. turnbulli* have been maintained experimentally for more than 10 years, and *G. salaris* for more than 5 years in the laboratory. With their short generation times and simple culture requirements, the entire trajectory of infection of these ectoparasites can be monitored in real time on a single host. However, the most important resource for evolutionary biologists is the huge diversity of gyrodactylids on bony fishes, representing a wide range of evolutionary interactions. The temporary evasion of host responses by host transfer may increase the abilities of *Gyrodactylus* species to colonise new host species and may partially explain the high rate of diversification demonstrated by viviparous gyrodactylids (Boeger *et al*., 2005). Amongst the 400 recorded species, there are examples where speciation may have occurred in the last few thousand years, through to species which are

several millions of years old. By reviewing gyrodactylid biology here, we aim to increase the profile of these parasites not just among helminthologists, but also for those with a wider interest in evolution.

Part 1. Gyrodactylid autecology

2. MORPHOLOGY

Gyrodactylids are amongst the smallest monogeneans (Figure 2) and some, such as *Isancistrum* (see Llewellyn, 1984), are similar in size to oncomiracidium larvae (~200 μm). The fusiform body has a posterior opisthaptor armed with marginal hooks, hamuli and bars (see Section 2.3). Anteriorly, two conspicuous cephalic processes bearing

Figure 2 Scanning electron micrograph of a heavy *Gyrodactylus salaris* infection on the skin of an Atlantic salmon parr (K. Kvalsvik, unpublished).

adhesive glands and spike sensilla are involved in attachment but are also important in the sensory biology of gyrodactylids (see Bakke *et al.*, 2004a). The transparent body is dominated by the F1 generation embryo curled within the uterus (Figures 1 and 3); in gravid worms, all other body organs are compressed by the sheer volume of the developing embryonic mass.

The internal anatomy of gyrodactylids has been extensively misinterpreted because they lack many structures present in oviparous monogeneans. Egg-laying monogeneans produce large tanned egg capsules (Smyth, 1954) which hatch to a (normally) swimming oncomiracidium. This larva has external, ciliated locomotory cells and usually four eye spots, and on hatching swims to a new host, where it sheds ciliated cells and settles to a sedentary existence, usually spending its entire life on the same host. The female reproductive system of these oviparous monogeneans is separated into a germarium, producing oöcytes and vitellaria, producing vitelline cells which contribute to the egg shell and provide nourishment for the developing embryo. These cells, with sperms, mix within the oötype to form a mature egg, which is then extruded and laid. Several reviews have been dedicated to different aspects of the biology of oviparous monogeneans, but gyrodactylids have barely any characteristics in common with these organisms, and their structure was consequently poorly understood for many years. Harris (1983) was the first to recognise egg laying in *Ooegyrodactylus farlowellae*, which allowed the identification of derived structures from plesiomorphic characters in gyrodactylids.

2.1. The Importance of Progenesis and Viviparity

The term progenesis is often confused with neoteny. Neoteny is the retention of larval characters beyond the normal developmental stage to allow continued exploitation of a particular habitat. Duration of the life cycle is not shortened (for further clarification, see Gould, 1977). Progenesis, on the other hand, is the acceleration of the life cycle to allow an organism to reproduce as a juvenile or larva. The early-maturing precocious larvae of *Polystoma* spp. are excellent examples of

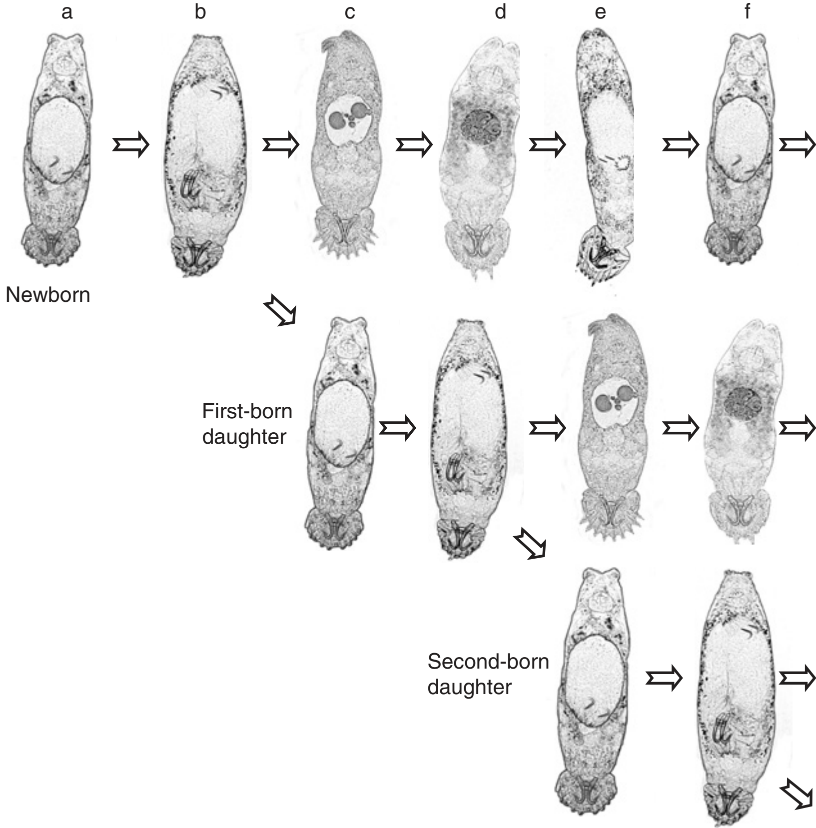


Figure 3 Light micrographs of *Gyrodactylus salaris* showing embryonic development during the life cycle. (a) Following birth (newborn, first born and second born daughters as shown), the hamuli of the next generation are visible towards the posterior axis of the F1 embryo close the Egg Cell Forming Region (ECFR) of the parent. (b) The hamuli of the F2 embryo lie at the anterior pole of the parental uterus, whereas the sclerites of the F1 embryo, at the posterior pole, are fully developed in near term adults. (c) Following birth, the contracted uterus contains two dividing cells which have originated from a single cell that has entered the uterus from the ECFR. (d) F1 embryo is a mass of ~50 cells that fills the uterus. No evidence of embryonic sclerite development. (e) A ring of primordial marginal hooks and rod-like hamuli primordial within the F1 embryo. (f) The marginal hooks have separated so a ring structure is no longer visible and curvative of the hamuli apparent in F1 haptor. The F1 embryo in this post-first birth worm is similar to that of a newborn parasite.

progenesis (although confusingly referred to as neotenics), as they reproduce when only weeks old, whereas "normal adults" (otherwise known as slow-growing worms) reproduce after 3 years. The retention of larval eye spots by the adult *Pseudodiplorchis* (see Cable and Tinsley, 1991) may be a neotenic adaptation allowing a light response when the toad hosts emerge from hibernatory burrows. Paedogenesis is as a general term used to describe reproduction in larvae, but often life-cycle duration is overlooked, so examples (such as salamanders) that have a life cycle of normal length or extended, should actually be considered neotenic. The viviparous gyrodactylids are highly progenetic (Harris, 1983) and the mature *Gyrodactylus* closely resembles larval and young (male) *Ooegyrodactylus.* The first birth of *Gyrodactylus* occurs after days (e.g. Jansen and Bakke, 1991), whereas in *O. farlowellae* there is a generation time of weeks (Harris, 1983). This abbreviation of the life cycle in viviparous gyrodactylids can be attributed to progenesis.

Viviparous gyrodactylids are amongst the most successful progenetic groups. Progenesis is also thought to have been important in the evolution of other minor phyla including rotifers, gnathostomulids and kinorynchs, groups which contain at most hundreds to thousands of species. If we are correct in estimating ~20 000 *Gyrodactylus* species (see above), the gyrodactylids would be one or two orders of magnitude more species-rich than these other groups.

Cable and Harris (2002) described the effects of progenesis on the female reproductive system. In other monogeneans, including the egg-laying gyrodactylids, this system has extensive vitellaria with a complex oötype and glandular apparatus. In the viviparous gyrodactylids however, the female system never fully develops and remains structurally simple, characterised by syncytial structures. Viviparous gyrodactylids are protogynous hermaphrodites, the female system maturing before the male, whereas all other monogeneans, including the oviparous gyrodactylids, are protandrous. This is further evidence of precocious maturation of the simplified female system, which becomes active before the male system has followed its normal trajectory to maturation. However, substantial differences exist in the extent of progenesis in different gyrodactylids. *Macrogyrodactylus* appears least progenetic, undergoing considerable post-embryonic

differentiation, particularly in the putative glandular tissue/subtegumental cells of the posterior body (see Cable *et al*., 1996, 1997, 1998; El-Naggar and Cable, in press). By contrast, *Isancistrum* may represent extreme progenetic abbreviation of the life cycle; the haptor lacks hamuli and bars, and other organ systems appear simplified.

Coincident with progenesis in other organisms, such as bdelloid rotifers, is eutely (a constant number of nuclei, usually established in the juvenile stage), and this is also seen in gyrodactylids. Gyrodactylids such as *G. gasterostei,* contain ~1000 cells, with all non-reproductive cell division being completed before birth. The only major changes which occur post-birth are within the male reproductive system. This may not be the case for all genera, especially for the very large and relatively long-lived *Macrogyrodactylus*. The exact point at which eutely is established must represent the switch from development of the parent to development of the next-generation embryo, and is therefore an important determinant of reproductive rate.

As in other progenetic, eutelic groups, gyrodactylid body size and cell number are insufficient to support the development of extensive internal organ systems. Functions such as internal transport and homeostasis are carried out by syncytial layers; many of the internal boundaries in gyrodactylids are syncytial, allowing transport of material within, but not necessarily across, layers. Syncytia characterise this group and are found throughout the body including the external tegument, the absorptive layer of the intestine, the lining of the Egg Cell Forming Region (ECFR; see Jones *et al*., 1997, 1998; Cable *et al*., 1996, 1997, 1998), and the uterus.

Viviparity is the second major reproductive adaptation of gyrodactylids. Uniquely they give birth to fully grown young which already contain developing embryos *in utero* (Figures 1 and 3). Embryos may originate in one of two ways. The second-born and subsequent daughters develop from mature oöcytes in the same manner as in any other metazoan (although oöcytes may be fertilised by sperm or develop apomictically). However, the first-born daughter always develops asexually in the centre of its parental embryo as a group of cells that become differentiated from their parent. It is impossible therefore to track the first-born daughter back to a single cell. In this respect, viviparity in gyrodactylids, at least the development of the

first-born daughter is another expression of the large potential for asexual reproduction in platyhelminths. A plausible hypothesis for the origin of viviparity is that an oviparous gyrodactylid acquired an adaptation whereby an embryo *in ovo* developed daughter embryos asexually, to be born almost directly after the larva had hatched. Such an adaptation would be favoured because of the reduction in generation time achieved, and eventually loss of egg shell would lead to the assumption of an entirely viviparous life cycle. All other aspects of morphology must be interpreted in the context of the extreme progenesis and viviparity shown by gyrodactylids.

2.2. Tegument

The characteristic syncytial tegument of monogeneans has been described for *Gyrodactylus gasterostei* (see Lyons, 1970; Cable *et al.*, 1996), *Gyrodactylus eucaliae* (see Kritsky, 1971; Kritsky and Kruidenier, 1976) and for *G. turnbulli* and *G. bullatarudis* (see Cable *et al.*, 1996). Embryonic gyrodactylids have a nucleated epidermis, which in other monogeneans is replaced by an outer anucleated cytoplasmic layer connected to subtegumental cell bodies that usually lie beneath muscle blocks. Surprisingly, no intact cytoplasmic connections linking the outer tegument of mature gyrodactylids to the putative subtegument have been described and there is considerable confusion as to the identity of the subtegumental cells themselves. However, there are a series of lateral cells, which are assumed to be subtegumental cells as they contain secretory vesicles similar to those in the surface layer of the adult tegument (e.g. El-Naggar and Cable, in press). In *G. eucaliae*, a single type of subtegumental cell appears to produce two distinct tegumental vesicles (Kritsky and Kruidenier, 1976) whereas in *Macrogyrodactylus clarii* three different secretory vesicles are each manufactured in a distinct cell type (El-Naggar and Cable, in press). In both gyrodactylids, these putative subtegument cells are restricted to particular regions of the body. The extent of subtegumental variation in other gyrodactylids is unknown, but the basic structure of the tegument appears similar to that of other monogeneans. The outer surface is surrounded by an outer plasma membrane amplified with numerous microvilli, between which

often lies a prominent carbohydrate-rich glycocalyx. The outer cytoplasmic layer is usually devoid of cell organelles, as nuclei, mitochondria, Golgi bodies, and endoplasmic reticulum tends to be restricted to the subtegumental cells. It rests on a basal membrane overlying a basal lamina complex and muscle layers; in gravid worms this tissue is often greatly compressed against the uterus (Cable *et al.*, 1996). The contents of tegumental vesicles are secreted on to the surface of *Gyrodactylus* spp. (Cable *et al.*, 1996; Bakke *et al.*, 2006) and there is presumably quite a high turnover of vesicles as indicated by the manufacture of large numbers of vesicles in the putative subtegumental cells. Even the surface layer of near term F1 embryos contains numerous tegumental vesicles, but their secretion has only been observed after birth.

2.3. Attachment and Musculature

Gyrodactylids primarily attach using the opisthaptor armed with 16 peripheral articulated marginal hooks and usually with a single pair of ventrally orientated hamuli, linked by separate dorsal and ventral bars. As in the acanthocotylids, *Enoplocotyle*, the anoplodiscids, bothitremids and tetraonchoidids, the marginal hooks have articulated blades (sickles), which can move relative to their shafts. The marginal hooks are arranged around the periphery of the haptor within finger-like tegumental papillae (Figure 4). The marginal hooks are capable of considerable mobility, moving freely within each tegumental papilla, in addition to the shafts being capable of extension in and out of the haptor. The papillae have an extensive musculature, allowing each individual marginal hook to work independently of its neighbours, but the roof of the haptor is also well provided with radial fibres which bind the hooks together and allow them to act as a unit. Fibres attaching to the marginal hooks may be strengthened and therefore more visible than normal musculature. The ‘‘sickle filament loop’’ (Figure 5) of Malmberg (1970) is an open-ended loop which fits over the marginal sickle blade and transmits force from effector muscles located deep within the papilla (Shinn *et al.*, 1993), while ‘‘shank ligaments’’ connect the marginal hooks into the deeper haptor. The marginal hooks represent the major attachment organs of

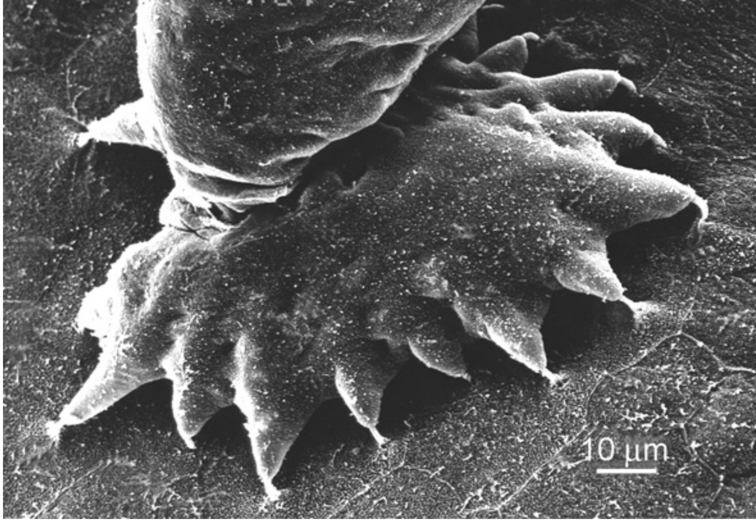


Figure 4 Scanning electron microscopy of the opisthaptor of *Gyrodactylus salaris* attached to the skin of an Atlantic salmon.

gyrodactylids and, with the hamuli and ventral bar, represent the main taxonomic structures. Shinn *et al.* (2003) showed that their gaffing action, taken in concert, firmly attach the gyrodactylid to the fish surface. They are also essential in other behaviours, most notably helping to attach to smooth surfaces, such as glass (personal observation) and the surface water film (Cable *et al.*, 2002a).

The single pair of hamuli lies ventral to the marginal hooks. In skin parasites, the hamuli are fish-hook shaped, connected by the dorsal bar which is fused at its ends to processes on the surface of the hamuli and a ventral bar loosely attached to the hamuli (Figure 6). The anterior tips of the hamulus roots are bound to large fibrous ligaments running anteriorly and eventually join the body wall musculature. Some 30–50 µm anterior of the hamulus roots these ligaments pass through a fibrous yoke which ensures that the force exerted by the body wall musculature acts at an angle to pull the hamulus roots forward and into the mid-line. The dorsal bar antagonises these forces and maintains the spacing between the hamulus shafts (Figure 7). When the body wall musculature relaxes, the hamuli rotate slightly (Shinn *et al.*, 2003) and move relative to the ventral bar. This

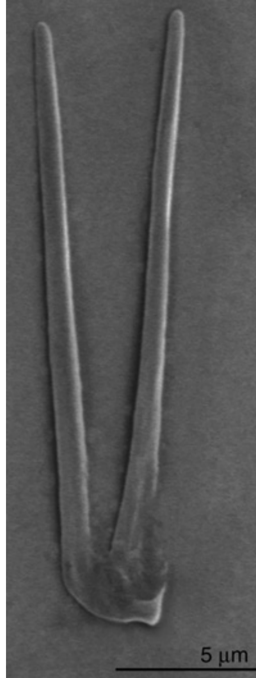


Figure 5 Scanning electron micrograph of the sickle filament loop of the marginal hook of *Gyrodactylus salaris* from Atlantic salmon, often mentioned in species descriptions. In our opinion, this structure, often detached entirely from the sickle during digestion, is of little value in *Gyrodactylus* taxonomy.

sequence of movements forces the hamulus points against the fish skin, exerting pressure to lift the roof of the haptor, while tensioning the marginal hooks. The ventral bar (Figure 8) acts as a pressure pad preventing slippage of the anterior part of the haptor and the hamuli (Shinn *et al.*, 2003). In skin parasitic gyrodactylids (on which Shinn *et al.*, 2003 based this model of attachment), the hamulus points normally do not extend from the tegument (Wagener, 1860; Cone and Odense, 1984) or gaff the fish skin (Figure 7). Instead, pressure is applied by the whole length of the point, forced down onto the fish skin but not penetrating it. This is therefore similar to the mechanism of attachment of skin-parasitic capsalid monogeneans (e.g. Kearn, 1971) in which damage to the host epithelium is minimised and attachment is achieved while the musculature is relaxed.

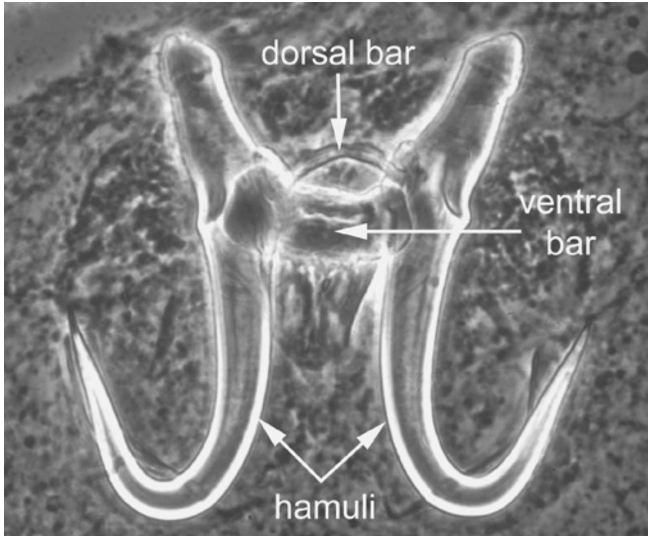


Figure 6 Light micrograph of flat mount preparation of the hamuli and bars of *Gyrodactylus salaris* (Lierelva strain) from Atlantic salmon as seen from the ventral side.

The apparent uniformity of gyrodactylid attachment hides a huge diversity of detail. In gill-parasites, with their characteristic strongly curved hamulus shafts, the hamuli often penetrate the gill tissue to some depth. *Ooegyrodactylus farlowellae*, despite appearing similar to other skin parasitic gyrodactylids, also gaffs skin tissue deeply, and marginal hooks are relatively unimportant in attachment (Harris, 1983). This may be because the large body size requires more robust attachment than the smaller *Gyrodactylus* species or it may be an adaptation to the bony external surface of loricarid catfish. More subtle diversity of attachment also exists. Harris and Cable (2000) noted that *G. poeciliae*, with slender marginal hook sickle points less than 1 μm in length, all fell from the host following fixation, whereas *G. milleri*, with more robust marginal hook sickles, remained attached to the same poeciliid host. Cone and Odense (1984) found that *G. avaloniae*, *G. adspersi*, *G. bullatarudis* (possibly *G. turnbulli*, see Harris, 1986) and *Gyrodactylus* sp. from goldfish (close to *G. gurleyi* according to Cone and Wiles, 1984) all had a superficial attachment mechanism in which the marginal hooks hardly penetrated the

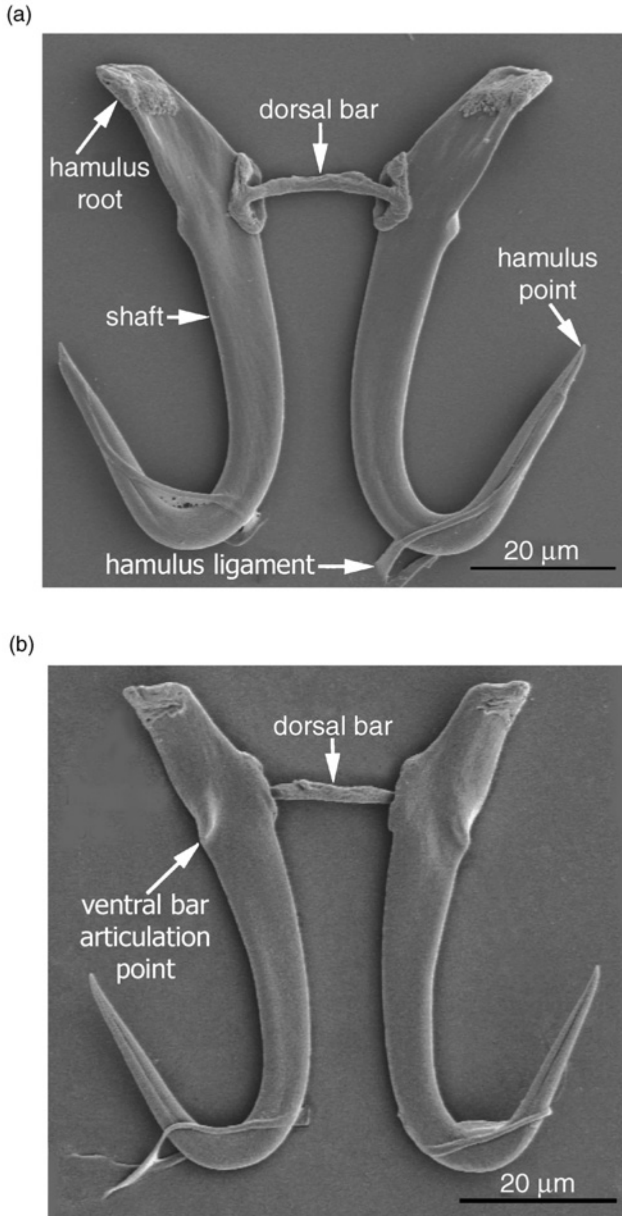


Figure 7 Scanning electron micrographs of the dorsal side (a, Lake Bullaren strain from rainbow trout) and ventral side (b, River Rauma strain from salmon) of the hamuli (anchors) and dorsal bar of *Gyrodactylus salaris.*

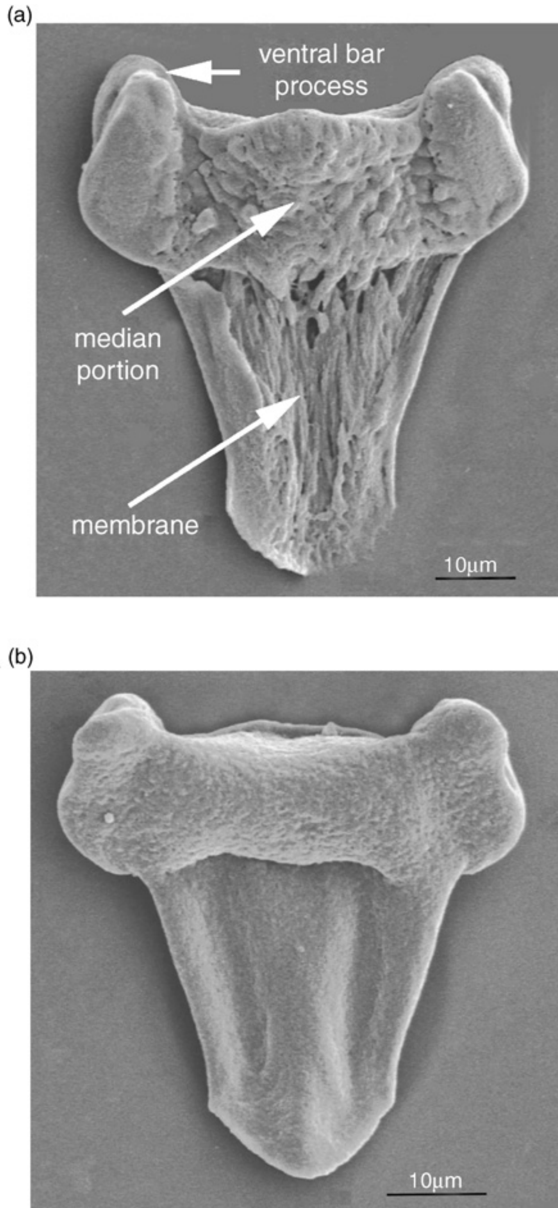


Figure 8 Scanning electron micrographs of the dorsal (a) and ventral (b) sides of the ventral bar of *Gyrodactylus salaris* (River Rauma strain) from Atlantic salmon.

epithelium. In contrast, *G. salmonis* penetrated host cells deeply (Cone and Cusack, 1988). In *G. salaris*, the sickle points are approximately 6 µm in length and too short to penetrate the epidermis into the blood supplied dermis (Figures 9 and 10; see Sterud *et al.*, 1998).

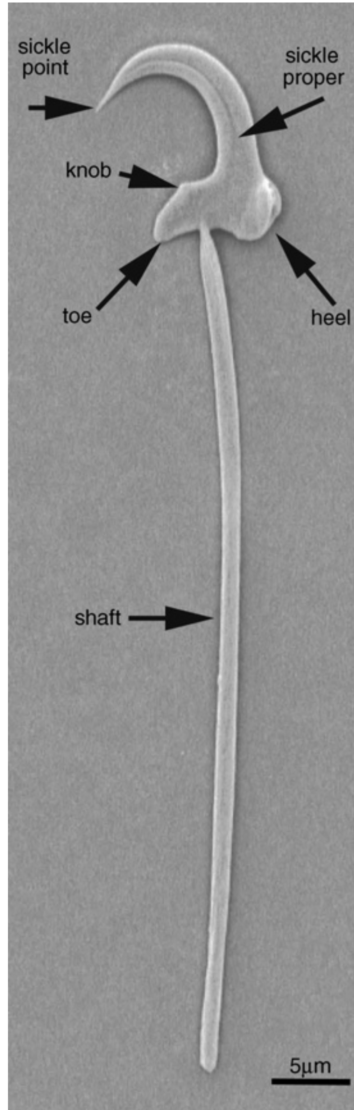


Figure 9 Scanning electron micrograph of a marginal hook of *Gyrodactylus salaris* (River Rauma strain) from Atlantic salmon.

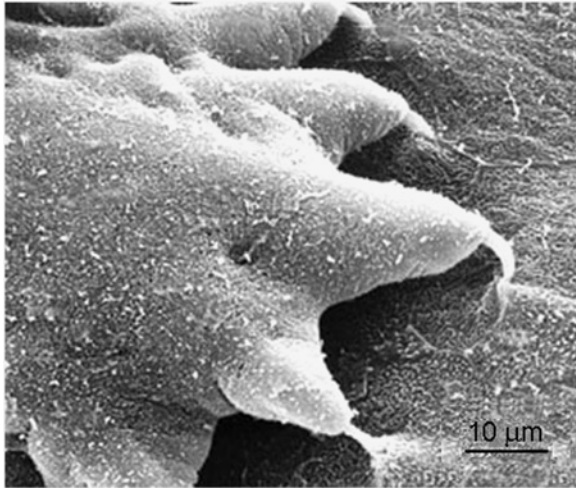


Figure 10 Scanning electron micrograph of the marginal hooks of *Gyrodactylus salaris* penetrating the skin of an Atlantic salmon. The sickle points are ~6 µm in length and too short to penetrate the epidermis into the blood supplied dermis.

Overall, *Gyrodactylus* species seem to have been selected to minimise damage to the host epithelium, trending towards superficial attachment via the marginal hooks and the hamuli used as a pressure pad.

The structure and organisation of the haptor has been the main character used in describing new genera, and different genera therefore have different attachment strategies. The simplest modification is the complete loss of the hamuli and bars. This appears to have occurred independently at least twice; in *Anacanthocotyle* parasitising characins in Central America (Kritsky and Fritts, 1970) and in *Isancistrum* infecting squids in the North Atlantic (de Beauchamp, 1912; Llewellyn, 1984). In these genera attachment relies on the marginal hooks only, in *Isancistrum* possibly because of the very small body size (100–300 µm length; Llewellyn, 1984) or the fragile thin epidermis of the host squid (Polglase *et al.*, 1983). Loss of hamuli and bars may also be due to progenesis, which reduces developmental time available for hamulus development and migration. Progenesis may also explain the peduncular location of hamuli in *Acanthoplacatus* (see Ernst *et al.*, 2001a) which do not migrate into the haptor as the parasite matures. It is difficult to see how the hamuli could

function in their peduncular position and they appear rudimentary in the illustrations of Ernst (1999).

The larger-bodied gyrodactylids such as *Macrogyrodactylus*, *Swingleus*, *Polyclithrum* and *Mormyrogyrodactylus* (Luus-Powell *et al*., 2003) all use shallow suctorial attachment. The marginal hooks are separated into anterior and posterior groups (recognising the stresses placed on the haptor when the host fish moves), and the haptor may be strengthened with accessory plates or bars. In each of these genera, the haptor has evolved independently for suctorial attachment suggesting strong common selective forces on gyrodactylid evolution. The functional parallels with the capsalid attachment mechanism (Kearn, 1971) are striking. In *Fundulotrema*, *Swingleus* and *Mormyrogyrodactylus*, an accessory peduncular plate is present to allow the anterior of the haptor to be pressed against the host (Cone and Odense, 1988; Billeter *et al.*, 2000; Luus-Powell *et al*., 2003). Suctorial attachment has also evolved in *Gyrdicotylus gallieni,* from the mouths of clawed toads (*Xenopus*). The entire haptoral rim has become modified into a sucker, partially separated into two units by the hamuli (Harris and Tinsley, 1987). The membraneous edge of the haptor extends beyond the marginal hooks and lacks the papillae characteristic of the genus *Gyrodactylus*, instead being modified into a marginal valve (Harris and Tinsley, 1987). Attachment is to the highly contractile oral epithelium and the suckers have converged upon those of polystomatid monogeneans, which attach to the extensible epithelium of the amphibian urinary bladder.

The attachment of several gyrodactylid genera has not been fully analysed. The haptors of *Gyrodactyloides* and *Laminiscus* were originally thought to be identical, but are clearly not, even though both are adapted for gill attachment. *Laminiscus* is most similar to *Archigyrodactylus* (Mizelle and Kritsky, 1967) and has an additional plate within the haptor, the function of which remains unknown. It is unfortunate that we understand so little about the diversity of gyrodactylid attachment, as the minutiae of hamulus and bar structure still form the basis for classification of the group. There are many descriptions of haptoral structures which we do not understand functionally; *G. anudarinae*, *G. tibetanus* and *G. luckyi* all have a ventral bar membrane (an essential component of the pressure pad)

modified into two filaments; *G. aksuensis* has hamuli barely larger than the marginal hooks; and many species have hamulus roots folded inwards to the mid-line of the haptor. This has evolved independently at least twice (once in loach gyrodactylids such as *G. pavlovskyi*, *G. jiroveci* and *G. incognitus* and the second time in *G. pleuronecti*, *G. flesi* etc. infecting flatfishes), suggesting that it confers specific advantage to skin parasites.

Hamuli and bars of individual species are invariant. In *G. gasterostei,* the variance of hamular dimensions in natural populations was no greater than that within inbred laboratory lines, with variance being only ~5% of the mean (Harris, 1998). This work, and our unpublished observations on the guppy parasites *G. turnbulli* and *G. bullatarudis*, implies that the hamuli and bars are tightly controlled genetically, although their size may vary according to environmental influences. The most important environmental factor affecting hamulus and marginal hook size is temperature, an increase in which results in smaller hamuli and marginal hooks (Kulemina, 1977; Mo, 1991a, b, c, 1993; Dávidová *et al*., 2005; see also Section 9.1). This is general in all species in which it has been studied. Why then is gyrodactylid morphology so constant within, but consistently different between, species? The evidence, particularly from the *G. salaris* species complex is that morphology can change following host switching and isolation, as populations of *G. salaris* from salmon (and rainbow trout) are morphologically different to specimens from grayling (Lindenstrøm *et al*., 2003a; Shinn *et al*., 2004; Olstad *et al.*, unpublished), despite their suggested recent origin by host switching (see Meinilä *et al*., 2004). These anecdotal observations suggest that the rate of morphological evolution in gyrodactylids changes when a host shift occurs, in addition to being influenced by environmental factors.

The body wall musculature in *G. eucaliae* is typical of other invertebrates with one to three discontinuous layers (Kritsky, 1971); an outer circular, an intermediate longitudinal and an inner diagonal layer. The outer two layers were present throughout the body whereas the inner was usually restricted to the cephalic and peduncular regions of the parasite. Two types of myofilaments with an electron-dense sarcoplasm were non-striated and orientated longitudinally in the fibres (Kritsky, 1971). More recently, El-Naggar *et al*. (2004a) confirmed

the triad of muscle layers and using confocal scanning microscopy were able to show the intricate lattice-like arrangement of muscle fibres throughout the body and haptor of *Macrogyrodactylus clarii*.

2.4. Digestive System

All gyrodactylids are epidermal browsers that occasionally will take dermal cells as well. Goblet mucous cells may be digested with epithelial cells, and as a thin layer of mucus covers the epidermis (Whitear, 1970), their diet is usually cited as epidermal cells and mucus. Gyrodactylids with black guts have invariably been grazing on melanocytes (Cable *et al*., 1997, 2002b), rather than on blood.

The ultrastructure of the bilobed, blind ended gut of gyrodactylids is well documented, especially the characteristic syncytial gastrodermis (Kritsky *et al*., 1994; Cable *et al*., 1996, 1997, 1998; El-Said Arafa, 1998; Olstad *et al*., 2006). This pattern is common throughout the group except in *Isancistrum*, in which the two caeca fuse behind the testis forming a ring. The gastrodermis lies on a thin basal lamina complex, often close to the surface of the parental uterus enclosing the embryo. Nuclei are sparse, and often associated with mitochondria and Golgi bodies, while much of the cytoplasm is packed with a range of different digestive vesicles (Kritsky *et al*., 1994; Cable *et al*., 1999). Microvilli project into the gut lumen or are compressed together in newborn worms or specimens which have recently evacuated their gut contents.

The pharynx, lying just above the union of the two gut caeca, is less well described. It consists of a ring of eight large cells, constricted by muscle blocks (Figure 11). Malmberg (1970) made the distinction between species with long pharyngeal processes (e.g. *G. salaris*; Figure 12) and those with short processes. However, the processes extend anteriorly from the pharyngeal cells, their difference in length being one of degree. The pharynx is protruded through a bell-like valve onto the host epithelium (Figure 12) during feeding, and the processes placed against the host epithelium. This can be seen more easily in species with long processes, but the feeding mechanism appears identical irrespective of process length.

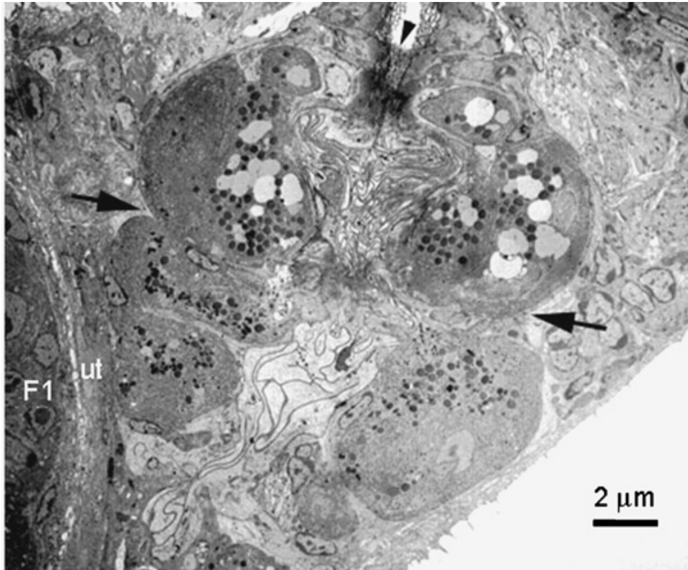


Figure 11 Transmission electron micrograph of the mouth (arrow head) and pharynx of *Gyrodactylus gasterostei*. The pharyngeal bulb constricted by muscle blocks (arrows) lies in close proximity to the parental uterus (ut) and F1 embryo. [Reproduced from Cable *et al*. (2002b) with permission of Cambridge University Press.]

The pharyngeal cells are packed with secretory granules, and, by analogy with *Entobdella soleae* (see Kearn, 1971) and other skin-feeding monogeneans (Halton, 1997), we assume that digestive enzymes are released directly onto the skin of the fish within the chamber sealed off by the pharyngeal valve. This begins digestion, and partly digested epithelial cells are drawn up into the gut for further intra-cellular digestion by the gastrodermis. Proteolytic activity has never been demonstrated in the pharyngeal cells of gyrodactylids, but using SEM potential feeding pits are detectable in the host epidermis (Figure 13). These circular lesions appear to heal rapidly by epidermal growth and regeneration, and probably leave the dermis intact (see Figure 13; Kearn, 1963). Within the intestine, digestion proceeds rapidly. The actively phagocytic intestinal syncytium takes up fragments of host cells. Using previously starved parasites (the only feasible experimental scenario), feeding recommenced within 1 min of starved *G. gasterostei* being returned to a host

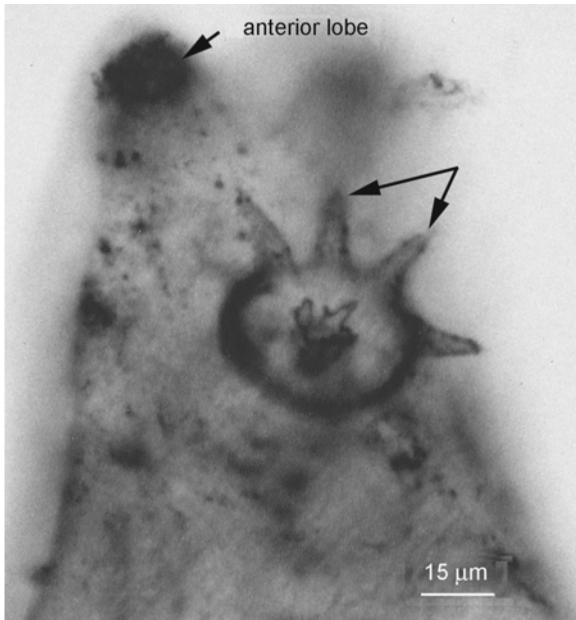


Figure 12 Light micrograph of the extended pharynx with the eight pharyngeal lobes spread out in a *Gyrodactylus salaris* specimen. (K.B. Nilsen, unpublished.)

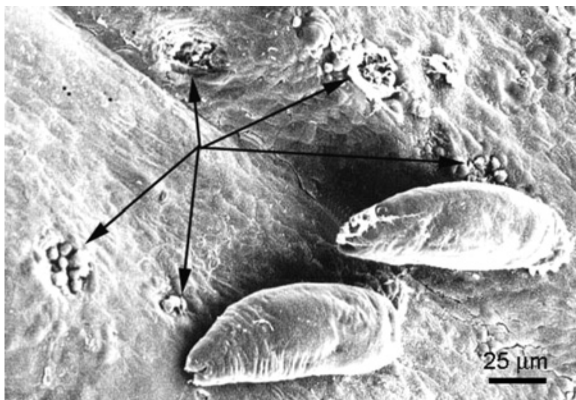


Figure 13 Scanning electron micrograph demonstrating the wounds (see arrows) on the epidermis of an Atlantic salmon parr caused by the feeding activity of *Gyrodactylus salaris.*

(Cable *et al.*, 2002b). The ingested material was highly particulate suggesting that the initial homogenisation of contents during the extra-corporeal phase of digestion is effective at breaking up host cells, although occasionally, almost intact host cells and host melanin are detected in the gut lumen (Figure 14). Within 5 min of first feeding, material was being actively phagocytosed into the intestinal syncytium and a digestive cycle similar to that observed in *Calicotyle kroyeri* (see Halton and Stranock, 1976; Kritsky *et al.*, 1994) was then followed (Cable *et al.*, 2002b). In *G. gasterostei*, feeding occurs once every 15–30 min, a patch of epidermis containing ~30 cells being stripped away on each occasion (Harris, 1982).

Direct physical damage to the host caused by feeding has been most comprehensively documented in *G. salaris* (see Figure 13). Not all ingested material is either absorbed or directly excreted. Observations on the fate of melanin granules in *Macrogyrodactylus polypteri* (see Cable *et al.*, 1997), where these are a common dietary component, and

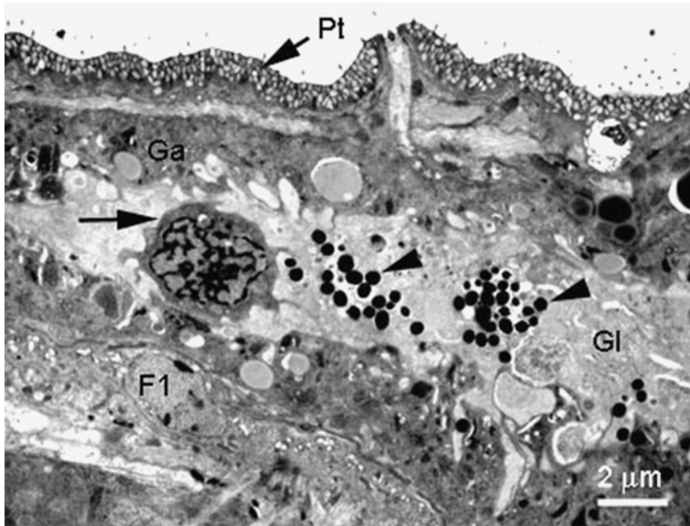


Figure 14 Transmission electron micrograph of the gastrodermis of *Gyrodactylus gasterostei*. The gut lumen (Gl) contains intact host cells (arrow) and melanosomes (arrow heads). The syncytial gastrodermis (Ga) is compressed between the tegumental surface layer of the parent (Pt) and the F1 embryo (F1). [Reproduced from Cable *et al.* (2002b) with permission of Cambridge University Press.]

in *G. gasterostei* (see Cable *et al*., 2002b) where they are not, indicate that the granules become phagocytosed by the gastrodermal syncytium and then remain *in situ* for long periods. The accumulation of host melanosomes in four distinct transverse bands in the intestine of *M. polypteri* is particularly intriguing (Malmberg, 1957; Khalil, 1970; Cable *et al*., 1997). Granules are also phagocytosed elsewhere in the gut, giving old animals an orange colouration between the black bands. Defecation has been observed in *M. polypteri* because melanin granules colours the expelled material.

2.5. Glandular System

Generally, monopisthocotyleans possess groups of bilaterally paired unicellular glands in the cephalic and trunk regions. In gyrodactylids, Wagener (1860) was the first to describe these glands and their distribution. Kathariner (1893) then observed ducts leading to the cephalic lobes and described these as cephalic glands. The cephalic glands release their contents individually onto the surface of the cephalic lobes. The first detailed account of these glands in any monogenean is that of Kritsky (1978, Figures 1–6), who described them in *G. eucaliae*. In this species, they consist of three distinct morphological types: three dorsal bilaterally paired groups producing elongated acidophilic secretions; one paired antero-ventral group producing basophilic secretions; and a group of postero-ventral glands immediately behind the pharynx secreting granular acidophilic secretions. All three glands are merocrine (the cell survives the cycle of secretory activity). The release of the contents of each granule is by union of its limiting membrane with the tegumental unit membrane (Kritsky, 1978). The presence of several types of cephalic glands indicates a multi-purpose role in *G. eucaliae*, but their primary function is considered to be adhesion (Kritsky, 1978; see Section 5.3).

Other components of the glandular system are described in Section 2.2 (tegumental secretions), Section 2.4 (secretions of the pharyngeal glands and the gastrodermis) and Section 2.8.2 (female reproductive system), but there are also unpublished thesis accounts of glands located throughout the body and associated with the haptor

(Kritsky, 1971; El-Said Arafa, 1998). Nothing is known of the chemistry of secretions from tegument and glands in gyrodactylids or in monogeneans in general (Whittington *et al.*, 2000a; Whittington and Cribb, 2001).

2.6. Excretory System

The excretory system consists of two longitudinal looped canals draining the two halves of the body and receiving smaller ducts from individual flame cells. The structure of individual flame cells and ducts is identical with that of all other platyhelminths in which they have been studied (Kritsky, 1971; Rohde, 1989; Cable and Harris, 2002) but overall the system is much simpler than in larger flatworms, presumably because of progenesis. Malmberg (1957, 1970) described the excretory system in considerable detail, and used it to subdivide the genus *Gyrodactylus* and establish the phylogeny of the group. The validity of Malmberg's (1970) classification based on excretory systems is discussed in Sections 4.1 and 5.2. A drawback to the use of the excretory system in taxonomy is that it can only be studied in living material, and apart from *Gyrodactylus*, only *Ooegyrodactylus* (see Harris, 1983), *Macrogyrodactylus* (see Malmberg, 1957), *Gyrdicotylus* (Vercammen Grandjean, 1960; Harris and Tinsley, 1987), *Swingleus*, *Polyclithrum* and *Isancistrum* (Malmberg, 1998) have been fully described.

2.7. Nervous System

Despite the wealth of descriptions of platyhelminth nervous systems visualised by immunofluorescence (e.g. Halton and Maule, 2004, and references therein), until recently the only accounts of the gyrodactylid nervous system were an early description of an unidentified species from the three-spined stickleback visualised using the thiocholine method (Lyons, 1969) and immunocytochemical demonstration of two neuroactive substances in *G. salaris* (see Reuter, 1987) revealing the now classical orthogonal pattern of nerve cords (Reuter *et al.*, 1998). However, confocal scanning laser microscopy has revealed with superb clarity the spatial arrangement of muscle and associated

cholinergic, peptidergic and aminergic innervations in *Macrogyrodactylus clarii* (see El-Naggar *et al*., 2004a).

Three types of putative sense organ have been described in gyrodactylids. Firstly, there are uniciliate tegumental receptors, consisting of a nerve bulb connected to the tegument by septate desmosomes and a terminal, free 9 + 2 axoneme with no basal body (Lyons, 1969), which in other invertebrates are thought to be mechanoreceptors (Lyons, 1973). In *G. salaris*, three distinct types of uniciliate sensilla occur in the head region of *G. salaris*: a tapering form, a cylindrical form and a form with a terminal bulb (see Bakke *et al*., 2004a). The length of sensilla axonemes is also highly variable reflecting growth or functional differences, and some may be retractable (Bakke *et al*., 2004a). Second, there are compound uniciliate tegumental receptors clustered to form the prominent pair of ''spike sensilla'' on the cephalic lobes of *Gyrodactylus* spp. (Lyons, 1969, 1973; Figures 15 and 16). The phylogenetic distribution of these receptors is unclear, but they are absent from most monogenean groups. Kearn (1993) recorded similar structures from an analogous position in *Enoplocotyle kidakoi* and Lyons (1969) found them in the same position in the oncomiracidium of *Entobdella soleae*, and from the pharynx of adult *Acanthocotyle lobianchi* (see Lyons, 1969). The phylogenetic distribution of these receptors warrants further examination. These

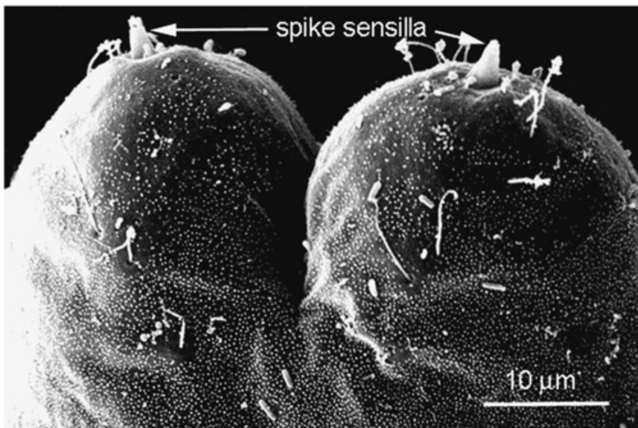


Figure 15 Scanning electron micrograph of the sensory apparatus on the anterior lobes of *Gyrodactylus salaris*, dorsal view.

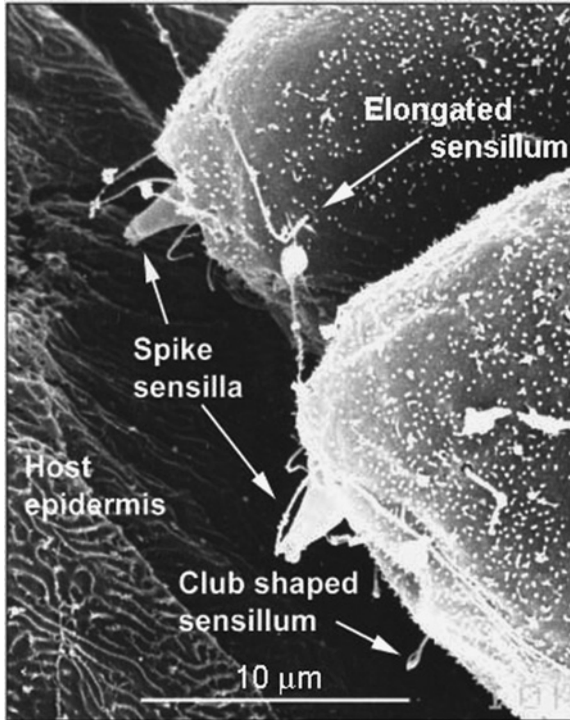


Figure 16 Scanning electron micrograph of the sensory apparatus at the anterior lobes of *Gyrodactylus salaris* actively searching the skin surface of an Atlantic salmon parr, anterior lateral view. (Adapted from Bakke *et al.*, 2004a with permission from *Folia Parasitologica*.)

receptors are thought to be chemosensory and are used extensively when gyrodactylids probe their substrate or surrounding environment prior to movement, feeding or interaction with another gyrodactylid (Figure 16). Third, there are sub-surface ciliary receptors, possibly photoreceptors, described by Watson and Rohde (1994) using TEM from just below the spike sensilla of an unidentified *Gyrodactylus* sp. from swordtails. Each receptor consisted of a single dendrite ending in an extra-cellular cavity containing a small number of modified cilia. Unlike most monogeneans, no pigmented eye spots have been described from *Gyrodactylus* species. In living *Ooegyrodactylus farlowellae*, a pale green, lenticular structure lies in the midline anterior to the pharynx, which persists throughout life although

it is most obvious in larvae (Harris, 1983). In fixed, stained specimens, this structure lies in the same position as the cerebral organ of *Macrogyrodactylus polypteri* (see Malmberg, 1957). Harris (1983) suggested that this may be a photoreceptor, but this has also never been investigated further.

Surface sensilla (uniciliate and compound uniciliate) are symmetrically distributed around the median longitudinal axis and can be mapped using silver nitrate that stains cell junctions. Shinn *et al.* (1997, 1998a, b) and El-Naggar *et al.* (2001) used this method to distinguish *Gyrodactylus* and *Macrogyrodactylus* species, but single specimens cannot be identified reliably. Staining is particularly unreliable in the haptoral region due to the presence of other argentophilic structures and host mucus (Shinn *et al.*, 1997; Bakke *et al.*, 2004a), and the sensilla pattern on the body can sometimes be obscured by a large *in utero* embryo (Shinn *et al.*, 1997). Bakke *et al.* (2004a) questioned the temporal stability of the sensilla pattern at specific locations, and the pattern may change with age. Bakke *et al.* (2004a, b) concluded that mapping sensilla was useful in investigating variation in gyrodactylids, but was not diagnostic. Chaetotaxy may prove to be a reliable indicator of higher taxon relationships (Shinn *et al.*, 1998a; El-Naggar *et al.*, 2001) based on the branching structure of the nerve systems in relation to sensilla pattern, but perhaps more interesting would be to assess whether there is any relationship between the abundance and distribution of sensilla and gyrodactylid life history strategies, such as mode of transmission and behaviour on the host.

2.8. Reproductive System

The reproductive system of viviparous gyrodactylids is highly simplified because of progenesis (see Kritsky, 1971 for ultrastructural details; and Section 2.1). Reproductive structures and modes were reviewed by Kearn (1996) and more recently by Cable and Harris (2002), but here we briefly consider the differences in reproductive morphology between genera, focussing on the viviparous forms unless otherwise specified.

2.8.1. Male Reproductive System

The gyrodactylid male reproductive system consists of a single sac-like post-ovarian testis with flat epithelial cells resting on a thin basal lamina enclosing a central mass of loosely packed germinal cells, from which spermatozoa pass forward to a penis, localised just behind the pharynx. In viviparous genera, the male system only becomes functional after the parasite has given birth once. In *G. gasterostei*, sperms begin to mature just after the first birth and then accumulate in the anterior seminal vesicle, located just behind the penis (Harris, 1985; Cable *et al*., 2001) (Figure 17, which is plate 3.17 in the separate Colour Plate Section). Malmberg (1970) and Kritsky (1971) depicted a *vas deferens* connecting the testis to the penis (following either the left or right gut caeca, respectively) but we have never identified this in whole mount preparations or sections, although the accumulation of sperms in the seminal vesicle is very clear. We suspect that in some species sperms migrate anteriorly via tissue layers (Harris *et al*., 1997; Cable *et al*., 1998, 2001). The structure of the male reproductive system varies in different species and appears more complex in *Macrogyrodactylus polypteri* (see Malmberg, 1957). This genus is least affected by progenesis and therefore additional development of the male system may take place.

Considerable diversity exists in the structure of the male intromittent organ, which forms an important character for generic diagnosis. Previous nomenclature, including Kritsky (1971), refers to the intromittent organ as a cirrus, a structure that is inverted when not in use. Strictly speaking a penis is not inverted when stored, and may be protruded or extended during copulation. The confusion arises from the fact that the spherical intromittent organ of *Gyrodactylus* is similar in structure to that of rhabdocoels (Baer and Joyeux, 1961), which was known to be everted during use and is therefore a cirrus. Observations on copulation in *Gyrodactylus* show that the structure does not evert (Braun, 1966; Cable and Harris, unpublished); it is thrust out of the body and used the hook into the tegument of the partner. The structure is therefore technically a penis. Amongst the egg-laying gyrodactylids, *Ooegyrodactylus* also has a penis, in this case tubular, and Harris (1983) captured images of this structure

extended. We have therefore previously favoured use of the term "penis" for the intromittent organ of gyrodactylids and have considered that an evolutionary trend from tubular to spherical has taken place. The ball-like penis of *Gyrodactylus* comprises a muscular bulb enclosing both the prostatic reservoir and a short ejaculatory duct (Kritsky, 1971), and is armed with a large hook and small spines. This situation has recently become much more complex. Amongst the viviparous genera, published descriptions suggest that some African *Gyrodactylus* species have tubular penes (Paperna, 1979) and we have seen similar structures in undescribed species of *Gyrdicotylus* (Harris, unpublished). *Mormyrogyrodactylus* has a tubular structure characterised as a cirrus (Luus-Powell *et al*., 2003), but this organ may be extended from the body, functioning partly as a penis. The recent description of several new egg-laying gyrodactylids by Kritsky *et al*. (2007) has also demonstrated the diversity of the male intromittent organ. Although *Ooegyrodactylus farlowellae* appears to have a conventional penis (Harris, 1983), the intromittent organ of *Phanerothecium caballeroi* is coiled and covered entirely in a layer of hardened tissue, making it difficult to imagine how it could function as either an eversible cirrus or an extensible penis. In some genera, such as *Hyperopletes*, the intromittent organ is lined with fine spines, suggesting that it everts as a cirrus, much like *Mormyrogyrodactylus* (Luus-Powell *et al*., 2003). Amongst the other genera of egg-laying gyrodactylids, diversity of the intromittent organ approaches the bizarre. *Nothogyrodactylus*, *Aglaiogyrodactylus* and *Onychogyrodactylus* all have a system of accessory sclerites around the penis, the function of which is entirely unknown. Oviparous genera may also have two seminal vesicles of various shapes (e.g. Kritsky *et al*., 2007), in contrast to the single seminal vesicle of viviparous forms. In *Onychogyrodactylus sudis*, copulation was observed (Kritsky *et al*., 2007) similar to that of *O. farlowellae* (see Harris, 1983). The accessory sclerites did not appear to be used for hypodermic impregnation in this case, or to be inserted into the partner's female tract.

The spermatozoa of monopisthocotyleans show considerable morphological variation (Justine *et al*., 1985; Justine, 2001). There is a relatively large database on monogenean spermiogenesis and sperm

ultrastructure covering more than 60 species; however, few attempts have been made to study gyrodactylid spermatozoa (Justine, 1993). The mature 30 μm-long filiform spermatozoa of *G. eucaliae* conform to the typical flatworm structure, being spindle-shaped, of a nearly uniform diameter (0.7 μm) except at its gradually tapering ends (Kritsky, 1971; see also Malmberg and Lilliemarck, 1993). The nucleus extends from within its lobe towards the centre of the spermatozoa, the two axonemes are bilateral on either side of the nucleus and the mitochondrial lobe opposes that of the nucleus (Kritsky, 1971). The sperms are differentiated from those of other flatworms by the lack of free flagellae and/or peripheral or marginal microtubules beneath the plasmalemma (Figure 17); they most closely resemble those of turbellarians and digeneans. More recently, Schmahl and Elwasila (1992) described spermatogenesis of *Macrogyrodactylus polypteri* as similar to that of other monopisthocotyleans, but clearly different from that of polyopisthocotyleans.

2.8.2. Female Reproductive System

The female reproductive system is also apparently simple, belying complexity at a subcellular level. The egg-laying gyrodactylids have a female system similar to that of other oviparous monogeneans, although they possess characters linking them with *Enoplocotyle* and the acanthocotylids: the ovary is in the mid-body and develops after the testis. The testis of *Ooegyrodactylus* becomes less apparent and recrudesces when the female system matures (Harris, 1983; Kritsky and Boeger, 1991). In young *O. farlowellae*, with a mature male system, the female system consists of a small germarium, immediately posterior to a large seminal receptacle. This opens to the exterior via the common female duct, which functions as a vagina in young worms. The male and female systems of *O. farlowellae* open side by side in the mid-region of the body, and insemination occurs when the penis of one individual is inserted into the common female duct of a partner (Harris, 1983). As the male system starts to recrudesce, the female germarium grows substantially. Four rows of post-germarial vitelline follicles appear, and the seminal receptacle and posterior part of the common female duct begins to function as an oötype. The

oötype region is surrounded by large, diffuse glandular tissue, a synapomorphy with *Enoplocotyle* (see Boeger *et al*., 1994). Eggs develop one at the time and are retained in the common female duct before being laid, when they are glued to the substrate via the adhesive droplet (Harris, 1983; Kritsky *et al*., 2007).

In the viviparous forms, the female system is modified, but the basic pattern of oviparous forms can still be discerned. As a result of progenesis, the full developmental expression of the female system is never attained; the "vitellaria" never fully developed and never produce egg-shell precursor proteins. The vitelline cells appear reduced to patches of glandular syncytia within the posterior part of the body (Cable *et al*., 1996), which have no connection with the other parts of the female system, but our understanding of the function and physiology of these tissues is rudimentary, and to complicate matters further, some of these "vitelline" cells (at least in *Macrogyrodactylus*) may actually be subtegumental cells (El-Naggar and Cable, in press). The most distinctive feature of the female system of the viviparous genera is the uterus, which develops as an expansion of the common female duct, anterior to the large seminal receptacle. The germarium is reduced to a patch of tissue on the posterior wall of the seminal receptacle. The main function of the germarium/seminal receptacle appears to be egg cell maturation, and for this reason it is termed the Egg Cell Forming Region (ECFR) (see Jones *et al*., 1994, 1997).

In *Macrogyrodactylus*, there is much more complexity to the posterior female system, much of which develops after the parasite has given birth for the first time. Patches of tissue in longitudinal rows have the same configuration as the vitellaria of *Ooegyrodactylus*. However, different cell groups have a different microscopic appearance (Malmberg, 1957) suggesting a different function. A further complication in *Macrogyrodactylus* is the appearance of a putative secondary seminal receptacle that lies to one side of the primary receptacle/ECFR. This structure was noted by Malmberg (1957) and has been confirmed by further light microscope studies on *M. clarii* by El-Abbassay (2001). This structure also appears to be present in the other large-bodied genera *Polyclithrum* (Ernst *et al*., 2000, 2001a) and *Swingleus* (Billeter *et al*., 2000).

The diffuse glandular tissue which characterises the oötype region of the egg laying gyrodactylids and *Enoplocotyle* is not seen in viviparous forms. However, there are groups of cells at the junction of the ECFR and the uterus, which may be homologous with this glandular tissue. These cells are not well developed in *Gyrodactylus*, but are noticeable in, for example, *Gyrdicotylus* (see Harris and Tinsley, 1987).

A consequence of progenesis, and a remarkable feature of the gyrodactylid reproductive system, is a general lack of ducts. There appear to be no permanent ducts connecting the separate parts of the female or male reproductive systems. As noted above, at least in some species, self-sperm seem to migrate to the seminal vesicle through body tissues (Cable *et al*., 1998). Presumably, all sperm acquired from a partner during hypodermal impregnation that are not injected directly into the ECFR, must migrate through tissues. Similarly, different parts of the female system are not directly connected. When a daughter is born, it appears to break through the body wall (Jones *et al*., 1998; see Figures 3C and 19A in Cable *et al*., 1996 and Cable and Harris, 2002, respectively, for images of birth pore/plug), which then heals over, leaving no permanent female opening. Similarly, there is no permanent connection between the ECFR and the uterus. After a daughter is born, the next oöcyte enters the uterus via the so-called cap cells at the junction of uterus and ECFR (Cable and Harris, 2002). As noted above, there are also no ducts linking the structures identified as vitelline cell homologues. Any secretory product from these cells must disperse in some other way (Cable *et al*., 1996).

2.8.3. *Embryology*

The early research on gyrodactylid germ cell lineages is remarkably accurate. Using mid-19th century light microscopy, Wagener (1860) in particular achieved results which were not surpassed until the advent of electron microscopy some 100 years later. The high quality of early osmium-fixed sectioned material prepared by Kathariner (1893, 1904) and Gille (1914) was demonstrated when Cable and Harris (2002) published comparable images prepared using Feulgen-stained chromosome spreads. The improved technology does not give

substantially better insight into gyrodactylid reproduction, and Gille (1914) was able to show both mitotic and meiotic chromosomes within the gyrodactylid oöcyte.

Katharíner (1904) undertook the work which led to *Gyrodactylus* being considered a classic example of polyembryony. The myth of *Gyrodactylus* development grew from this observation: following cleavage of the zygote, one of the cleavage products became quiescent, while the other forming the F1 generation grew around it. Eventually, the quiescent cell became activated to form the F2, when one cell produced by the first division of the F2 became quiescent, eventually re-activating to form the F3 (see Cable and Harris, 2002). *Gyrodactylus* thus appears a neat example of segregation of the germ cell lineage, which is then transferred intact to the next generation. Braun (1966) revisited this system and more or less confirmed Katharíner's (1904) results (although he claimed to observe meiosis within the quiescent cell) and demonstrated for the first time that reproduction (at least of the first-born daughter) can continue for up to 30 generations without the need for a sexual partner. This has subsequently been repeated with *G. gasterostei* (see Harris, 1998). Our own observations, however, suggest a re-interpretation of Katharíner (1904) and Braun (1966). In particular, we note the difficulty of tracking the F2 generation embryo back to a single cell, and note that the large, pale staining cells of the embryo (which Katharíner and Braun interpreted as quiescent) actually contain up to 4*n* copies of DNA, suggesting they are about to divide (Cable and Harris, 2002). It appears that the F1 generation embryo within the embryo cluster develops when the uterine wall of its parent appears, separating off cells at the centre of the embryo to become the next generation.

This mechanism remains poorly understood, partly because of the small size of gyrodactylids, the fact that two different developmental routes exist within the life cycle (the first daughter develops at the centre of an embryonic mass, whereas all subsequent daughters develop from an oöcyte), and because it is unique with no parallels in the Animal Kingdom. However, several interesting features have emerged over the past 15 years (reviewed in Cable and Harris, 2002). Perhaps, the most interesting concerns the role of "blastomere anarchy" in gyrodactylid development. In most invertebrates,

cleavage is a precise process resulting in an embryo capable of developing into an adult organism. This precision is usually based upon the intra-cellular architecture of the oöcyte, with its specific localised mRNAs allowing structural differentiation. However, in neoophoran platyhelminths (in which yolk and oöcyte functions reside in separate cells), the pattern of cleavage is a property of the precise embryonic architecture and vitelline cells provide ‘‘scaffolding’’ within which the blastomeres can differentiate accurately. Bresslau (1909), coined the term ‘‘blastomeren anarchie’’ to describe this apparent totipotency of the blastomeres in rhabdocoels – only the position of blastomeres, determined by the scaffolding vitelline cells, could limit their developmental pathway. In gyrodactylids, this same phenomenon seems to apply, but vitelline cells are not available to form scaffolding. Instead, the early embryo can be highly plastic, with blastomeres separating and rejoining in different configurations. Only after the embryo has achieved a certain complexity, does re-organisation and re-shaping cease, and development proceed along a fixed pathway. These cellular re-organisations have not been observed during development of the first-born offspring, which occurs within a cell mass where cell movements are restricted. One consequence of blastomere anarchy is that it has proved impossible to identify a particular blastomere as the progenitor of the first-born daughter.

In the absence of scaffolding vitelline cells, the function of organising the embryo appears to have been taken over by the syncytial uterine lining. Cable *et al.* (1996) describe this layer in detail, finding it to be metabolically very active, with a great deal of protein secretion, and, judged by fluorescence microscopy, abundant RNA transcription. At the anterior and posterior poles of the uterus are cell bodies, termed cap cells by Cable *et al.* (1996). The role of these cell bodies and the associated syncytium is unclear, but is almost certainly related to nutrition of the embryo and to co-ordination of its early development (Cable and Harris, 2002).

There are surprisingly few studies on gyrodactylid internal structure from across the spectrum of species, and most studies have concentrated on easily obtainable forms from guppies, goldfish, salmonids and sticklebacks. The diversity of gyrodactylid internal structure has been underestimated, although several studies suggest that it might be

considerable. In particular, the glandular tissue posterior to the ECFR and the extent of the fully formed testis, seem variable (Ernst *et al*., 2001a, b) as does the construction of the pharynx and penis spines. This diversity has never been fully investigated or placed within a phylogenetic context. There is also diversity in reproductive strategy, an extreme example being *G. gemini*, in which a pair of daughters develops *in utero* (Ferraz *et al*., 1994). No details are available as to the sequence of development in *G. gemini,* and it is not known whether these offspring are twins (i.e. derived from a single oöcyte) or sisters (originating from separate oöcytes). The intra-uterine generation (i.e. the first born daughters) presumably develop asexually as in conventional *Gyrodactylus* species (see Cable and Harris, 2002), but again whether this is synchronous or asynchronous development is unknown (Ferraz *et al*., 1994). We have been unable as yet to obtain new material of this parasite, but the factors controlling embryogenesis would be particularly interesting to examine. The original description of *G. trairae*, another South American species, also shows subtle differences in the orientation of the embryo cluster (Boeger and Popazoglo, 1996), which suggest a difference in embryology.

3. ETHOLOGY

3.1. General Behaviour

Almost all behavioural observations have been made on viviparous gyrodactylids. Harris (1983) maintained *Ooegyrodactylus farlowellae* in culture for a short period, and additional behavioural observations have been provided by Kritsky *et al*. (2007). The viviparous gyrodactylid monogeneans exhibit a limited behavioural repertoire and with the morphological uniformity of *Gyrodactylus* spp., researchers might be excused the belief that all species are very similar. In fact, the limited range of behaviours is combined in ways that adapt gyrodactylids to the wide range of hosts and habitats exploited. As the most diverse vertebrate group, fishes may be shoaling or territorial, lotic or lentic, pelagic or benthic, marine, brackish or freshwater (Bakke *et al*., 1992a). Some hosts are semelparous (e.g.

Oncorhynchus or squid), others are anadromous (e.g. Atlantic salmon) or catadromous (e.g. European eel), while many acquire resistance, forcing gyrodactylids to transfer to other hosts (see Sections 8.1 and 8.2). Wherever investigated, there is growing evidence that the different behaviours are combined into repertoires that maximise transmission even where host ecology is extreme. The range of basic behaviours exhibited by gyrodactylids includes:

3.1.1. Locomotion

When moving on the host, the anterior glands cement the head temporarily to the substrate while the opisthaptor is released and drawn up to the head. The head is then released. This may be repeated for sustained periods, or just one or two steps may be taken before settling down to a single position again.

3.1.2. Swimming

Recently, El-Naggar *et al.* (2004b) reported swimming in *G. rysavyi*. This skin and gill parasite exhibits coordinated, unidirectional wriggling movements when detached from its host, the Nile catfish (*Clarias gariepinus*). This questions the doctrine that gyrodactylids have no specific transmission stage, but so far this behaviour has not been observed in other gyrodactylids, most of which sink in an outstretched position if released in the water column. Specimens of *G. turnbulli* and *G. salaris* if forcibly detached may thrash back and forth until reaching a solid substrate, but such behaviour is not unidirectional. The specific transmission behaviour of *G. turnbulli* (in which detached parasites migrate into the water film, see Section 3.3; Cable *et al.*, 2002a) is not a "swimming behaviour" (cf. Huyse *et al.*, 2003).

3.1.3. Questing

All gyrodactylids spend much of their time questing, in which the body is extended away from the substrate, with the cephalic lobes frequently stretching and spreading out. At intervals, the parasite bends to allow

the cephalic lobes to touch the surrounding substrate. Parasites may attach to another substrate or host, or may interact directly with other parasites leading sometimes to copulation. When questing is undertaken immediately before feeding, brushing of the host's epidermis with the cephalic lobes may involve mechanical or chemical assessment of the host skin. In all cases, questing behaviour seems to allow the spike sensilla and uniciliate receptors of the cephalic lobes to sample the environment, host or another parasite (see Figure 16).

Questing may be spontaneous, but it also increases substantially in response to external stimuli, such as touching the body with a fibre. Questing behaviour has been quantified in *Gyrdicotylus gallieni* (see Harris and Tinsley, 1987), which responds to vibration with increased activity and to water currents with violent thrashing movements. The response to vibration has also been noted anecdotally in other species. Responses to light or shadowing have not been tested. Generally, gyrodactylids alternate between phases of movement with stationary periods and periods of questing.

3.1.4. Feeding

Our understanding of the mechanics of feeding is discussed in Section 2.4. In *G. gasterostei*, *G. turnbulli*, *G. salaris* and *M. polypteri*, individual parasites lie in a characteristic pose with the anterior portion of the body extended and flattened against the host epidermis while firmly attached by the opisthaptor, often with the anterior lobes raised (Harris, 1982; Cable *et al.*, 2002b, unpublished). The pharynx is protruded and brought into close contact with the host's epithelium. Pumping of the pharynx can be observed, with slight waves of contraction passing along the body. Feeding lasts a few minutes, or even seconds, after which the worm straightens up but is contracted and less active for a few minutes.

3.1.5. Copulation

This has been described in detail for viviparous genera (Malmberg, 1957; Braun, 1966) and regularly observed during routine experimental

infections (Harris, 1989; Cable *et al.*, unpublished). Parasites quest with conspecifics until they impale their penis into the chosen partner. Once copulation is initiated, the partner may react by grasping the initiator with its own penis and mutual insemination may follow (Harris, 1989). Normally, viviparous gyrodactylids copulate with their bodies aligned or entwined for a few seconds or minutes, until insemination into the seminal receptacle, or other region of the body, has occurred via hypodermic impregnation. The parasites then part. Unilateral copulations, in which one partner does not reciprocally inseminate, are also common, at least in *G. turnbulli* (Cable *et al.*, unpublished). In oviparous gyrodactylids, the copulatory organ of one copulant is inserted directly into the uterine pore of its partner (Harris, 1983; Kritsky *et al.*, 2007). Considering the diversity of male copulatory organs (Kritsky *et al.*, 2007), it is possible that egg laying gyrodactylids also display a range of associated behaviours.

3.1.6. Birth and Oviposition

Only the viviparous gyrodactylids give birth to fully grown young. The best description of birth in *Gyrodactylus* is Braun (1966). It was also described in *Macrogyrodactylus polypteri* (see Khalil, 1970), but in this case the worms were in suboptimal conditions and the births may have been pathological. In healthy worms attached to a fish, birth occurs rapidly. The daughter breaks out through a ventral birth pore close to the pharynx. A parasite about to give birth can be identified by its gravid appearance and the slow waves of muscular contraction passing along the parent's body. The first part of the daughter to emerge, as a bleb of tissue, is the central region of the folded body (see Figures 1 and 3), which is quickly followed by the anterior portion of the worm (see Figure 18). The head of the daughter attaches using sticky secretions from the cephalic glands and pulls the rest of the body out of the mother onto the skin of the host, where the haptor attaches. The mother then shrinks down and is quiescent for a period before activity is resumed, when she normally moves away from the immediate vicinity of the daughter, often towards the anterior of the host (Cable *et al.*, 2000). If for some

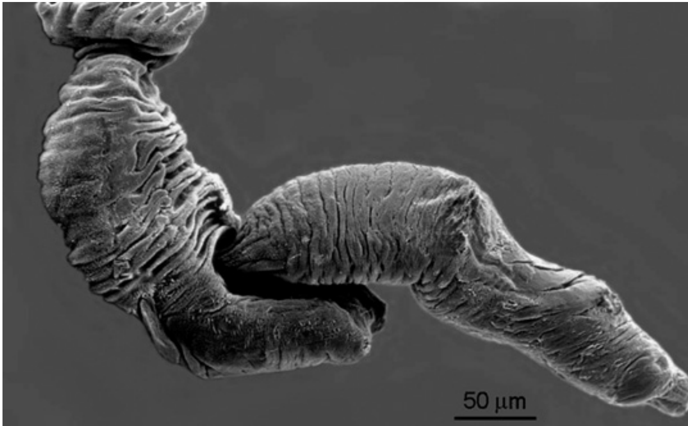


Figure 18 *Gyrodactylus salaris* individual giving birth to a pregnant daughter as large as itself. Such a daughter potentially could give birth itself 1–2 days later depending on temperature. (T.A. Mo, unpublished.)

reason, the mother becomes detached during the early stages of birth and the daughter has no host substrate onto which it can attach its anterior glands, both worms usually die as the daughter is unable to escape from its mother's uterus. If, in these instances, the daughter is manually pulled free using watchmaker's forceps and a fine pin, the mother can survive. Thus, muscular activities of both parent and offspring are essential for successful birth. If parasites are detached for prolonged periods, abortion of young embryos is common (Cable *et al.*, 2002b).

Oviparous gyrodactylids lay eggs within the environment, where they adhere by the sticky droplet either to the surrounding substrate (*Ooegyrodactylus farlowellae*; see Harris, 1983) or to the surface of the fish itself (Kritsky *et al.*, 2007). Several egg-laying forms retain eggs *in utero* (Kritsky *et al.*, 2007) in a manner reminiscent of *Acanthocotyle greeni* (see Llewellyn, 1984).

3.1.7. Transmission

Little is known about transmission of oviparous gyrodactylids. All stages of the life cycle can transmit (at least in *Ooegyrodactylus*; see Harris, 1983), and eggs are placed on (Kritsky *et al.*, 2007) or near

(Harris, 1983) the sedentary host, leading to the build up of infrapopulations. The relative importance of individuals of different ages in transmission is unknown although Harris (1983) found both very young larvae and adults able to move between hosts. In viviparous gyrodactylids, the egg stage is entirely absent, and development is curtailed by progenesis at the "young male" stage of the egg-laying gyrodactylids. Part of the selective pressure for progenesis in the viviparous genera may therefore be to maintain maximum transmission potential in the adult parasite. Continuous transmission and the infection of new hosts throughout the life cycle enhances colonisation of new host resources (Boeger *et al.*, 2005) and favours host shifts. Viviparous gyrodactylids are renowned for transferring quickly from host to host, and some can move between fish during the most fleeting of contacts (Bychowsky, 1961). However, if given the opportunity to transfer under optimal conditions, many make no attempt to leave their current host, probably as most parasites only transfer when necessary (see Section 3.2). As noted by Scott (1985a), transmission is risky and results in high mortality. However, transmission may also influence population performance by reducing the death rate caused by host responses (Boeger *et al.*, 2005). Not surprisingly, gyrodactylids show considerable diversity of strategies when detached from the host and we are becoming increasing aware of a range of different transmission strategies related to host ecology.

Following prolonged detachment or host death, individuals of *G. salaris* may hold their extended body (two or three times its length) at right angles to the substrate gently swaying back and forth. They rarely probe the surrounding substrate but can transfer immediately if a suitable host comes into contact. In *G. salaris* attached to dead hosts, two distinctive modes are recognised: (i) stationary transmission mode (STM), in which parasites are motionless with their slightly extended bodies held at right angles to the host tissue; and (ii) search mode (SM), in which the parasites' body is highly extended (at least $4\times$ its normal length) and makes constant circular movements and contractions around the motionless haptor (Olstad *et al.*, 2006). Thus, individuals of *G. salaris* in the laboratory remain on a dead host (Olstad *et al.*, 2006), possibly similar to *G. pungitii* (see Malmberg, 1970) but in marked contrast to *G. rarus* and

G. cryptarum (see Malmberg, 1970), *G. gasterostei* (see Harris, 1982) or *G. turnbulli* (see Cable *et al*., 2002a), which all leave the host shortly after its death. *G. gasterostei* and *G. pungitii*, infecting the three- (*Gasterosteus aculeatus*) and nine-spined sticklebacks (*Pungitus pungitus*), respectively, thus employ quite different transmission strategies although their hosts occupy the same macro- and microhabitats (e.g. Copp and Kovac, 2003). Future studies should examine the behaviour of these parasites on sympatric hosts and under the same macroenvironmental conditions.

3.2. Site Specificity and Migrations on the Host

Some gyrodactylids show pronounced site specificity, but this is highly variable between species. Most infect the skin and fins, but some also occur on the gills (*G. masu*, see Ogawa, 1986; *G. arcuatus*, see Harris, 1993). Amongst gill specialists, there may be further subdivision. Some (e.g. *G. aeglefini* and *G. unipons*) infect only the gill filaments, others may occur on gill filaments and on gill arches and in the pharynx (*G. branchicus*), some utilise the pharynx but also occur on filaments and arches (*G. cernuae* and *G. perlucidus*), while *G. cryptarum* inhabits the preopercular sensory canals of the head (Malmberg, 1970). *Isancistrum subulata* occurs mainly on the tentacles and suckers of the squid, *Alloteuthis subulata,* and rarely enters the mantle cavity, in contrast to *I. loliginis* which infects the gills (see Llewellyn, 1984). Such segregation of different species on the same host is common and may occur even within skin parasites. The two common gyrodactylids of guppies (*Poecilia reticulata*) display marked site preferences, with *G. bullatarudis* occurring rostrally, especially on the head and mouth at low densities (Harris and Lyles, 1992), while *G. turnbulli* occupies caudal regions (Harris, 1988). In mixed infections on salmonids, *G. colemanensis* almost exclusively attaches to the margin of fins and *G. salmonis* the head, body and the broad surfaces of the fins (Cone and Cusack, 1988).

Initial colonisation appears random, followed by migration to a specific site. In *G. colemanensis* on rainbow trout fry, parasites attach anywhere on the body surface but then relocate posteriorly, with

most migrating to the caudal fin followed by the pectoral and pelvic fins. The length of the fin margin and fin activity appeared to influence this distribution (Cone and Cusack, 1989). Under experimental conditions, *G. turnbulli* most commonly infects guppies via fins (Harris, 1988), but individual parasites then migrate to the peduncle and caudal fin where dense aggregated populations develop. After a few further days a return migration to the fins occurs, possibly to facilitate transmission (Harris, 1988). At low intensities, the dorsal fin of salmon parr was the principal site for attachment for *G. salaris* (see Jensen and Johnsen, 1992; Koski and Malmberg, 1995), followed by pectoral and anal fins. This pattern is also seen in the field. *G. salaris* on salmon parr from Batnfjordselva (Mo, 1992) occurred mainly on the fins (34.4% on the dorsal, 27% on the pectorals and 24.7% on the other fins). The remainder occurred on the body (7.8%), head (3.5%) and gills (2.6%). The parasite population only increased in size significantly on the caudal fin and body, suggesting these are the most suitable areas for parasite feeding. In a later study of Batnfjordselva, Appleby and Mo (1997) found 15% of *G. salaris* on the gills, predominantly on the filaments. In some years, up to 40% of the parasites were found on the gills (up to 300 per fish). In a few fish with small infections, all parasites were found on the gills. The proportion infecting the gills appeared higher in summer than winter and was also greater on older (2+) fish (Appleby and Mo, 1997). The reason for this difference between these two investigations in the same river system is not explained, even though both the intensity and age of infection were similar (see Harris, 1988, 1993). However, pragmatically, these data illustrate the importance of examining the gills for *G. salaris,* which has generally been assumed to be a skin parasite when monitoring epidemics in Norway (Appleby and Mo, 1997) and elsewhere in the EU.

Parasite distribution may also vary with infection intensity. At intensities of less than 100 parasites, more *G. salaris* were located on the dorsal fin, followed by the pectoral and anal fins, but at intensities of greater than 100 parasites more *G. salaris* were located on the caudal fin, and when exceeding 1000 parasites the body of the fish is also heavily infected (Jensen and Johnsen, 1992). Mo (1992, 1997) speculated that the preferred dorsal, pectoral and pelvic fins may be

related to transmission, which is primarily via the substrate by detached parasites while the fish rest, and not via host-to-host contact (Bakke *et al*., 1991a, b; Jensen and Johnsen, 1992). The preference for the dorsal fin might be due to physical contacts between fish as they nip each other while defending the territory against intruders (Mo, 1992). Appleby (1996a) noted increased abundance of *G. callariatis* on the skin in very heavy infections, which he interpreted as a migration of parasites out of the gill chamber for transmission. Conversely, in small infections, most worms were found on fins, suggesting they had recently arrived on the cod.

Individual gyrodactylids tend to move short distances on the host to feed (see Section 3.1), but at high parasite densities, intraspecific competition and resource depletion may induce a switch in site preference on a particular host. *Isancistrum subulatae,* which occurs on the arms and tentacles and suckers of the squid *Alloteuthis subulata*, is found on the surface of the head and the eyes only in heavy infections (>1000), and only in extremely high parasite loads does it enter the mantle cavity (Llewellyn, 1984). Crowding of growing infrapopulations of *G. salaris* on salmon may also result in dispersal of the parasite over wider areas of the host's skin and fins (Appleby and Mo, 1997).

Site specificity may be influenced directly or indirectly by external factors. Salinity influences site selection by *G. callariatis.* In salt water it parasitises the host's body, whereas in brackish water it is found mainly on the gill arches (Malmberg, 1970; Appleby, 1996a). This is also observed in *G. arcuatus*, although in this case, interaction with other gyrodactylid species may be as important as the direct impact of salinity. This species can infect gill arches, filaments, pharynx or skin and fins. In UK freshwater, it is normally restricted to the gills, with *G. gasterostei* present on the skin surface. In UK marine environments, *G. arcuatus* is often found on the skin surface, with *G. branchicus* in the gills (Harris, 1993). An increase in the proportion of *Gyrodactylus* individuals on the gills of cyprinids (goldfish and golden shiners) with increasing temperatures has also been observed (Anthony, 1969; Kirby, 1981). However, in these studies, individuals were not accurately identified, and the most likely explanation of this observation is that different species were present on the gills and skin that responded differently to temperature.

Morphological and physiological host parameters may also influence site specificity. Buchmann and Uldal (1997) infected four different salmonids (rainbow trout, brown trout, and Baltic and Atlantic strains of salmon) and found differences in site selection on each. Similarly, the site preference of the Danish Gx morph of *G. salaris* differed between rainbow trout and salmon (Lindenstrøm *et al.*, 2003a). Microhabitat preferences of *G. salaris* in relation to susceptibility status of different salmonid strains has also been tested, with the highest proportion of parasites found on the fins and head of all fish species and strains (Heinecke, 2005). However, over time they found an increasing percentage of parasites on the caudal fin of susceptible East Atlantic salmon strains and a converse tendency of decreasing proportion on the tail of the responding Swedish Lule salmon. The factors triggering migration of gyrodactylids are unknown but are probably related to resource availability, infrapopulation density, parasite age, reproductive status, avoidance of the host's immune response, and finally inbreeding or hybridisation avoidance.

The age of individual gyrodactylids may be important in migrations. Young *G. gasterostei* placed on the head or pectoral fins of three-spined sticklebacks almost inevitably migrate towards the caudal fin (Harris, unpublished). Activity is closely linked to reproductive status and increases in post-first and post-second birth mothers that contain small embryos. This was observed both from living attached worms (Harris, 1982) and deduced from the age structure of detached parasites in sediment (Harris, 1993). Older *G. gasterostei* (post-second birth) develop a swollen, empty uterus which is filled with a clear fluid (Harris, 1985, 1997; Cable and Harris, 2002), and wander extensively upon the host, being found at widely different points on different days. It is not clear whether this is a change in behaviour as parasites become more active, or whether these older individuals readily become dislodged (see Lester, 1972) but in confined experimental situations can reattach again to different parts of the host. In *G. salaris,* most newborn parasites move only short distances but those which do migrate (defined as movement from one region to another) most commonly re-locate immediately after each birth. The majority migrated anteriorly when placed on the caudal fin

and did not return to posterior sites; the migration and distance from the original site increased with parasite age (Cable *et al*., 2000). Worms migrating from the tail were normally found on the peduncle (42%) or the anal or adipose fin. Only post-second birth or older worms migrated as far as the pectoral or more rarely the pelvic or dorsal fins. In *G. salaris*, the level of activity of individual worms after birth of the first daughter varies according to the particular stock of salmon that is infected (Cable *et al*., 2000), illustrating the complex interplay between factors influencing migration on the host. Movement of gill parasites is particularly interesting as it must be undertaken for transmission to occur. In UK lowland populations of *G. arcuatus*, where the parasite is normally found in the stickleback gill chamber, migration of older specimens onto the skin was noted during epidemic population growth (Harris, 1993). This suggests a movement of parasites from the gill chamber to achieve transmission. For obligate gill parasites (e.g. *G. rarus*), such migrations are a prerequisite for transmission and must take place at some point in the life cycle. *Isancistrum loliginis* from the squid *A. subulata* (see Llewellyn, 1984) must leave the gills to achieve transmission, but has only rarely been found among *I. subulatae* on the arms and tentacles. If transfer occurs during squid copulation both species may transfer to the arms but later migrate to their respective site-specific sites. The only gyrodactylids in which migrations have been directly observed are *Gyrdicotylus* spp., from the African clawed toad *Xenopus* spp. Entry to the mouth is normally via the nostril, and parasites experimentally placed on the skin orientate towards and enter the nostril, emerging into the mouth shortly after (Harris and Tinsley, 1987). The reverse migration, out of the mouth cavity, has not been observed directly, although specimens of *Gyrdicotylus* do appear in the water in old infections, suggesting migration away from the toad (Harris and Tinsley, 1987; Jackson and Tinsley, 1994).

Much migration behaviour may relate to immunological changes in the host epidermis. *G. derjavini* on rainbow trout normally prefers the caudal, pectoral, pelvic and anal fins, but as infections progress the proportion of worms on the pectoral fins declines (Lindenstrøm and Buchmann, 1998). In corticosteroid-treated immunosuppressed fish, this trend is reversed (Lindenstrøm and Buchmann, 1998).

Olafsdóttir *et al.* (2003) noted aggregation of *G. derjavini* on the cornea (said to be an immunologically privileged site), with up to 30% localising on this relatively small area. This aggregation is reduced if hosts are immunosuppressed with dexamethasone (Olafsdóttir *et al.*, 2003). Interestingly, Cable (unpublished) have noted similar behaviour by *G. bullatarudis*, but not by *G. turnbulli* which occurs on the same poeciliid host. Similarly, Pie *et al.* (2006) have suggested that migrations by *G. anisopharynx* on the surface of *Corydoras* sp. are not adaptive in terms of transmission, but may be concerned with evading either the immune response or intra-specific competition. It has been suggested that mucous cell densities, which differ over the fish surface (Pickering, 1974), may influence behaviour and survival of gyrodactylids on rainbow trout, as they may select mucus cell-rich areas during initial colonisation but escape these areas during the host response phase (Buchmann and Bresciani, 1998). These results strongly suggest that mucous cells play a decisive role in gyrodactylid site selection. On abnormal hosts, distribution may change. Mo (1997) found *G. salaris* mainly on the gills and mouth of the strongly resistant brown trout. However, mucous cell density decreases when exponentially growing *G. salaris* populations are present, suggesting that more complex factors may influence site selection (Sterud *et al.*, 1998). Matejusová *et al.* (2006) support this, suggesting very local expression of particular genes in relation to damage by parasites. *G. vimbi* on roach aggregates around the anus, but this species apparently migrates to the gills during the host response, returning to the skin when the acquired immune response has declined (unpublished). In *Macrogyrodactylus polypteri*, parasites may aggregate around inflamed areas of epidermis (Khalil, 1970), while surrounding skin is unexploited. These aggregations move from day to day and periodically disperse, but always appear to be associated with inflamed host tissue (Harris, unpublished). Appleby and Mo (1997) explained the frequent occurrence and relative high intensity of *G. salaris* on salmon gills as a possible evasion of the immune response. Generally, changes in the gyrodactylid site selection have been interpreted as being an escape from localised immune reactions (Richards and Chubb, 1996; Buchmann and Bresciani, 1997; Buchmann and Uldal, 1997; Buchmann, 1998a, b).

There is a temptation to regard site selection by gyrodactylids as niche restriction or specialisation to reduce competition with other species. Site selection can also enhance reproductive isolation, by ensuring that conspecifics find, and mate with, only their peers. This was originally suggested to account for the restriction of egg-laying polyopisthocotylean monogeneans to particular gill arches (Llewellyn, 1956; Rohde, 1979). There is little consensus about site selection and restriction by parasites (e.g. Friggens and Brown, 2005), and it is worth remembering that we know very little of the factors controlling gyrodactylid distributions. There is little consistent evidence that gyrodactylids can exhaust nutritional resources, as, for example, salmon can tolerate infections of several thousand *G. salaris* without immediate death (Mo, 1992), while guppies may support less than 20 *G. turnbulli* before dying (Madhavi and Anderson, 1985; Cable *et al.*, unpublished). Different gyrodactylids all exploit the same carbon source when infecting a fish; over-exploitation leading to host death by one species will kill individuals of another gyrodactylid species on the same host, even if in a different microhabitat. It could be argued that microhabitat segregation allows one species to avoid a host response induced by another gyrodactylid. However, Richards and Chubb (1996) found that the host response affected both *G. turnbulli* and *G. bullatarudis* in similar ways, despite their differences in microhabitat. There is also no evidence that gyrodactylids of minnows, for example, which support more than 15 gyrodactylid species show any greater niche specialisation than do those of fishes, such as tench, which support only two gyrodactylid species. Arguments from reproductive biology are also not compelling; in general, gyrodactylids do not show the extent of microhabitat specialisation of dactylogyrids or ancyrocephalids, and they can also reproduce asexually (Section 2.8). Aggregation for the purposes of sexual reproduction is therefore unlikely.

Adaptive variation of the attachment organs is clearly important in the evolution of site specificity, as skin parasites generally have different haptoral morphology to gill parasites, and host shifts occur more readily than site shifts. Nevertheless, there are members of skin-parasitic groups which have become specialist gill parasites (e.g. *G. tincae*), although in this case there is no pressure to avoid competition with other gyrodactylids. The shift in the distribution of *G. arcuatus* relative to other

species present may on the other hand be due to competitive interactions. However, there are many more distributions which cannot be explained in this way and there is much to learn about gyrodactylid site specificity before we can speculate on issues of niche restriction.

3.3. Migration Between Hosts: Lack of a Dispersive Phase

In most parasites, specific transmission stages or larval adaptations for dispersal have evolved. In contrast, gyrodactylids have no specific transmission stage as the newborn individual attaches itself to the same host as its mother and host transfer can occur at any life stage. Transmission must be linked either to the development of a hostile microenvironment due to host immunity or death, to occasional chance contacts with other hosts but related to the daily and seasonal host behaviour and ecology, to eventual density-dependent mechanisms within parasite infrapopulations themselves, or to accidental dislodgement.

Gyrodactylids may survive varying periods away from the host. Old accounts broadly refer to survival of 48 h (Guberlet *et al.*, 1927; Bychowsky, 1961; Tripathi, 1957), but this depends on temperature, species and physiological condition. At 15°C, Lester and Adams (1974a) observed a mean life expectancy of 1.8 days in *G. alexanderi* detached from *Gasterosteus aculeatus*. In *Gyrodactylus gasterostei* at 10°C, mortality was constant for the first 60 h of detachment, after which it increased substantially until the end of life, suggesting worms could survive until the exhaustion of energy reserves (Cable *et al.*, 2002b). However, embryos of parasites long detached from a host tend to be aborted or show developmental abnormalities, suggesting that energy reserves are withdrawn from the embryo to sustain the parent (Cable *et al.*, 2002b). In *G. salaris*, survival is improved by remaining attached to a dead host. At 18°C, maximum survival of worms attached to glass was only 27 h. On dead salmon, however, worms survived much longer, up to 72 h (Olstad *et al.*, 2006). Parasites were particularly active on dead hosts, moving short distances on the fins during the 24 h after host death. A high proportion of *G. salaris*

remain with their dead hosts, even after decay had begun (cf. *G. turnbulli*; Cable *et al.*, 2002a). After 72 h, they could re-infect naïve hosts much more successfully than worms attached to glass. Clearly, *G. salaris* is specialised to remain with the host after death, achieving survival advantage from doing so (Olstad *et al.*, 2006), although it can also drift in the water column, attaching to salmon both high in the water column or close to the bottom substrate (e.g. in rivers with water flow 0.25 m/s; Bakke *et al.*, 1992a; Soleng *et al.*, 1999a).

Detached *Gyrdicotylus gallieni, Gyrodactylus turnbulli* and *G. bullatarudis* survive for similar periods (Harris and Tinsley, 1987; Cable, unpublished) at tropical temperatures (25°C), and *Macrogyrodactylus polypteri* can survive for up to 5 days away from the host (Khalil, 1970; Harris, unpublished) at 25–30°C. Under these circumstances, energy conservation is paramount, and although the swimming behaviour of *G. rysavyi* (see El-Naggar *et al.*, 2004b) may be a high-cost transmission strategy, most gyrodactylids adopt a "sit and wait" strategy, with limited movement until a host approaches. A substantial proportion of the total *M. polypteri* population can be detached at any one time (Harris, unpublished), and movement between the fish and the substrate appears a normal part of this host–parasite interaction. This is also true of *G. salaris,* which may survive for prolonged periods off the hosts, for example, attached to the walls (plastic) of fish tanks or net (metal) of grid boxes, and later infect newly introduced fishes (Bakke *et al.*, 1991a, 2002, unpublished; Olstad *et al.*, 2006). A high proportion of the *G. salaris* suprapopulation may, at any moment, be off the salmon due to transmission hazards in lotic habitats and the death of heavily infected hosts. Normally, however, the proportion of the population detached in other *Gyrodactylus* species is probably small. Harris (1982, 1988) used dishes on the tank floor to quantify detachment in *G. gasterostei* from sticklebacks and *G. turnbulli* from guppies. In both cases, although substantial numbers of parasites were recovered, they still represented a relatively small proportion compared to the number still attached to the hosts. Specific behaviours facilitate transmission in *G. turnbulli* (see Cable *et al.*, 2002a). This species crawls into the meniscus of the water column to hang from the surface film, increasing opportunities for transmission when the surface-feeding host guppy skims the surface film for floating food items.

Transmission from living hosts is parasite age and stage dependent. In both *G. turnbulli* on guppies (Harris, 1989) and *G. salaris* on salmon (Harris *et al*., 1994), the detached parasite population recovered from the water column contains an excess of older (post-first) birth worms. In the case of *G. salaris* (Harris *et al*., 1994), this could be refined to show excess detachment of individuals 1–2 days after they had given birth for the first, second or third time. This coincides with an increase in activity seen in individuals of this age in *G. gasterostei* (see Harris, 1980, 1982) and presumably results in increased transmission of these age/stage classes. This phenomenon has also been deduced from observations of transmission of *G. sphinx* on the Black Sea Blenny (*Aidablennius sphynx*) where transmission is linked to development of the male reproductive system (Dmitrieva, 2003).

Vertical transfer of gyrodactylids may also occur. *G. salaris* cannot feed on salmon eggs, but they can attach and survive for up to 2 days (Mo, 1987; Bakke *et al*., 1998) making eggs potentially important in transnational dispersal by Man. However, *G. salaris* display a strong preference for newborn alevins as oppose to salmon eggs (Mo, 1987). Transmission may take place during adult–fry interactions, either during birth (*G. turnbulli* on guppies; Cable, unpublished) or brooding (*G. gasterostei* on three-spined sticklebacks; Cable, unpublished). Gyrodactylids are particularly common on fish with highly developed brood care, and the possibility of transfer from parent to offspring at birth (poeciliids) or within the nest (sticklebacks, gobies, blennies) is likely to be increased compared to fishes that show little parental investment or live in age-structured shoals. However, the introduction and spread of *G. salaris* and *G. vimbi* within the new salmon and roach generation, respectively, in spring occurs early and rapidly (Jansen and Bakke, 1993a; Appleby and Mo, 1997; unpublished). Harris (1982) demonstrated rapid acquisition of *G. gasterostei* and other ectoparasites by young stickleback fry, which presumably took place from male fish to fry in the nest. Transmission from these individuals to other stickleback fry then took place rapidly in the large shoals of young fish in river margins.

Transmission strategies must also take into account the population structure of the host population. This is very clear in *G. salaris*, a

parasite of wild salmon parr (Johnsen, 1978; Johnsen and Jensen, 1988, 1992, 1997, 2003; Jansen and Bakke, 1993a; Appleby and Mo, 1997). The length of the freshwater parr phase varies significantly, from ~9 months in southern Britain and Spain to 5 years in Northern Norway (Nicieza *et al*., 1994; Bakke and Harris, 1998). Although parasite dispersal to new salmon populations probably occurs via adult ascending and spawning salmon and parr (Soleng *et al*., 1998), or between precocious male parr, transmission between parr generations is also important in sustaining a *G. salaris* infection in a river. Clearly, there is greater potential for intra-parr transfer in the North of Norway than there is in southern Europe. The pathogenic potential of *G. salaris* is bound to be related to salmon population structure, and the parasite is unlikely to be highly pathogenic in rivers at the southern limit of the range. Here, there is a distinct break between parr generations, salmon are entirely absent from the river for a short period (Bakke and Harris, 1998) and *G. salaris* cannot survive in salt water (see Section 9.2).

These specific transmission behaviours are clearly an important part of gyrodactylid evolutionary biology and worthy of further investigation, especially given the ecological diversity of gyrodactylid hosts. Insufficient parasite species have been examined, but trends towards particular transmission strategies may occur, for example, in species infecting solitary compared to shoaling fishes, lotic vs. lentic hosts, or pelagic vs. benthic hosts. Finally, understanding the processes that facilitate transmission may be important in determining speciation mechanisms, as they are important in host switching.

Part 2. Gyrodactylid systematics, phylogeny and evolution

4. PHYLOGENY: THE FAMILY ALBUM

4.1. Taxonomic History

Attempts to subdivide the viviparous gyrodactylids (see below), have until recently been based entirely on morphology, primarily that of the attachment hooks and bars. This has run parallel with discussions

over the species concept in *Gyrodactylus*. Although most authors tend to split species, a few have taken a wider view. Wagener (1860), for example, grouped all similar gyrodactylids from cyprinids into a single species, which was never formally described. Sproston (1946) similarly recognised only "*G. elegans*" or "*G. medius*", discriminating on hamulus characters now known to be a fixation artefact. Although this approach has long been discredited, it explains many spurious records [e.g. *G. elegans indicus* (see Tripathi, 1957) and *G. medius* from *Ciliata mustela* (see Srivastrava and James, 1967)].

Malmberg (1970) was the first to systematise relationships within *Gyrodactylus* and the Gyrodactylidae based on morphology. His scheme was based on the excretory system, supplemented with observations on marginal hook type, and assumed that the excretory system had evolved from greater to lesser complexity by the loss of flame cells and secondary canals (Malmberg, 1957, 1964, 1969, 1970). Six subgenera (*Gyrodactylus*, *Mesonephrotus*, *Paranephrotus*, *Metanephrotus*, *Neonephrotus* and *Limnonephrotus*) were formally described (Malmberg, 1970) using these characters and host group/habitat. Malmberg (1970, 1993) also established species groups within each subgenus based on marginal hook morphology. The gyrodactylid subgenera were linked to the larger host phylogenetic categories within Greenwood *et al.*'s (1966) teleost classification. For example, species of the subgenus *Gyrodactylus* (according to Malmberg, the most primitive subgenus), infect ostariophysans, placed by Greenwood *et al*. (1966) at the base of the teleost stem. Within this subgenus, two species groups, the *G. elegans* and *G. phoxini* groups were recognised. The *G. elegans* group are gill parasites with a thin, spine-like ventral bar membrane, while *G. phoxini*-type species are skin parasites with a broad, spoon-like ventral bar membrane.

Malmberg's (1970) contribution in establishing the foundation for subsequent phylogenetic and taxonomic studies of this diverse group cannot be over emphasised. However, his scheme suffers from an assumption of host–parasite co-evolution. Originally based on a now outdated fish phylogeny (Greenwood *et al*., 1966), Malmberg (1998) updated his work using Nelson's (1994) fish classification, but problems remain. The most significant weaknesses are the lack of an independent character system to establish the direction of excretory

system evolution, and the assumption that *Macrogyrodactylus polypteri* has co-evolved with the chondrostean *Polypterus* and therefore the evolution of viviparous gyrodactylids predated the appearance of the teleosts. In fact, most *Macrogyrodactylus* species infect catfishes (Prudhoe, 1957; Paperna, 1979; El-Naggar and Serag, 1987), suggesting the association with chondrosteans is due to a host switch. Bakke *et al.* (2002) could find little convincing evidence for any co-evolutionary relationships between gyrodactylids and their fish hosts, and certainly not before the evolution of the Ostariophysi or Clupeomorphs. Molecular work has shown the importance of host switching which has occurred even between families (Ziętara *et al.*, 2002) as well as orders (Huyse *et al.*, 2003) of fishes in gyrodactylid evolution (see Section 6.1) and at the species level attempts to identify co-evolutionary trends have failed. Nevertheless, there is some support for groupings based on morphological, ecological and biogeographical trends, and although Malmberg's (1970) species groups have no formal taxonomic status, they have been important for handling the complexity of this genus.

Malmberg's subgeneric analysis was restricted to Scandinavian species and has never been fully adopted by other researchers, probably because the excretory system can only be studied in living worms. Only Gläser (1974) extended the excretory system analysis to describe new species groups within *Gyrodactylus*, again, for North European species and only from gobies. A great number of North American and Eastern Eurasian species may belong to existing species groups, but without redescription or molecular analysis their relationships cannot be confirmed. The danger of basing species groups on morphology and host group only is shown clearly by the *G. salaris* group, created by Malmberg (1993) to accommodate *G. wageneri*-like forms from salmonids. This notion of a separate radiation on salmonids is the simplest co-evolutionary hypothesis and accounts for those few *G. wageneri*-like forms that are found on salmonids in North America (Cone *et al.*, 1983). However, molecular phylogenies fail to support a separate *G. salaris* group (Cable *et al.*, 1999) and infer strongly that salmonids have instead been infected on several occasions by *G. wageneri*-like gyrodactylids (Matejusová *et al.*, 2003; see Section 5.3.2).

4.2. The Family Gyrodactylidae

The Gyrodactylidae (Beneden and Hesse, 1864), as originally constituted included only the viviparous genus *Gyrodactylus*. As explained by Bychowsky (1961), Beneden and Hesse (1864) referred to the "Gyrodactylides" rather than the Gyrodactylidae, and it was left to later authors (principally Cobbold, 1864) to implement the formal family name. This explains the confusion in the literature over the authority for the family "Gyrodactylidae". We agree with Bychowsky (1961) that the origin of the grouping in the modern sense lies with Beneden and Hesse (1864) and these authors should therefore be credited with establishing the family. A series of subfamilies have been erected piecemeal to accommodate genera based on haptor morphology. The Isancistrinae was erected by Furhmann (1928) for *Isancistrum*, lacking all bars and hamuli. Rogers (1967) erected the Polyclithrinae, for *Polyclithrum*, with numerous supporting bars in the haptor. All other genera have been assigned to the catch-all subfamily Gyrodactylinae. We deprecate the use of these subfamilies, which are based on morphology only and may reflect convergence rather than true relationship. They have received little support in either molecular (e.g. Matejusová *et al*., 2003) or morphological (e.g. Malmberg, 1998) phylogenies. However, we do recognise the division between the viviparous genera and the oviparous forms, which are so profoundly different biologically, and agree with Boeger *et al*. (1994) that viviparity evolved once within one group of the Gyrodactylidae. The family therefore includes both egg-layers and live-bearers, and the family Ooegyrodactylidae of Harris (1983) should be allowed to lapse.

4.3. The Genera

Based on morphological criteria, 30 genera have now been described (7 oviparous and 23 viviparous; see examples of genera in Figures 19 and 20). Several viviparous genera are however invalid. *Paragyrodactylus* Szidat is invalid as the name was preoccupied by *Paragyrodactylus* Gvosdev and Martechov. The genus was renamed as

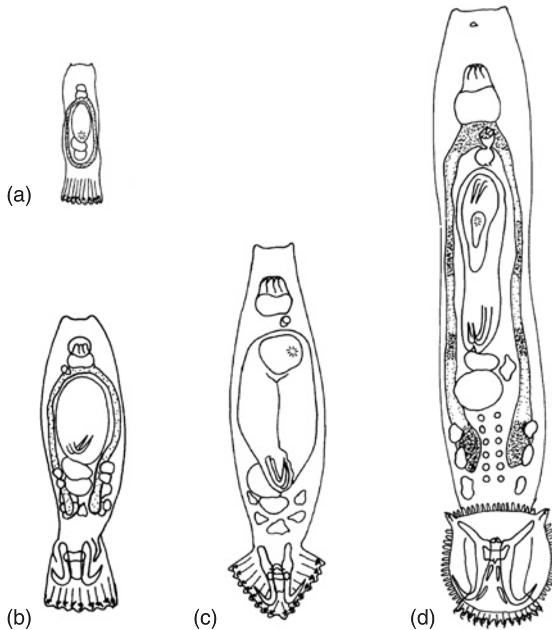


Figure 19 Relative body size and morphological complexity amongst the viviparous gyrodactylids. (A) *Isancistrum*, from squid (body length ~0.1 mm). Redrawn from Llewellyn (1984) and original observations; (B) *Acanthoplacatus*, from tropical marine fish (body length ~0.3 mm). Redrawn from Ernst *et al.* (2001a); (C) *Gyrodactylus* (body length ~0.6 mm, although much smaller and larger species are also known in this genus, 0.1–1 mm). Original; (D) *Macrogyrodactylus* from African fish (body length 1.0–1.5 mm). Redrawn from Malmberg (1956) and El-Naggar and Serag (1987). All drawn to scale.

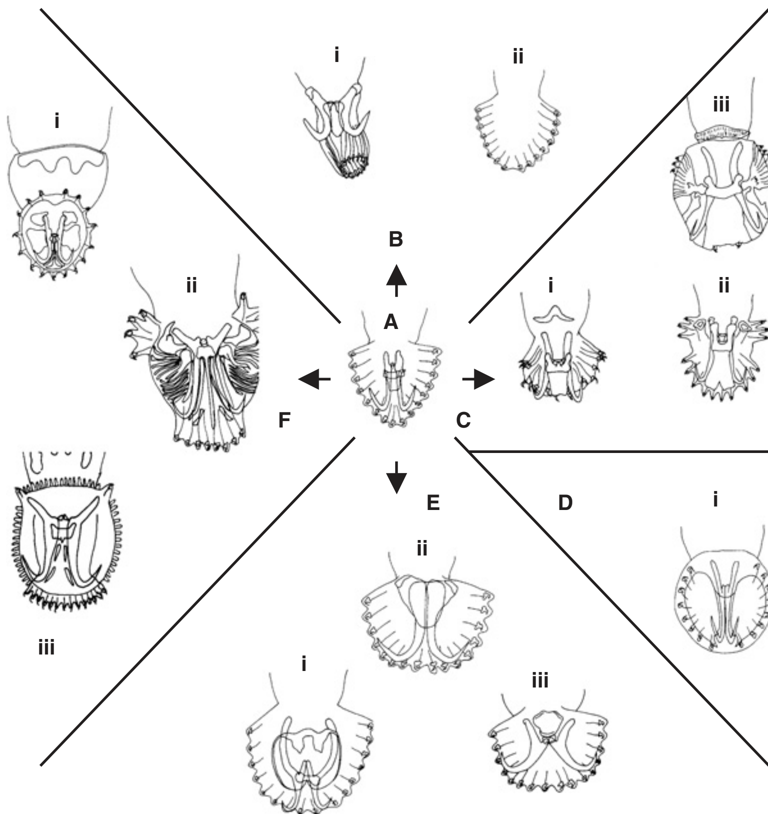
Paragyrodactyloides (Szidat), but the species concerned is now regarded as a *Gyrodactylus* (Popazoglo and Boeger, 2000), making the generic name a junior synonym. *Neogyrodactylus* Prudhoe is similarly a junior synonym of *Macrogyrodactylus. Neogyrodactylus* Baugh, 1957 was pre-occupied and therefore should not be used. Yamaguti introduced *Metagyrodactylus* (Baugh) as a replacement. *Micropolyclithrum* is considered a junior synonym of *Polyclithrum* (Ernst *et al*., 2000). We also recommend that *Fundulotrema* Kritsky and Thatcher and *Metagyrodactylus* (Baugh) Yamaguti be considered as junior synonyms of *Gyrodactylus* (see below). *Afrogyrodactylus*

was synonymised with *Gyrodactylus* by Paperna (1979). The taxonomic status of *Swingleus* Rogers remains uncertain.

The following genera make up the family Gyrodactylidae Beneden and Hesse, 1864:

4.3.1. Oviparous Genera

The oviparous *Phanerothecium* was originally described as viviparous (Kritsky and Thatcher, 1977), but its true significance was recognised by Harris (1983), who erected the Ooegyrodactylidae to accommodate this genus and *Ooegyrodactylus*. The Ooegyrodactylidae is paraphyletic (Boeger *et al.*, 1994) and so the oviparous forms are considered here as part of the Gyrodactylidae. The egg-laying genera



possess a unique combination of traits (16 articulated marginal hooks, single pair of hamuli with connective dorsal bar, ''spike'' sensilla) characteristic of the gyrodactylids. They are all protandrous and lay eggs with a sticky droplet that hatch to release unciliated crawling larvae with prominent spike sensilla. Seven genera have been described, including three new genera proposed by Kritsky *et al.* (2007), based on differences in the structure of the male reproductive system and the vitelline system [although Kritsky *et al.* (2007) comment that the taxonomic significance of the vitelline follicles and ducts should be re-evaluated].

These parasites infect South American catfish, predominantly loricariids. Some infect pimellodellids and Kritsky *et al.* (2007) also recorded undescribed forms from trichomycterid catfish. These often live within the gill chamber of loricariids and the parasites may be

Figure 20 Haptor diversity in the viviparous gyrodactylids. (A) Basic haptoral type with two hamuli, linking dorsal bar, ventral bar and 16 marginal hooks. Exemplified by *Gyrodactylus*. (B) Successive diminution of the haptor, in (i) *Acanthoplacatus* (hamuli and bars fail to migrate) and (ii) *Isancistrum* and *Anacanthocotyle* (attach to fish and cephalopod skin), hamuli and bars lost. (C) The ''*Swingleus*'' group (attach to fish skin). Hamular and ventral bar morphology suggests these genera are related. (i) *Fundulotrema*, peduncular bar and grouping of marginal hooks (three pairs anteriorly). This genus differs from species of *Gyrodactylus* infecting *Fundulus* only in the peduncular bar (ii). *Swingleus*, as *Fundulotrema*, but grouping of marginal hooks is more prominent, accessory plates present. (iii) *Accessorius*, as *Swingleus* but peduncular bar absent, accessory plates absent and two sucker-like sclerotised structures present. (D) Modification of haptor into discrete suckers in genera that attach to amphibian buccal epithelium. (i) *Gyrdicotylus*. (E) Marine forms (attach to fish gills), hamuli with two roots, accessory plate present in haptor, function unknown. (i) *Gyrodactyloides*; (ii) *Archigyrodactylus*; (iii) *Lamniscus*. (F) Large parasites (attach to fish skin), entire haptor modified into flattened sucker. (i) *Mormyrogyrodactylus*. Peduncle with peduncular bar present and functioning in attachment; (ii) *Polyclithrum*. Marginal hooks grouped anteriorly and posteriorly, accessory bars present; (iii) *Macrogyrodactylus*. Marginal hooks grouped anteriorly and posteriorly, different arrangement of accessory bars present. Note that these groupings are not exhaustive (*Swingleus* could equally be placed within group F), and that phylogenetic relationship is not implied by similarity of haptoral mechanism.

utilising the trichomycterid as a phoretic host. The loricarid catfish were probably one of the most recent groups of bony fishes to differentiate, making them unlikely primary hosts for such a large and diverse group as the gyrodactylids. Boeger *et al.* (2003) use the occurrence of egg-laying genera on loricariids to suggest that gyrodactylids diversified from a South American origin some 60 million years ago. We prefer to suggest that the presence of oviparous gyrodactylids on catfish is the result of an ancient host switch.

(1) *Aglaiogyrodactylus* Kritsky, Vianna and Boeger, 2006: Species of this genus, recovered from loricarid (*Kronichthys*) catfish in Brazil, possess a complex accessory piece and a male copulatory organ enclosed within the copulatory sac.
(2) *Hyperopletes* Boeger, Kritsky and Belmont-Jégu, 1994: Parasites of loricariid catfishes in Brazil, *Hyperopletes* is distinguished from the other egg-laying gyrodactylids by a copulatory organ armed with fine spines and by the arrangement of seminal vesicles; otherwise, it is very similar to the other oviparous forms.
(3) *Ooegyrodactylus* Harris, 1983: Collected from the loricariid catfish (*Farlowella*) from Brazil/Peru, this genus can be distinguished by an entirely muscular penis with small basal bulb. The growth from small crawling larva through male phase to the large egg-laying females was described by Harris (1983) and it is the only oviparous genus so far to have been maintained in laboratory culture. Kritsky *et al.* (2007) consider this genus most similar to *Phanerothecium*, separated by characters including short egg-filament, separate seminal receptacle, absence of pregerminal vitelline follicles and inverted U-shaped vitelline ducts. The original name (*Oögyrodactylus*) was modified to *Ooegyrodactylus* in accordance with the International Code on Zoological Nomenclature.
(4) *Phanerothecium* Kritsky and Thatcher, 1977: *Phanerothecium* differs from *Ooegyrodactylus* in having the intromittent organ supported by a thin external layer of hardened protein. Some species of *Phanerothecium* retain egg clusters *in utero*, have a long egg filament, the seminal receptacle is absent or intra-germarial and pre-germarial vitelline follicles are

present. Species have been described from the loricariid catfish *Plecostomus* (see Kritsky and Boeger, 1991) and *Hypostomus* (see Boeger *et al.*, 1994), suggesting that this genus also primarily infects loricariid catfish, although the type species was described from the pimellodellid *Zungaro zungaro*.

(5) *Phanerothecioides* Kritsky, Vianna and Boeger, 2006: Species from this genus infect *Hypostomus* catfish in Brazil. The parasites lack haptoral bars, there are no pre-germinal vitelline follicles and the vitelline ducts take the form of an inverted U-shape. Species have a conspicuous testis, a syncytial prostatic gland and a reduced copulatory sac.

(6) *Nothogyrodactylus* Kritsky and Boeger, 1991: Described from the loricarid catfish *Ancistrus* from Brazil, this genus was originally distinguished by the presence of accessory sclerites on the penis; these sclerites have subsequently also been found in *Onychogyrodactylus* and *Aglaiogyrodactylus*.

(7) *Onychogyrodactylus* Kritsky, Vianna and Boeger, 2006: This genus is similar to *Nothogyrodactylus* with species of both infecting *Ancistrus* spp. Species are characterised by the spine-like accessory sclerites lying within a separate pouch to the copulatory sac.

4.3.2. Viviparous Genera

The viviparous genera are remarkably uniform, with little variation in internal anatomy. Differences tend to be either in the presence of accessory sclerites on the haptor (*Polyclithrum*, *Swingleus*, *Fundulotrema* and *Macrogyrodactylus*) or loss of hamuli and bars (*Anonchohaptor* and *Isancistrum*), or in penis structure (*Gyrdicotylus*, *Mormyrogyrodactylus* and *Scleroductus*). Molecular evidence (Cable *et al.*, 1999; Ziętara *et al.*, 2002) indicates fundamental differences even within the genus *Gyrodactylus*, which are not reflected in morphology.

(8) *Afrogyrodactylus* Paperna, 1968: Erected for *A. characinis* from *Alestes*, this genus has a tubular, spinous penis and hamuli

with a developed dorsal root. Synonymised with *Gyrodactylus* in Paperna (1979), this species would repay further examination as the penis structure suggests that it may represent a different genus, more akin, for example, to *Mormyrogyrodactylus* described by Luus-Powell *et al.* (2003).

(9) *Accessorius* Jara, An and Cone, 1991: This viviparous taxon from the South American characin, *Lebiasina maculata*, has two tubular sucker-like structures within the haptor. These appear to be reinforced, rather than simply muscular, and their function remains unclear. Jara *et al.* (1991) grouped *Accessorius* with *Polyclithrum* and *Swingleus* in the Polyclithrinae, but there is probably not a close relationship between the latter genera and there is certainly no reason to link *Accessorius* to them. The ventral bar morphology resembles that of *Gyrodactylus costaricensis* and *G. poeciliae*, but the detailed relationships of *Accessorius* remain unclear.

(10) *Acanthoplacatus* Ernst, Jones and Whittington, 2001: Recorded from the skin of *Siganus* spp. from tropical reefs, this genus is characterised by hamuli that remain embryonic and fail to migrate back down the peduncle into the haptor. Surprisingly, this genus is recorded as lacking spike sensilla (Ernst *et al.*, 2001a).

(11) *Anacanthocotyle* Kritsky and Fritts, 1977: A genus which lacks hamuli and bars: the haptor is armed only with 16 marginal hooks. Erected for *Anonchohaptor anonchohaptor* from a South American fish, this genus is poorly characterised and there seems little justification for it. Apart from host group, this genus cannot be distinguished from *Isancistrum*, and it appears to be a *Gyrodactylus*-type in which the hamuli and bars have been secondarily lost.

(12) *Archigyrodactylus* Mizelle and Kritsky, 1967: Recorded from the Pacific tomcod *Microgadus*, this genus has an elaborated ventral bar membrane, with wings spreading back around the haptor, probably to reinforce the suctorial disc. Insufficient is known about this genus to comment on its validity; however, the resemblance with *Laminiscus*, from gadids in the Atlantic, is striking.

(13) *Fundulotrema* Kritsky and Thatcher, 1977: This genus was erected by Kritsky and Thatcher (1977) to accommodate five *Gyrodactylus* species infecting *Fundulus* and related fishes in North America: *G. foxi* Rogers, 1973, *G. megacanthus* Wellborn and Rogers, 1967, *G. stableri* Hathaway and Herlevich, 1973, *G. trematoclithrus* Rogers, 1967 and *G. prolongis* Hargis, 1955. All possess a characteristic peduncular bar, which is thought to act as a pressure plate during attachment (Kritsky and Thatcher, 1977; Cone and Odense, 1988). There is little doubt that *Fundulotrema* is derived from *Gyrodactylus* species; indeed there is a close relationship between the hamulus and bar structure of *Fundulotrema* and that of *G. funduli* or *G. stegurus*, which also infect *Fundulus* but lack the peduncular bar. To this extent *Fundulotrema* cannot be regarded as valid, but should rather be seen (with the species lacking peduncular bars) as part of a species group of *Gyrodactylus* in the sense of Malmberg (1970).

(14) *Gyrdicotylus* Vercammen Grandjean, 1960: First recorded from the stomach of *Xenopus* toads in Kivu by Vercammen Grandjean (1960), this genus was redescribed by Harris and Tinsley (1987). These parasites infect the mouth and pharynx of *Xenopus* and the related pipid toads, *Silurana* and *Hymenochirus* (see Tinsley, 1996), and have a suctorial attachment mechanism in which the ventral and dorsal bars are absent, the hamuli have two roots which form the dorsal wall of the suctoral haptor, and the marginal hooks pin down a marginal valve around the edge of the haptor. The penis has a single complete row of large spines. *Gyrdicotylus* species have now been recovered from throughout sub-Saharan Africa (Harris and Tinsley, 1987; Tinsley 1996; J.A. Jackson, personal communication).

(15) *Gyrodactyloides* Bychowsky, 1947: Bychowsky established this genus for species parasitic from marine teleosts (gadids, osmerids and marine phase salmonids). The ventral bar lacks a membrane, the hamuli have two roots and the dorsal bar is absent. Instead, a thin, membraneous structure extends around from the anterior of the haptor to just posterior to the ventral bar.

(16) *Gyrodactylus* von Nordmann, 1832: The original genus described by von Nordmann (1832). Von Nordmann grouped both *Gyrodactylus* and what we now know as *Tetraonchus* and *Dactylogyrus,* within the same genus. It was left to Wagener (1860) to reformulate the generic description to include only viviparous forms. *Gyrodactylus* now contains over 480 species descriptions, ~400 of which are valid (Harris *et al.*, 2004).

(17) *Isancistrum* de Beauchamp, 1912: *Isancistrum loliginis* held the distinction of being the only monogenean from a cephalopod mollusc when described from the squid *Loligo* at Roscoff by de Beauchamp (1912). It was also characterised by the absence of hamuli and bars, the haptor being armed only with 16 marginal hooks. Subsequent workers were unable to rediscover this parasite until Llewellyn (1984) redescribed it from *Alloteuthis subulata*. Llewellyn also described *I. subulatae*, distinguished from the original species on the basis of site of infection. *Isancistrum* is probably derived from *Gyrodactylus*, although it shows a number of intriguing features. It is the smallest known gyrodactylid, with a body size of only 0.1–0.2 mm. Unlike *Anonchohaptor*, *Isancistrum* possesses a ring intestine. The extent to which *Isancistrum* has radiated onto cephalopods is not clear and molecular data are urgently needed for this genus.

(18) *Macrogyrodactylus* Malmberg, 1956: This genus includes the largest known gyrodactylids, attaining a maximum length of ~1.5–2 mm and clearly visible to the naked eye, infecting the skin of African freshwater fishes. Coincident with Malmberg's description, Prudhoe (1957) described a catfish-infecting form as *Neogyrodactylus*, which is therefore a junior synonym. The haptor is armed with two hamuli, ventral and dorsal bars and 16 marginal hooks. However, the anterior marginal hooks are reflected forwards, while the other seven pairs are arranged in a row along the posterior margin of the haptor. Additional hardened struts within the haptor reinforce the suctorial disc, which is also characterised by small, possibly sensory, peg-like tegumental extensions.

The penis is armed with flat plates (one larger than the others), rather than the hooked spines of other gyrodactylids.

There is also a secondary seminal receptacle behind the ECFR (Malmberg, 1957; El-Naggar and Serag, 1987; El-Abbassay, 2001) and numerous unidentified, lateral cells in the body, some of which have been identified as putative subtegumental cells (El-Naggar and Cable, in press), although the function of others remains unknown. In *M. polypteri*, the gut is banded (Malmberg, 1957), due to accumulation of melanin granules from the host *Polypterus* (see Cable *et al.*, 1997), but other species of *Macrogyrodactylus* have a transparent intestine. Having described *M. polypteri* from the primitive fish *Polypterus*, Malmberg (1957) considered this genus to be primitive. Most other species, including the form described by Prudhoe (1957), have, however been described from catfish, and it seems most likely that *Polypterus* is infected as the result of a host-switch. At a molecular level, *Macrogyrodactylus* appears to group with the African *Gyrdicotylus* from amphibians (see Matejusová *et al.*, 2003), but many more African species need to be included in the analysis to draw conclusions about the primitiveness and relationships of these genera.

(19) *Mormyrogyrodactylus* Luus-Powell, Mashego and Khalil, 2003: Described from the primitive African fish *Marcusenius macrolepidotus* in South Africa, this genus may be close to the forms described as ‘‘*Gyreteroncus*’’ by Euzet and Birgi (1988) in a conference abstract, but never properly described. Unfortunately, the internal structure of this genus was not fully described, and the homology of organ systems to those of other gyrodactylids is unclear. The significance of this genus is that it has many characters which appear primitive and it may be basal to many other genera. The haptor is reinforced to function as a shallow sucker, but anteriorly there is a broad peduncle, including a peduncular bar, which is also involved in attachment. Additional work on this genus is awaited with interest.

(20) *Neogyrodactylus* Prudhoe, 1957: A junior synonym of *Macrogyrodactylus* Malmberg, 1957.

(21) *Neogyrodactylus* Baugh, 1957: This genus was erected for a gyrodactylid from the parasitic crustacean *Argulus* in India.

According to the description, the ventral bar is absent. Current knowledge would suggest that this represents a *Gyrodactylus* species using *Argulus* as a phoretic host. However, the description is poor and does not correspond with any known description of a fish-infecting gyrodactylid. The name is preoccupied by Prudhoe (1957) and was therefore replaced by *Metagyrodactylus* Yamaguti 1963.

(22) *Metagyrodactylus* (Baugh) Yamaguti, 1963: Yamaguti (1963) used this name to replace the preoccupied *Neogyrodactylus* Baugh, 1957. However, there is little to separate this genus from *Gyrodactylus*, and we suggest that this genus be considered a junior synonym of the latter.

(23) *Polyclithrum* Rogers, 1967: Another genus with accessory supporting struts on the haptor and an asymmetrical distribution of marginal hooks (like *Macrogyrodactylus* and *Swingleus*). *Polyclithrum* has been recorded extensively from mullets (Mugilidae). As in *Macrogyrodactylus* (see above), there is a distinct seminal receptacle, lying to one side of the ECFR (Ernst *et al.*, 2000), which was not identified by Rogers (1967). This sets *Polyclithrum* apart from the *Gyrodactylus* species infecting mullets, and links it with *Macrogyrodactylus* and *Swingleus*. This may indicate relationship between these genera, or may simply be because they are the largest and least progenetic of the viviparous genera. Molecular data are needed to answer this question.

(24) *Micropolyclithrum* Skinner, 1975: Described to accommodate *Polyclithrum*-like forms from the mullet *Mugil cephalus* from Florida: considered a synonym of *Polyclithrum* by Ernst *et al.* (2000).

(25) *Scleroductus* Jara and Cone, 1989: A South American form from pimellodellid and auchenipterid catfish, *Scleroductus*, is characterised by two hardened ribs along the ejaculatory duct within the bulbous penis (Jara and Cone, 1989). Although resembling *Gyrodactylus*, Kritsky *et al.* (1995) agreed that the structure of the copulatory organ was sufficiently different to justify the separate genus. The intromittent organ has well-developed prostatic reservoirs and a seminal vesicle, and

appears intermediate between the tubular primitive penes (in *Mormyrogyrodactylus* and the oviparous genera) and the globular copulatory organ of *Gyrodactylus*. Further study of this genus could help resolve important aspects of the evolution of primitive gyrodactylids.

(26) *Swingleus* Rogers, 1969: *Swingleus* was erected for a gyrodactylid from *Fundulus* in which the haptor has one pair of marginal hooks shifted anteriorly while the remainder are grouped posteriorly. The haptor bears support struts and superficially resembles *Macrogyrodactylus* or *Polyclithrum*. However, *Swingleus* also possesses a peduncular bar, linking it with both *Fundulotrema* and those *Gyrodactylus* species (*G. funduli* and *G. stegurus*), with similar hamulus morphology that infect *Fundulus*. However, *Swingleus* also has an accessory seminal receptacle (Billeter *et al*., 2000), which may link it with *Macrogyrodactylus* and *Polyclithrum. Swingleus* potentially sheds light on the plasticity of the gyrodactylid haptoral apparatus; this radiation onto fundulids has resulted in considerable variation in haptoral armature.

(27) *Paragyrodactylus* Gvosdev and Martechov, 1953: A parasite from loaches from the central Asian Ili River basin, separated from *Gyrodactylus* because of the greater complexity of the attachment apparatus. Further study needed to confirm whether these differences are fundamental, or whether this represents a local diversification within *Gyrodactylus*.

(28) *Paragyrodactylus* Szidat, 1973: Erected for *Paragyrodactylus superbus*, a parasite of *Corydoras* from southern South America, the genus name was preoccupied by *Paragyrodactylus* Gvosdev and Martechov, 1953; the genus was therefore renamed *Paragyrodactyloides* by Nunez (1975).

(29) *Paragyrodactyloides* Nunez, 1975: Renamed because of preoccupation of *Paragyrodactylus*, *Paragyrodactyloides* was synonymised with *Gyrodactylus* by Popazoglo and Boeger (2000).

(30) *Laminiscus* Palsson and Beverly Burton, 1983: The haptor of *Gyrodactyloides gussevi*, from the capelin *Mallotus villosus*, differs significantly from that of other *Gyrodactyloides* species, having a rounded shield-like plate between the hamulus roots.

Palsson and Beverly Burton (1983) erected *Laminiscus* for this species and others (e.g. *G. strelkowi*) with a rounded plate between the hamulus roots. *Laminiscus* closely resembles *Archigyrodactylus* from gadids.

4.4. Evolutionary Affinities of the Gyrodactylids

Gyrodactylids are very different from other monogeneans, lacking structures and organ systems that may help determine their affinities. It is important to distinguish: (i) plesiomorphic characters which differ because ancestral oviparous gyrodactylids were different to other monogeneans, and (ii) apomorphic characters that have arisen due largely to progenesis and viviparity. Monogeneans typically have the highly modified, ciliated, swimming oncomiracidium larva, and Llewellyn (1981) discussed viviparity in *Gyrodactylus* from the viewpoint that this larva had been secondarily lost. Absence of a swimming larva was seen as an uninformative apomorphy resulting from the viviparous lifestyle. However, it then became apparent that gyrodactylids primitively lack a ciliated oncomiracidium (Harris, 1983), and that, like the terrestrial lice, all stages in the life cycle could transfer between hosts. The larva of *Ooegyrodactylus* is therefore plesiomorphic with the primitive unciliated crawling larva of the acanthocotylids, of *Enoplocotyle* and of *Udonella*. Plesiomorphic characters of gyrodactylids include: (i) a pair of multiciliate ‘‘spike’’ sensilla, one on each cephalic lobe (Lyons, 1969; see Figures 15 and 16), present throughout life; (ii) male and female reproductive systems with separate openings, but no secondary vaginae. The primitive intromittent organ is tubular, reinforced with rings, accessory sclerites or spines; (iii) an unciliated crawling juvenile, and (iv) pigmented eye spots absent throughout the life cycle. A combination of these plesiomorphic characters is shared with other primitive monogeneans, including the Acanthocotylids, *Enoplocotyle* and *Udonella*, although only *Enoplocotyle* shares all of these characters. Other characters, including the hinged marginal hooks (Boeger and Kritsky, 1997) also link the gyrodactylids with the acanthocotylids and *Enoplocotyle*, but these may be shared, derived characters.

The most striking resemblance is between the egg-laying gyrodactylids and *Enoplocotyle*, a small monogenean from the skin of the moray eel. In this genus, the haptor is armed with 16 small articulated marginal hooks, identical to *Gyrodactylus*. The larva is a primary unciliated "crawlaway", lacking eye spots and armed only with marginal hooks. The three species of *Enoplocotyle* are normally linked with the acanthocotylids, which also, have 16 marginal hooks, but develop a large pseudohaptor immediately in front of the haptor proper, armed with rows of plates which, in *Acanthocotyle*, engage with the dermal denticles of the host (Malmberg and Fernholm, 1991). In the related *Myxinocotyle*, the pseudohaptor is suctorial, allowing attachment to the smooth-skinned hagfish (Malmberg and Fernholm, 1989). *Enoplocotyle* has been poorly studied since first described by Tagliani (1912), but *E. kidakoi* is highly reminiscent of the ooegyrodactylids (Kearn, 1993), with four longitudinal rows of vitellaria, a post-germarial testis separate male and female systems, and a large fertilisation chamber (cf. the ECFR of gyrodactylids) adjacent to the germarium. Kearn (1993) also notes the presence of compound ciliary sensilla "at the anterior tip of each cone-shaped head lobe". No micrographs are available, but the described resemblance to the spike sensilla of gyrodactylids is considerable. Boeger and Kritsky (1997) also link the diffuse glands surrounding the fertilisation chamber of *Enoplocotyle* with those of egg-laying gyrodactylids. We consider it likely that enoplocotylids are probably the closest described monogeneans to the egg laying and viviparous gyrodactylids.

Frustratingly, acanthocotylids and *Enoplocotyle* (and the ooegyrodactylids) have not yet been included in molecular phylogenies. Using 18S rDNA sequence data, Olson and Littlewood (2002) linked the gyrodactylids with *Udonella*, the hyperparasite of caligid copepods which lacks opisthaptoral hooks and has often been treated as convergent with, rather than closely related to, the Monogenea. The morphology of *Udonella* is also very similar to that of *Enoplocotyle* and the oviparous gyrodactylids although the male and female systems share a common opening. Remarkably, despite all the molecular analyses of *Gyrodactylus*, no deposited 28S rDNA sequences were available to allow Olson and Littlewood (2002) to place *Gyrodactylus* more

precisely. Boeger and Kritsky (1997) using morphology, link the gyrodactylids with the acanthocotylids (including *Enoplocotyle*), forming a sister group to the anoplodiscids, another primitive group with hinged marginal hooks. This analysis, however, is based on the premise that *Enoplocotyle* is indeed an acanthocotylid; *Udonella* was not considered. Matejusová *et al.* (2003), using a wider range of gyrodactylid 18S rDNA sequences, also achieved a close sister group relationship (>90% bootstrap support) between the gyrodactylids and *Udonella*, with capsalids again the sister group to the *Gyrodactylus*/*Udonella* clade. These appear to represent the surviving terminal branches of an earlier monogenean radiation, prior to the appearance of the modern dominant groups such as the microcotylids and polyopisthocotyleans. An argument against the basal position of the ooegyrodactylids in gyrodactylid ancestry is the identity of the host group; the loricariid catfish are relatively recently evolved (Boeger *et al.*, 1994, 2003). However, other apparently primitive egg-laying monogenean groups also occur on modern fishes, presumably as a result of host switching. For example, *Udonella* is a hyperparasite of *Caligus* parasitic on a range of teleosts, *Enoplocotyle* is found on *Muraena*, while the acanthocotylids occur on elasmobranchs and agnathans.

The formal taxonomic position of gyrodactylids remains confused, partly because our perception of the position of these groups within the Monogenea is poorly developed. Today, the Monogenea are usually treated as a class of the Platyhelminthes (e.g. Brusca and Brusca, 1990 (see Tree of Life Project); Olson and Littlewood, 2002), distinct from the class Trematoda, which includes the Aspidogastreans and Digeneans. Although Odhner's (1912) classification into Monopisthocotylea (based on the adults fairly simple attachment mechanisms, and which feed on skin and mucus) and Polyopisthocotylea (based on the adults complex attachment mechanisms involving clamps and suckers, and which are generally gill feeders on blood) is well known and intuitively simple. However, the classification of Bychowsky (1961), who divided the Monogenea into the Polyonchoinea (with numerous marginal hooks in larvae, roughly corresponding to Monopisthocotylea) and the Oligonchoinea (reduced marginal hooks in larvae, roughly corresponding to the Polyopisthocotylea), is also widely used. The

difficulties with all classifications of the Monogenea is that they fail to reflect the fact that the Monopisthocotylea (Polyonchoinea) are a much more diverse group than the Polyopisthocotylea, which most authors accept include only two natural groups, the Oligonchoinea proper and the polystomatids (Polystomatoinea) from amphibians. For an informative and acerbic discussion of these nomenclatural issues, see Olson and Littlewood (2002). The Polyonchoinea include such clearly natural monopisthocotylean groupings as the capsalids and the microcotylids, as well as a wide range of smaller groups, some (*Enoplocotyle, Udonella*) represented by only single taxa. To reflect this, Malmberg (1990) created the subclass Articulonchoinea, to include eight families with articulated marginal hook sickles (the Gyrodactylidae, Enoplocotylidae, Anoplodiscidae, Acanthocotylidae Bothitrematidae, Tetraonchoididae, Ooegyrodactylidae and Sundanonchidae) which he considered primitive. Shinn *et al.* (1998a), using patterns of sensilla distribution, also reflected these ideas, proposing that gyrodactylids were closer to the polyopisthocotylean line of monogenean evolution, and formed a separate subclass between monopisthocotyleans and polyopisthocotyleans. Boeger and Kritsky (1993), in the first cladistic analysis of the Monogenea rejected the Articulonchoinea as polyphyletic, as the Acanthocotylidae (including *Enoplocotyle*) grouped with the Capsalidea Lebedev, 1988. Later, Boeger and Kritsky (2001) demonstrated that the monophyly of the Order Gyrodactylidea Bychowsky, 1937 was supported by six synapomorphies of which three were non-homoplasious: the presence of two seminal vesicles, large vitelline follicles and hinged marginal hooks. Boeger and Kritsky (1993, 1997) additionally suggested that the Gyrodactylidea and the Dactylogyridea were sister groups within the Polyonchoinea. Boeger and Kritsky (2001) suggested an independent origin for the six families of the order Gyrodactylidea which comprises the Gyrodactylidae, Anoplodiscidae, Bothitrematidae, and Tetraonchoididae, with the Udonellidae Taschenberg, 1879 and the Acanthocotylidae. Further molecular and morphological studies on the Gyrodactylidea, including other taxa such as *Enoplocotyle*, and overcoming the prejudice to consider *Udonella* as not a monogenean, are required to resolve these competing hypotheses.

5. SYSTEMATICS: THE BIOLOGICAL BEDROCK

5.1. Morphological Conservatism

Gyrodactylid alpha taxonomy is based on morphology, predominantly upon the morphometrics of the attachment apparatus, but may implicitly rely heavily on the host identity. The hamuli and bars represent a remarkable taxonomic resource. They are composed of keratin-like proteins (Kayton, 1983; Shinn *et al.*, 1995a), secreted with a remarkable degree of precision, and the complexity of their shapes provides much taxonomically useful information. The structures are stable in shape, for the most part fully formed at birth (but see Jackson and Tinsley, 1995), tough and not easily distorted, and resistant to most chemical fixatives. However, as 3D structures they present challenges to capture the subtleties of their shape. They are also composite structures, reflecting differences in the proteins secreted during their synthesis. The hamulus points and marginal hook sickles are particularly resilient, surviving prolonged proteinase digestion. These structures are also rigid, whereas hamulus roots (Figure 7) and the ventral bar membrane and processes (Figure 8) are far more fragile and easily damaged during preparation. These structures are also flexible and may distort badly in formalin or ethanol fixed material, or even in air-dried specimens for SEM, when they may appear hollow and flattened (cf. Veltkamp *et al.*, 1996 for a freeze substitution method which minimises dehydration artefacts). This becomes a particular problem in those species with hamulus roots folded naturally into the midline (e.g. *G. nemacheili* and allies), and hence it is important that observations on preserved specimens are supplemented by observations of living gyrodactylids. Ventral bar shape is particularly crucial in identifying a novel gyrodactylid, although current analyses tend not to capture the complexity of this structure (Figures 8 and 21). Similarly, the shape of the dorsal bar can be diagnostic (Figure 8), but there is little need for measurements. On the other hand, critical examination of marginal hook sickles (Figure 9), and to a lesser extent the hamuli, is necessary to establish the finer points of relationship of a gyrodactylid, especially in such

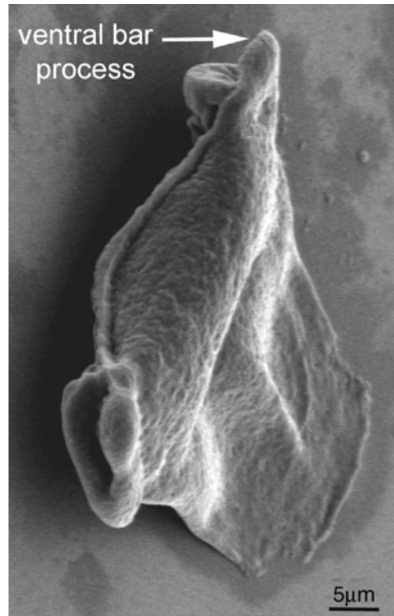


Figure 21 Scanning electron micrograph of a ventral bar (tilted anteriorly) of *Gyrodactylus salaris* (River Rauma strain) from Atlantic salmon.

difficult groups as the *G. wageneri* species complex (Malmberg, 1970), or within variants of *G. salaris* and *G. thymalli.*

Early gyrodactylid taxonomy relied on light microscopy of Canada Balsam mounted specimens. Marginal hooks and hamuli could be resolved with great accuracy in these preparations, and Wagener's (1860) descriptions can be instantly recognised today. However, many other taxonomists lacked this skill and their descriptions are so incomplete (e.g. Kathariner's 1904 description of *G. gracilis*) that the species cannot be re-identified. The viscosity of Canada Balsam makes it difficult to find properly flattened marginal hooks, while the refractive index is too close to that of the marginal hooks to make fine observation easy. American systematists used formalin and other reagents as a bath treatment to remove parasites from hosts prior to mounting (Parker and Haley, 1960; Putz and Hoffman, 1963). This generated many new species descriptions because of the ease with which fish could be screened (and is still in use e.g. Billeter *et al.*, 2000;

Kritsky *et al.*, 2007). However, formalin fixation may distort the hamulus roots, pulling them into the midline and, making their true shape difficult to discern.

With these difficulties in mind, Malmberg (1957, 1964, 1970) experimented with alternative mountants allowing adequate flattening and an appropriate refractive index. He developed saturated ammonium picrate mixed with glycerin as a combined fixative-mountant allowed to seep under the coverslip to fix a living, flattened gyrodactylid at the point of death. This revolutionised gyrodactylid taxonomy, offering significant improvements in flattening, refractive index and visibility, and combined with phase contrast illumination has become the method of choice for taxonomy of lower monogeneans. Although robust, it is not without disadvantages. Ammonium picrate mounts can dry out, but can subsequently be revived by allowing distilled water to seep under the cover slip. Mounts are fragile and contamination with immersion oil is a real problem, and so ringing with varnish is a necessity. They must also be stored flat. There are other disadvantages. Most notably, dried ammonium picrate is highly explosive, with severe restrictions on its supply and transportation and the viscosity of this medium can still be too great to allow full flattening of small marginal hooks. In addition, the limitations of resolution of the light microscope do impose limits on the use of marginal hooks in gyrodactylid taxonomy. For example, *Gyrodactylus poeciliae* has marginal hook sickles which are so small that their tips cannot be resolved with light microscopy (Harris and Cable, 2000). Suggestions for improvements of Malmberg's (1957) method include Ergens (1969) method for transferring *Gyrodactylus* specimens from ammonium picrate-glycerin into Canada balsam, Kritsky *et al.*'s (1978) method for staining the connecting bars of formalin-fixed *Gyrodactylus* specimens with Gomori's trichrome, and Richards and Chubb's (1995) technique using Mallory stain during the transfer of *Gyrodactylus* specimens from ammonium picrate-glycerin into a permanent mountant. Deposition of ammonium picrate-glycerin specimens is permissible within most major museums, a concession to the value of the technique.

With the increasing use of molecular techniques, the main disadvantage of ammonium picrate-glycerin is its inadequacy for

alcohol-fixed specimens, which often show significantly distorted hamulus roots preventing simultaneous high-resolution morphometry of the same specimens. This is particularly noticeable in the description of *G. teuchis* (Lautraite *et al.*, 1999), the morphometrics of which are much less adequately described than the molecular sequence (Cunningham *et al.*, 2001). This led to experimentation with various methods for examination of haptors of individuals, the bodies of which had previously been used for molecular analysis, and we highly recommend combined morphological and molecular descriptions of gyrodactylids in all taxonomic work (Harris *et al.*, 1999). Scanning electron microscopy (SEM) has been used for monogenean taxonomy since Maillard *et al.* (1982) disrupted soft anatomy with sodium carbonate to free the copulatory organ of *Diplectanum aequans*. Mo and Appleby (1990) and Shinn *et al.* (1993) used proteases (principally trypsin), with ultrasound (sonication) to liberate hooks from gyrodactylids. This gave a poor yield, it was impossible to assign individual hooks to particular specimens, and if the body/uterus was not removed hooks from the parental and progeny generations were mixed. Harris *et al.* (1999) finally resolved these difficulties, developing proteinase K/sodium dodecyl sulphate (SDS) digestion of individual worms for both SEM and molecular analysis. This has been used to describe gyrodactylids from poeciliids (Harris and Cable, 2000; Cable *et al.*, 2005), gobies (Longshaw *et al.*, 2003), bullheads (Winger *et al.*, 2005) and salmonids (Shinn *et al.*, 2004; Robertsen *et al.*, 2007a) including the description of the rainbow trout-infecting *G. salaris* variant (Lindenstrøm *et al.*, 2003a). Another advantage is that, providing the surrounding tissue is suitably digested, application of variable pressure when applying the coverslip does not significantly alter measurements. A major drawback of digestion techniques remains their inability to deal with formalin-fixed material. This would be extremely valuable, as gyrodactylids can frequently be collected from museum specimens of their hosts. Analysis of material collected in this way is probably the only feasible method for collection of gyrodactylids from certain host groups, for example, those from the deep sea, from squids or from African freshwater fishes and amphibians.

A number of authors have looked to other morphological characters that could be used in gyrodactylid alpha taxonomy, most notably the mapping of sensory structures (chaetotaxy) (Shinn *et al.*, 1992, 1997, 1998a, b; Bakke *et al.*, 2004a; see Section 2.7). However, the use of haptoral measurements persists with associated advances in statistical analysis. Multivariate statistical analyses and automated methods applicable to a single hook are greatly improving the objectivity of these methods (Shinn *et al.*, 1996, 2000; Kay *et al.*, 1999; Bakke *et al.*, 2004a, b), although the most fundamental issue is to determine the most appropriate measurements to record.

Most authors have a favoured suite of measurements, which have evolved from the work of Malmberg (1957, 1970) and Ergens (1957) and centre on the hamuli and marginal hooks. Prior to this, the Soviet Russian school used dimensions such as "hamulus length", "marginal hook length", and so on, but these were poorly defined and remain open to interpretation. Malmberg (1957) was the first to use an explicit measurement system. A similar, but slightly different, system evolved in the United States (Mizelle and Kritsky, 1967) and an unresolved problem is that Nearctic and Palaearctic gyrodactylids have been measured using different systems. Eastern Palearctic species described by Gussev (1953, 1955) follow the Soviet measuring system, while most western Palearctic species follow the systems of Malmberg and Ergens. Harris (1985, 1986) followed Malmberg's system fairly closely, but found some characters, for example, dorsal bar measurements, relatively meaningless because they were susceptible to distortion. Shinn (1994) reviewed the measurement of gyrodactylids critically, and revised Malmberg's (1970) range of measurements to give greater precision and utility. In particular, he argued that measurements such as "marginal hook sickle distal width" were uninformative because they could not be measured accurately using the light microscope. Others, such as "marginal hook sickle filament length" are too variable. Shinn *et al.* (2004; see their Figures 1–4) developed a standard suite of light microscope measurements for gyrodactylids of salmonids with new measurements on the ventral bar added by Olstad *et al.* (unpublished). Light microscopy measurements should, where possible, be supplemented by SEM observations (e.g. Figures 7–10). The use of measurements between established

landmarks allow rapid semi-automatic identification of gyrodactylids by relatively untrained operators (Kay *et al.*, 1999). This has been developed further to utilise the power of PC neural networks to learn identification of single gyrodactylids (McHugh *et al.*, 2000; Shinn *et al.*, 2000). At the same time, shapes, particularly of the marginal hook sickles, can be adequate to discriminate individuals where linear dimensions cannot. This was noted by Malmberg (1970), but had not been addressed objectively until Shinn *et al.* (1996) began to investigate whether computerised image analysis techniques could discriminate aspects of shape, rather than absolute size. However, it is evident that inaccuracy in the specification of landmark positions for linear measurements in gyrodactylid taxonomy still occurs, and non-comparable dimensions of closely related species are still selected by different taxonomists.

Significant seasonal variations occur in the size of gyrodactylid haptoral hooks and bars, and can be generated experimentally using different temperature regimes (see Mo, 1991a, b, c; Appleby, 1994, 1996a, b). Lower temperatures extend embryogenesis leading to the birth of specimens with larger hooks (Kulemina, 1977; Mo, 1991a, b, c; Dávidová *et al.*, 2005; see Section 9.1). This environmental effect can be so marked as to result in samples from the same population having non-overlapping size ranges at the warmest and coldest periods of the year (Mo, 1991b). Morphological variants are known (Harris, 1998; Geets *et al.*, 1999), and there may be significant differences between individual parasites, with quite distinct individuals occasionally present within the same population and molecular clade (Winger *et al.*, 2005; Robertsen *et al.*, 2007a; Olstad *et al.*, unpublished). Breeding of these individuals may lead to a reversion to type morphology (Harris, 1998). Host identity and site of attachment may also influence haptoral morphology (Huyse and Volckaert, 2002; Robertsen *et al.*, 2007a) in genetically characterised taxa. Dmitrieva and Dimitrov (2002) also showed an effect of salinity on hamulus and marginal hook size, but in this case, parasites were not characterised genetically and may have represented different genotypes.

Species descriptions in gyrodactylids present a number of problems which can, in extreme cases, make re-identification almost impossible. This is particularly so for older species descriptions, but can also

apply to recent descriptions which otherwise conform to ICZN protocols. We suggest that all new species descriptions of gyrodactylids include morphometric analysis based on proteolytic enzyme digested specimens using LM, preferably supplemented with SEM, and where possible rDNA sequence data spanning ITS1, 5.8S and ITS2 (Harris *et al.*, 1999; Bakke *et al.*, 2005). To some extent this suggestion has been followed, and recent descriptions such as those of *G. neili* (Le Blanc *et al.*, 2006) and *G. thlapi* (Christison *et al.*, 2005) do show much higher standards of description than many previous accounts.

5.2. Molecular Genetics

Molecular approaches to parasite taxonomy and systematics began in the early 1990s (Wilson, 1991) and with the threat posed by further range extension of *G. salaris*, it became apparent that conventional morphological methodologies were insufficient for identification of this pathogen. Cunningham and co-workers therefore developed molecular probes to differentiate *G. salaris* from other, non-pathogenic species infecting salmonids (Cunningham *et al.*, 1995a, b, c, 2000, 2003; Cunningham, 1997, 2002; Cunningham and Mo, 1997; Cunningham and Johnston, 1998; Collins and Cunningham, 2000; Collins *et al.*, 2000; Matejusová *et al.*, 2001; Sterud *et al.*, 2002; Matejusová and Cunningham, 2004). The first marker developed was the V4 region of the 28S large ribosomal subunit (LSU), chosen because of the availability of highly conserved flanking regions that could be used to design universal primers. This locus contained amplifiable restriction fragments that distinguished *G. salaris* from non-pathogenic *G. truttae* and *G. derjavini* (see Cunningham *et al.*, 1995a, b) but not from *G. thymalli* or *G. teuchis* (see Cunningham *et al.*, 2001). This led to the sequencing of the internal transcribed spacers (ITS1 and ITS2) and 5.8S rDNA of the ribosomal gene cassette (Cunningham, 1997). The 5.8S rRNA sequence is highly conserved and may therefore be a good marker of the deeper divisions within the genus (Ziętara and Lumme, 2002; Huyse *et al.*, 2003). Cable *et al.* (1999) sequenced ITS from non-pathogenic species, allowing the first molecular phylogeny for the genus. Molecular

phylogenies of the genus *Gyrodactylus* inferred from rDNA ITS regions were used to evaluate the validity of subgenera and species groups (Ziętara *et al.*, 2002; Matejusová *et al.*, 2003), and the subdivision of species of gyrodactylids into groups with long and short ITS1 (Cable *et al.*, 1999; Ziętara *et al.*, 2002). ITS1 and ITS2 have proved more useful markers of gyrodactylid genomic variation than the V4 region. They are more variable than the structurally conserved LSU, and because of concerted evolution (Elder and Turner, 1995), they homogenise within sexually panmictic populations. ITS markers have proved remarkably robust and 125 species (over one quarter of the described species) have now been sequenced at this locus, principally by Matejusová *et al.* (2000a, 2001, 2003), Ziętara *et al.* (2000, 2002) and Ziętara and Lumme (2002, 2003, 2004). This data set is particularly useful for its emphasis on the freshwater Eurasian species, especially the *G. wageneri* group species.

ITS sequences have proved sensitive markers of the boundaries between different gyrodactylid taxa, with even small sequence differences signalling different species identity. For example, *G. rarus* and *G. branchicus*, which are almost identical by morphometry but infect different hosts and have different ecologies, differ by only 0.9–1.3% along 774 nucleotides of ITS (Ziętara and Lumme, 2003). Within a species, sequences of PCR amplified ITS regions appear invariant; for example, Ziętara *et al.* (2000) examined populations of several species from White Sea and Baltic Sea drainages without finding any differences in ITS. Cable and Harris (unpublished) have found identical ITS in *G. gasterostei* from different UK sites between which direct interbreeding is impossible. This is in marked contrast to other invertebrates where multiple ITS sequences from the same individual, revealed by cloning, render this data unusable for phylogenetic inference (e.g. Harris and Crandall, 2000). ITS sequences have also proved extremely useful for identifying previously unsuspected species or species complexes. *G. teuchis*, a non-pathogenic gyrodactylid of salmonids, was not differentiated until its ITS was sequenced (Lautraite *et al.*, 1999; Cunningham *et al.*, 2001). Ziętara and Lumme (2003) in particular have used this approach to great effect, identifying *G. alexgusevi* from within specimens previously identified as *G. lotae* (host *Lota lota*) and *G. jussii* from within specimens

previously identified as *G. macronychus* (host *Phoxinus phoxinus*). In both cases, morphological differences were observed a posteriori with the confidence given by recognition of distinct ITS sequences. None of these cases represent incipient speciation as in all three the difference in sequence identity between pair members is considerable. Interestingly, Ziętara and Lumme (2003) also identified a third variant of *G. macronychus*, also with a sequence difference of some 5% from either *G. macronychus* or *G. jussii*, within the work of Matejusová *et al.* (2003).

The large number of ITS sequences available through GenBank is making *Gyrodactylus* a highly attractive option for testing evolutionary hypotheses. One of the first and most robust findings to emerge is that host switching, rather than co-evolution, is the dominant mechanism for gyrodactylid evolution (Cable *et al.*, 1999; Huyse, 2002; Huyse and Volckaert, 2002; Huyse *et al.*, 2003; Matejusová *et al.*, 2003; Ziętara and Lumme, 2003). This is at odds with the view that co-evolution of hosts and gyrodactylids has dominated (Malmberg, 1970, 1998). For example, *G. gasterostei* on the three-spined stickleback (*Gasterosteus aculeatus*) and *Gyrodactylus pungitii* on the nine-spined stickleback (*Pungitius pungitius*) were regarded as sister species by Harris (1985); ITS variation makes it clear however that the sister species to *G. gasterostei* is *G. aphyae*, infecting the minnow *Phoxinus phoxinus* (see Ziętara and Lumme, 2003). Indeed, all cases of close molecular relationship concern parasites from widely differing fish hosts (Table 1), whereas apparently similar species infecting the same host are found to be only distantly related when ITS loci are sequenced (Table 1). These represent the first objective tests of speciation patterns within *Gyrodactylus*, in which molecular evidence is used to corroborate and test hypotheses generated from morphological and host identity evidence.

Perhaps, the most surprising feature of molecular phylogenies based on ITS1, 5.8S and ITS2 rDNA sequences is the apparent antiquity of the genus *Gyrodactylus*, and the extent of their morphological conservation. Ziętara and Lumme (2002) highlighted this, noting that differences between 5.8S rDNA of *Gyrodactylus* species from different subgenera exceeds that seen between different nematode families. Ziętara and Lumme (2002), perhaps wisely, did not speculate on the

Table 1 Pairwise molecular distances of rDNA internal transcribed spacer sequences between closely related *Gyrodactylus* species

Species pair				Pairwise molecular distance (%)	Author
Species 1	Host 1	Species 2	Host 2		
Gyrodactylid species pairs from the same host					
Gyrodactylus teuchis	*Salmonids*	*G. salaries*		5.6	Lautraite *et al.* (1999)
G. alexgussevi	*Lota lota*	*G. lotae*		8.5	Ziętara and Lumme (2003)
G. macronychus	*Phoxinus phoxinus*	*G. jussii*		21.8	Ziętara and Lumme (2003)
G. macronychus	*Phoxinus phoxinus*	*G. sp.*		19.2	Ziętara and Lumme (2003)
G. rugiensis	*Pomatoschistus microps*	*G. micropsi*		16.2	Huyse and Volckaert (2002)
Gyrodactylid species pairs from different hosts					
G. branchicus	*Gasterosteus aculeatus*	*G. rarus*	*Pungitius pungitius*	0.9–1.3	Ziętara *et al.* (2001)
G. salaris	*Salmo salar*	*G. thymalli*	*Thymallus thymallus*	0	Cunningham (1997)
G. gasterostei	*Gasterosteus aculeatus*	*G. aphyae*	*Phoxinus phoxinus*	1.1–1.2	Ziętara and Lumme (2002)
G. rugiensis	*Pomatoschistus microps*	*G. rugiensoides*	*Pomatischistus minutus*	2.5	Huyse and Volckaert (2002)
G. arcuatoides	*Pomatoschistus pictus*	*G. flavescensis*	*Gobiusculus flavescens*	1.6	Huyse *et al.* (2004a)

Those infecting the same host are invariably less closely related than those infecting different hosts, suggesting that they evolved in different refugia. The most closely related forms infect different hosts, indicating the importance of host switching in gyrodactylid evolution.

reasons for the molecular differentiation with morphological conservation, but it is likely that this is an ancient genus, with origins relating to the differentiation of modern fish groups in the Triassic (Nelson, 1994). Morphological conservation is perhaps surprising, but larvae resemble each other more than their respective adults (Gould, 1977), and as a progenetic group close resemblance between species might be expected.

ITS sequences have proved an objective test of Malmberg's (1970) classification of subgenera and species groups based on excretory system and marginal hooks. Some of Malmberg's (1970) subgenera receive broad support (Zię̨tara *et al*., 2002), although there are many differences of detail. Thus, Matejusová *et al*. (2003) reject the subgenus *Gyrodactylus* as paraphyletic because *G. markakulensis* forms a sister clade to the remainder of the genus; however, although Malmberg (1970) assigned *G. markakulensis* to the subgenus *Gyrodactylus*, he was clearly uncertain about its status and considered it somewhat different to other members of the group. All other species placed within subgenus *Gyrodactylus* by Malmberg (1970) which have been included in molecular analyses have formed a monophyletic clade (Matejusová *et al*., 2003). The subgenus *Limnonephrotus* remains recognisable in molecular analyses, although one paranephrotid species group, the *G. rugiensis* group (Gläser, 1974) also falls within this subgenus according to molecular analyses, and the subgenus *Neonephrotus*, represented by *G. anguillae*, is a separate clade within the *G. rugiensis* group which has evolved following a host switch to eels (Huyse *et al*., 2003; Matejusová *et al*., 2003). There is no support for the subgenera *Metanephrotus*, *Mesonephrotus*, or *Paranephrotus* excluding the *G. rugiensis group*, which all group together in molecular analyses. However, despite these differences in detail, we argue that Malmberg's (1970) subgenus concept remains useful, although the precise boundaries of the subgenera are bound to change as molecular re-evaluation of the group continues.

Malmberg's (1970) "species groups", are not as well supported, mainly because of host switching. The notion of a "*G. wageneri*" group as distinct from a "*G. salaris*" species group (Malmberg, 1993) gains no support, as closely related species infect sticklebacks, bullheads, salmonids and cyprinids (Cable *et al*., 1999; Matejusová *et al*., 2003). The

problem with ITS phylogenies are that too many of the sequenced species (and perhaps up to one third of the described species) are from the *G. wageneri*/*G. salaris* species group, and too few species groups and subgenera, especially from North America and the tropics, have been included. Nevertheless, sufficient consensus from morphology, molecules and biogeography is possible to allow some crude, but robust grouping of *Gyrodactylus* species at the sub-generic level. We would recognise the following entities:

1) A grouping corresponding roughly to the Malmberg (1970) subgenus *Gyrodactylus*. This includes the gill parasites regarded by Malmberg (1970) as belonging to the *G. elegans* species group (curved hamuli, narrow spine-like ventral bar membrane) and the *G. phoxini* species group (straight hamuli, ventral bar membrane spathulate, often a boss on the ventral bar). Most species occur on ostariophysans (cyprinids and cobitids) in Eurasia, but Le Blanc *et al*. (2006) have confirmed Malmberg's (1970) suspicion that the subgenus has radiated widely in North America also, and host shifts onto the Esocidae have occurred. Some species with this morphology which appear to fall within this group (e.g. *G. markakulensis*) are, according to molecular analyses, not closely related. Further work is needed to establish their status.
2) A grouping corresponding to the Malmberg (1970) subgenus *Limnonephrotus*, including additionally the paranephrotid *G. rugiensis* species group and *G. anguillae*. This grouping is predominantly found in Eurasian freshwater, with extension into brackish water. Most species infect ostariophysans (predominantly cyprinids), but there are examples from most freshwater and brackish fish families in the Northern Hemisphere. There are some putative Nearctic species of this group [e.g. the *G. pungitii*-like form recorded by Cone and Wiles (1985) from Canadian sticklebacks], but these are rare and predominantly infect migratory fish such as salmonids or gasterosteids.
3) A third, catch-all group, including tropical and marine representatives of the subgenera *Mesonephrotus* and *Paranephrotus*, and *Metanephrotus*, many of which have a holarctic distribution,

possibly due to distribution with their hosts. This group undoubtedly will be subdivided further as more sequences become available. *Fundulotrema* may originate in this clade, according to sequence data presented by Kritsky and Boeger (2003). Although the overall grouping is holarctic in distribution, some species groups, such as the *G. eucaliae* group, are found predominantly in the Nearctic and Neotropical.

A problem with establishing robust gyrodactylid phylogenies has been the use of markers inappropriate for the purpose. ITS sequences are too variable for genus-wide phylogenies (although useful within species complexes). The 5.8S rDNA is more useful for genus-wide comparison, but is too short. The V4 region of the 18S rDNA (Cunningham *et al*., 1995a, b, c) contains sufficient variation to be informative, but is also short. Instead, a greater range of 18S and 28S sequences are needed to establish the wider familial phylogeny. This has not stopped several groups from making genus-wide comparisons using sequences which include both ITS1 and ITS2 (e.g. Matejusová *et al*., 2001; Kritsky and Boeger, 2003). Small differences in alignment necessitated by the difficulties of obtaining global alignments of ITS with limited numbers of indels, can make large differences to the final phylogeny. Even the use of the 5.8S rDNA for genus-wide phylogenies is not without problems. Ziętara *et al*. (2002) identified sequencing errors in a number of sequences within GenBank which they took time to correct; there is no assumption of correctness in many accessed entries, and there is no curatorial control of accurate morphological identification of species prior to molecular analysis. In only a few cases have voucher specimens of taxa used for molecular analysis been deposited in easily accessible and secure collections.

The absence of any nuclear genomic markers at the intra-specific level has bedevilled molecular analysis of gyrodactylids. This was partly addressed by the studies of the Intergenic Spacer (IGS) of the ribosomal gene cassette (Collins and Cunningham, 2000; Collins *et al*., 2000; Sterud *et al*., 2002; Cunningham *et al*., 2003). IGS remains under the constraint of concerted evolution and therefore does not evolve as rapidly as single locus markers. Cunningham and Mo (1997) employed random amplified polymorphism DNA (RAPD)

markers to differentiate *G. salaris* populations; this did show distinct differences between *G. salaris* populations which would not have been expected if the parasite had been introduced to Norway in the mid-1970s. More recently, Collins *et al*. (2004a) sequenced the β-tubulin gene from *G. salaris*, providing the first single copy nuclear marker for the genus. Variation was found, but predominantly appears to be between gene isoforms within a single genome. Other loci have not been characterised, and attempts to isolate microsatellite markers for the group have been painfully slow. As an alternative we have used amplified fragment length polymorphism PCR (AFLP; Vos *et al*., 1995) to analyse *G. salaris* stocks from rivers in Southern, Central and Western Norway, and compared this to diversity in *G. turnbulli* from guppies (unpublished). This method has proved highly useful in identifying polymorphisms between stocks, but suffers from the drawback at present that a minimum of 200 gyrodactylids is needed for the initial ligation reaction. Nevertheless, the method has great potential for identifying genomic markers, especially if polymorphic bands are subsequently cloned and sequenced (Cable *et al*., unpublished).

Mitochondrial markers for gyrodactylids lagged until Meinilä *et al*. (2002) published a preliminary sequence for the *G. salaris* mitochondrial cytochrome oxidase 1 (COI) gene, which was used to generate primers for a variety of species (Meinilä *et al*., 2002; Hansen *et al*., 2003, in preparation). This locus is proving indispensable in recreating patterns of gyrodactylid speciation (see Section 5.3.2) and has attracted much interest with the advent of DNA barcoding (Hebert *et al*., 2003), but two cautionary points should be considered. Firstly, Meinilä *et al*. (2004) examined mitochondrial diversity from a population (Lake Kitka) isolated by post-glacial uplift for 8400 years. Using such data, a rate of mitochondrial gene divergence of some 20% per million years was estimated (Meinilä *et al*., 2004). This may not be unreasonable considering the short generation time of this parasite, but it compares to a normal rate of 1–2% per million years in other invertebrates (Knowlton and Weigt, 1998; Gomez *et al*., 2000; Nieberding *et al*., 2005). This high rate of change has significant implications for the functionality of COI, and several haplotypes have been identified in which amino acid sequence was changed. So

little is known of the structure–function relationships of COI (e.g. Meunier, 2001) that it is unwise to expect all evolution at this locus to be selectively neutral, and with such high rates of mutation, one might expect biologically significant mutants to arise relatively frequently. Given the diffuse and often exotic consequences of mitochondriopathies in humans, the only species in which they have been systematically explored, the likelihood of significant effects from mitochondrial mutations in *Gyrodactylus* is high. Secondly, although the COI mtDNA is an invaluable comparative molecular dataset with which to test the validity of the rDNA ITS data, it is unclear to what extent it is influenced by NUMTs (nuclear copies of mtDNA genes or pseudogenes). Potential NUMT COI sequences are deposited in GenBank (Accession numbers AY225307-08), but Meinilä *et al*. (2004) did not mention the presence of these nuclear copies or discuss their significance. Work is currently ongoing to fully sequence and annotate the mitochondrial genomes of various strains of *G. thymalli* and *G. salaris*, respectively. The complete mitochondrial genome of *G. salaris* from Skibotnelva, Norway, will be published soon (Huyse *et al.*, 2007) and the complete mitochondrial genome of *G. thymalli* from Hnilec, Slovakia, shall follow shortly (Plaisance *et al.*, submitted). Once the sequence data are available, regions showing significant sequence variation within and between species can be determined and subsequently be used for strain differentiation and identification.

Almost all current GenBank gyrodactylid species were originally identified using morphology and host specificity, and it is still on this basis that they are deposited in the database. Only with a more comprehensive whole genomic approach to the entire genus will molecular data begin to rival morphological and experimental studies for understanding the gyrodactylid evolution. However, with appropriate markers there is tremendous potential for studying the molecular ecology of well-characterised gyrodactylids, such as *G. turnbulli* and *G. salaris*, which are readily available from natural populations and easy to maintain experimentally. These microparasitic worms circumvent many of the problems highlighted by Criscione *et al*. (2005), such as small infrapopulations and non-availability of adult stages, and offer almost endless opportunities for testing the impact of

different life history traits (such as reproductive modes and pathogenicity) on genetic diversity.

5.3. Host Specificity Among Gyrodactylids

Most monogeneans are restricted to one or a few host species, and more than 70% are considered to infect a single host species (Bychowsky, 1961; Rohde, 1979). The paradigm that gyrodactylids are narrowly host-specific (Malmberg, 1970) often suggested by published host records, was re-examined by Bakke *et al.* (1992a). After eliminating obvious misidentifications and synonymies and restricting analyses to those gyrodactylids which have featured in two or more field studies or have been used experimentally, the proportion occurring on a single host species declined to 30%, suggesting that gyrodactylids are less host-specific than commonly thought, and that narrow specificity is an artefact based on the numerous descriptions of species collected only once. The prominent differences in susceptibility/resistance between stocks of the same host species (Bakke *et al.*, 1990, 1996) and potential parasite strain differences in infectiousness (Lindenstrøm *et al.*, 2003a; Olstad *et al.*, 2005, 2007; Cable and van Oosterhout, unpublished), reinforces the uncertainty in estimating host utilisation of gyrodactylids based on field observations.

The mechanisms that contribute to host specificity in monogeneans operate at several levels: host localisation, recognition and attachment (ecological and behavioural mechanisms); and establishment, growth and reproduction (physiological mechanisms). Gyrodactylids live within a complex ecosystem on the surface of an aquatic animal. The epidermis of the host, and its products, may attract or be inhospitable to a gyrodactylid or may be refractory due to acquired immunity. Often overlooked, feeding and digestion probably play key roles in determining host specificity in gyrodactylids. These parasites appear to possess acute powers of host discrimination and preferentially infect particular hosts (e.g. Buchmann and Uldal, 1997; Buchmann *et al.*, 2003a, 2004). Given their highly developed sensory system, it is not clear whether they fail to feed on unsuitable hosts or feed but cannot assimilate host molecules. Specific inhibitory

molecules may be present that prevent non-adapted gyrodactylids from utilising a host; but equally it may also be that the parasites are behaviourally specialised not to attempt feeding on sub-optimal hosts.

The cephalic glands have a primary adhesive function (see Section 2.5) and may influence host specificity (Whittington *et al*., 2000a, b; Whittington and Cribb, 2001). Whittington and Cribb (2001) proposes an instant reaction between the adhesive secretions of monogeneans and host mucus to allow firm but temporary attachment during transmission and locomotion on the epidermis (Whittington *et al*., 2000a). During birth, a newborn viviparous gyrodactylid attaches directly to the fish epidermis, initially with its anterior adhesive organs. Whittington *et al*. (2000a) hypothesised that initial contact allowed: (i) mechano- and chemosensory receptors of the parasite to interact with the host's surface, and (ii) a chemical reaction between the host's mucus and the adhesives secreted by the parasite, representing a potential recognition mechanism. As the anterior cephalic lobes of gyrodactylids make initial contact with the host, it is not surprising that Buchmann (1998a, c) found this region immunologically active, being rich in mannose-rich glycoproteins which stimulate the alternative complement pathway (see Harris *et al*., 1998). Both host epidermis and parasite tegument may therefore contribute to maintenance of the specific parasite–host relationship by chemical interactions between them (Whittington *et al*., 2000a, b). An important point about such ''recognition'' models of host specificity is that only a small change in chemical signature of either host or parasite could bring about a major shift in host specificity, which could not be predicted a priori. As an example, Leberg and Vrijenhoek (1994) noted that *Gyrodactylus turnbulli* could infect pathogenically a single clade of the gynogenetic clone *Poecilopsis* sp. In this case, a presumed minor genetic alteration in the host allowed an entirely unpredicted gyrodactylid to exploit it (but see King and Cable, 2007).

Despite the relative simplicity of a direct life cycle, research on host identification or recognition by monogeneans has been limited since Kearn's (1967) experiments on *Entobdella soleae* (see reviews by Whittington and Cribb, 2001; Whittington *et al*., 2000a, b). Gyrodactylids are particularly suited for experimental studies on host

identification and host specificity (Bakke *et al.*, 2002), and we may expect a diverse range of cues to be involved in host specificity considering their diversity of hosts. However, although gyrodactylids are frequently referred to as generalists and specialists (e.g. Matejusová *et al.*, 2000b), their host specificity, with a few notable exceptions (see Section 5.3.3), remains a neglected area. Several species have been studied experimentally, but most data remain anecdotal, or, as in *Gyrdicotylus*, is restricted to published summaries of a much larger corpus of unpublished data (see Section 5.3.1.). A classic example concerns the common guppy parasites *G. bullatarudis* and *G. turnbulli*, considered generalist and specialist respectively based on their known host range (Harris *et al.*, 2004). Harris (1986) presented anecdotal evidence that *G. bullatarudis* existed as host-specific strains, but although *G. turnbulli* has been the subject of numerous studies (e.g. Madhavi and Anderson, 1985; Scott, 1985a, b; Harris, 1986; van Oosterhout *et al.*, 2003), its host specificity has only recently been experimentally tested (King and Cable, 2007). Even with limited material, Llewellyn (1984) was able to demonstrate that *Isancistrium subulatae* from *Alloteuthis subulata* could infect *Sepiola atlantic*a. Similarly, the specificity of *G. gasterostei,* regarded as a generalist by Matejusová *et al.* (2000b), has been studied, but most data are anecdotal only or unpublished. Gläser (1974) noted that *G. gasterostei* could incidentally infect cyprinids when shoaling with these fishes during the winter months and Harris (1982) showed that it could infect *Pungitius pungitius* and *Phoxinus phoxinus*, but at significantly reduced rates compared to its preferred host, the three-spined stickleback (*Gasterosteus aculeatus*). These observations all suggest this species is a specialist, which can at best establish transient infections on other hosts. It is therefore all the more surprising this parasite is considered common on the cyprinid *Leuciscus cephalus* in the Czech Republic (see Gussev, 1985; Moravec, 2001). Hoffmann and Putz (1964) also presented anecdotal observations on the specificity of *G. macrochiri*, finding this species relatively restricted to *Lepomis macrochirus*. This again is at odds with observations from the field (summarised in Harris *et al.*, 2004), which suggest that this gyrodactylid can infect a wide range of centrarchids. This highlights a common problem with gyrodactylid specificity studies; anecdotal

observations in the laboratory can have little relevance to the field, while accidental infections in the field inevitably broaden observed host ranges. This is best illustrated by Malmberg (1970), who noted species such as *G. errabundus* infecting almost every host with which it came in contact, and by Dmitrieva and Gerasev (1997) and Dmitrieva and Dimitrov (2002), who recorded *G. alviga* from 15 host species from the Black Sea, but again, the precise status of the parasite on most of these hosts is unknown. The three taxa which have been best studied are *Gyrdicotylus gallieni* from the clawed toad *Xenopus* (see Jackson and Tinsley, 1994; Tinsley, 1996), *Gyrodactylus salaris* (see Bakke, 1991; Bakke *et al*., 2002) and *G. derjavini* (see Buchmann and Uldal, 1997) from salmonids.

5.3.1. Case Study: Gyrdicotylus

The host specificity of *Gyrdicotylus* spp., from the clawed toad *Xenopus* has been studied in some detail, although only summaries have been published (Jackson and Tinsley, 1994; Tinsley, 1996). *Xenopus* contains ~30 species (Tinsley, 1996) and gyrdicotylids also infect the related genera *Hymenochirus* and *Silurana* (see Tinsley, 1996). Records from natural populations of these toads suggest that considerable diversity exists within *Gyrdicotylus*, although only one species, *Gyrdicotylus gallieni* from *Xenopus laevis laevis* (see Harris and Tinsley, 1987) and *X. l. victorianus* (Vercammen Grandjean, 1960) has so far been formally described. *G. gallieni* from *X. l. laevis* also infects *X. l. victorianus* (see Harris, 1982; Jackson and Tinsley, 1994), suggesting a single panmictic species infecting *X. laevis* and its subspecies across Central and Southern Africa. It can also infect the endemic South African *X. gilli*, but not *X. borealis* (see Harris, 1982; Jackson and Tinsley, 1994). Infection trials using allopolyploid hybrid *Xenopus* species, such as *X. wittei* and *X. vestitus* are particularly interesting. These taxa originated as hybrids of ancestors from lineages related to *X. laevis* and *X. fraseri* and contain copies of the genome of each parent. *X. wittei* and *X. vestitus* are octoploid, but other taxa are up to dodecaploid (Tinsley, 1996; Evans *et al*., 2005), derived as hybrids between an octoploid and a tetraploid species. These central

African polyploid taxa bear a rich *Gyrdicotylus* fauna, and different populations of *X. wittei* have at least two species of this parasite. Rwandan *X. wittei* is not susceptible to *G. gallieni* from *X. laevis*, although *X. wittei* shares half its genome with a lineage closely related to *X. laevis*. Similarly, parasites from *X. wittei* were unable to infect either of the *X. laevis* subspecies tested or *X. vestitus*, although the latter also shares half a genome with a lineage closely related to *X. wittei*. Parasites from *X. vestitus* were also unable to infect *X. wittei* (J.A. Jackson, personal communication). These experiments are particularly interesting in the light of *Gyrodactylus salaris* and *G. derjavini* infections of salmon-trout hybrids (Bakke *et al*., 1999; Section 8.2). *Xenopus laevis* develops prolonged acquired immunity to *Gyrdicotylus* spp. (Jackson and Tinsley, 1994), and this gyrodactylid is relatively insensitive to complement (J.A. Jackson, personal communication). It is possible therefore that immunity to *Gyrdicotylus* is mediated in a different way to that developed by fishes to *Gyrodactylus*. Clearly, this host–parasite interaction presents a fascinating model system with which to dissect the mechanisms for host specificity and resistance to gyrodactylids at a molecular level.

5.3.2. Case Study: The G. salaris Species Complex

Host specificity has been most intensively studied experimentally in *G. salaris* and its close relatives including *G. thymalli* and the Danish rainbow trout variants (Lindenstrøm *et al*., 2003a; Jørgensen *et al*., 2006). As a consequence, patterns of specificity within this species complex are fairly well known. Bakke *et al*. (1990) developed a protocol for experimental testing of salmonid susceptibility and resistance demonstrating that *G. salaris* (Lierelva strain) was pathogenic to River Lone (South West Norway) and River Altaelva (North Norway) salmon strains, but that infections of River Neva salmon from the Baltic were limited and eventually eliminated. This protocol has been greatly extended and the specificity of *G. salaris* Lierelva strain has been tested on many salmonids (see Tables 3 and 4 in Bakke *et al*., 2002; Karlsson *et al*., 2003; Bakke *et al*., 2004b). However, it is important to note that under aquarium conditions infection of

atypical hosts may be possible, as experimental modulation of the host response in salmonids using cortisol (mimicking aspects of the stress response) significantly influences both innate and acquired resistance to *G. salaris* (see Harris *et al*., 2000), and detached *G. salaris,* frequently observed in aquaria, are found to be less host selective and may attach to any available fish species (Bakke *et al*., 1991a, 1992a).

A fundamental difference exists between the behaviour of Lierelva strain *G. salaris* on salmonids and that on non-salmonids. On brook lamprey, European eel, flounder, minnow, perch, roach, and three- and nine-spined sticklebacks, no population growth occurs and parasites are rapidly lost. On eels (Bakke *et al*., 1991a), survival is only slightly better than on glass (Olstad *et al*., 2006) and parasites may not feed. Generally, parasites transfer poorly to these non-salmonids, and parasites may possess behavioural and physiological mechanisms to avoid these hosts. On all salmonids tested, however, some parasite growth and reproduction was observed, although the population growth rate varied greatly between host species. On salmonids, *G. salaris* can generally transfer, establish, feed and reproduce, although poorly in some species, for example, on lake trout (*Salvelinus namaycush*) and whitefish (*Coregonus lavaretus*) (Bakke *et al*., 1992b; Soleng and Bakke, 2001a). On Atlantic salmon, the usual host in the wild, and on other susceptible salmonids, there is considerable variation in resistance between species, strains and individuals of the same population, even full-sibs (Bakke *et al*., 1996, 1999; see Sections 8.1 and 8.2).

On brown trout (*Salmo trutta*), after *S. salar* the host most likely to be encountered by *G. salaris* Lierelva strain in the wild, population growth is particularly poor (Jansen and Bakke, 1995; Bakke *et al*., 1999, 2002), similar to population growth on the more distantly related *Salvelinus namaycush* and *Coregonus lavaretus* (see Bakke *et al*., 1992b; Soleng and Bakke, 2001a). The grayling, *Thymallus thymallus*, is also a relatively poor host for *G. salaris* Lierelva strain (Soleng and Bakke, 2001b; Sterud *et al*., 2002) compared to salmon, despite the supposed close relationship between *G. salaris* and *G. thymalli* (see Meinilä *et al*., 2004). Soleng and Bakke (2001b) found that *G. salaris* populations survived for only ~35 days on grayling and that infection rarely exceeded 50 parasites per fish, although 0+ fish were more

likely to be susceptible than older (1+) grayling. On rainbow trout *Oncorhynchus mykiss* and brook trout *Salvelinus fontinalis*, population growth of *G. salaris* Lierelva strain is effective (Bakke *et al.*, 1991b, 1992c) and, although infections eventually self-limit, *O. mykiss* in particular is capable of sustaining population growth for considerable periods. Given the discussion below (Sections 6.5 and 6.6) concerning the "rainbow trout strain" of *G. salaris* from Lierelva (and Drammenselva/Lærdalselva/Bullaren) (Hansen *et al.*, 2003), these experiments need to be repeated with other Norwegian *G. salaris* strains belonging to the other *G. salaris* clades (see Hansen *et al.*, 2003).

The position of the Arctic charr, *Salvelinus alpinus* as a host for *G. salaris* is particularly interesting. An isolated, land-locked Arctic charr population (Korssjøen, central Norway) proved almost entirely refractory to the Lierelva strain of *G. salaris*, eliminating infection within a few days, whereas a northern anadromous population, sustained parasites for long periods; many months in pooled host populations (Bakke *et al.*, 1996). The role of this host in the natural epidemiology of *G. salaris* therefore remains unclear, although Mo (1988) considered Arctic charr a possible reservoir host when rotenone treatment of the Skibotnelva failed. Relatively high infections of Arctic charr were later observed in this river (Kristoffersen *et al.*, 2005). Knudsen *et al.* (2004) have also recorded exceptionally high *G. salaris* infection on Arctic charr in the nearby Signaldalselva, where salmon are also infected by *G. salaris*. The *G. salaris* in these rivers belong to a different clade than the Lierelva strain (Hansen *et al.*, 2003). Arctic charr are also infected with *G. salaris* in five salmon-free lakes in central south Norway (Robertsen *et al.*, 2006, 2007b). This host seems to be able to support *G. salaris* in species-poor fish communities in the absence of Atlantic salmon or rainbow trout. Recent work by Robertsen *et al.* (2007a, b) has shown that the *G. salaris* strain isolated from charr in the lakes had the same mitochondrial haplotype as rainbow trout parasites isolated from Lake Bullaren, Sweden but was non-virulent to salmon (Olstad *et al.*, 2005). However, the ITS of *G. salaris* from Arctic charr showed a difference of one nucleotide to that previously observed in *G. salaris* populations (Olstad *et al.*, 2007).

The specificity of *G. thymalli* has also been examined, although in less detail. This parasite utilises Atlantic salmon even less effectively (Bakke *et al*., 2002) than *G. salaris* can exploit grayling (Soleng and Bakke, 2001b), but also appears less aggressive on its normal host, with slower population growth on grayling than *G. salaris* Lierelva strain exhibits on susceptible salmon strains (Bakke *et al*., 2002; Sterud *et al*., 2002). However, only the Norwegian River Glomma strain of *G. thymalli* has been used in susceptibility testing. Given the molecular heterogeneity of *G. thymalli* in Scandinavia (Hansen *et al*., 2003, 2007; Meinilä *et al*., 2004), and the occurrence of other grayling clades infected with *G. thymalli* outside Scandinavia (e.g. Denham and Longshaw, 1999; Hansen *et al*., 2007), this work should be repeated with other *G. thymalli* strains and grayling stocks. The final members of the *G. salaris* species complex that have been tested experimentally are the rainbow trout variants isolated by Lindenstrøm *et al.* (2003a) and Jørgensen *et al*. (2006) from Danish rainbow trout. These parasites failed to exploit salmon stocks (Baltic and East Atlantic), but reproduced successfully on rainbow trout. Unfortunately, neither grayling nor brown trout were included in these comparisons.

5.3.3. Case Study: Gyrodactylus derjavini

The most frequently encountered gyrodactylid on salmonids in Scandinavia is *G. derjavini*, infecting both *Salmo trutta* and *Oncorhynchus mykiss*. Infections of rainbow trout can only date back to the introduction of this fish into Europe at the end of the 19th century. The widespread occurrence and epidemiology of this parasite on brown trout in southeastern Norway (Mo, 1997; Bakke *et al*., unpublished) suggests that the original host in Northern Europe is actually *S. trutta*. The host specificity of *G. derjavini* has been extensively tested by Buchmann's group. Buchmann and Uldal (1997) investigated the susceptibility of four salmonids (rainbow trout, brown trout, a Baltic and an Atlantic strain of salmon) to a Danish isolate of *G. derjavini.* They found initial parasite attachment did not differ between host species but that populations

increased significantly faster and to a higher level on rainbow trout compared with other salmonids. Parasite selection of microhabitat also differed between the host species. Salmon are slightly susceptible to *G. derjavini* Sandvikselva strain but this is also dependent upon the salmon stock tested (Bakke *et al*., 1999, 2002). In an experimental study where detached parasites were offered a choice between salmon, rainbow trout and carp, Buchmann *et al*. (2004) found preferences in both *G. derjavini* and *G. salaris,* as after 2 days 90% of *G. derjavini* infected the rainbow trout and 60% of *G. salaris* were attached to the salmon.

6. EVOLUTION AND PHYLOGENY OF GYRODACTYLIDS

6.1. Models of Parasite Speciation

Gyrodactylus is one of several hyperdiverse monogenean genera that could provide important insights into parasite speciation processes. Originally, parasite speciation was viewed predominantly from the perspective of co-evolution (or co-speciation; Fahrenholz, 1913), a parasite group evolving in parallel with its host group. This was certainly considered so for monogeneans (Bychowsky, 1961; Llewellyn, 1963) and was extended to gyrodactylids by Malmberg (1970). Ironically, it is now realised that there are very few convincing cases of cospeciation because of the exacting requirements needed for its demonstration (see Page *et al*., 1996) and the difficulties of interpreting topological incongruence between host and parasite phylogenies (Page and Charleston, 1998). Indeed, if Raup (1994) are correct in estimating extinction rates of 10–15% of vertebrate species per million years, then it is hardly surprising that co-evolution is so difficult to recognize, as the probability that two related hosts could be found containing two related parasites more than a few million years after the initial divergence event is small. Even groups such as the lice, originally held to be strictly co-evolutionary (Rothschild and Clay, 1952), are now seen to be much more complex (e.g. Hafner and Nadler, 1988; Page *et al*., 1996). A more convincing case can be made for the importance of

host-switching in parasite speciation, a concept long accepted by researchers of plant–insect interactions (e.g. Walsh, 1864) and shown by Brooks and McLennan (1993) to be a dominant force in the speciation of animal parasites.

Host switching is counterintuitive as it appears to be an unjustified example of sympatric speciation. Mayr (1963) argued that speciation could only occur when populations were physically separated by extrinsic barriers (allopatric speciation), either by separation into two parts of a previously contiguous population (vicariant allopatric speciation), or by the peripheral isolation of a small part of the initially contiguous population, within which divergence occurs much more rapidly because of its relatively small size (peripheral isolates allopatric speciation). Sympatric speciation, the evolution of intrinsic reproductive barriers between incipient species where the populations are not physically separated, was originally not held to be possible, and is still considered very rare in vertebrate evolution. Lynch (1989) considered over 70% of vertebrate speciation events took place through vicariant allopatric speciation, with 15% via peripheral isolation. Only 6% of cases could possibly be accounted for by sympatric speciation. Host switching within a single geographical locality can be an apparently sympatric isolating mechanism, if gene flow between parasite populations on different hosts is reduced to the point where reproductive barriers can evolve. It has been suggested many times for plant parasitic insects, most notably and persistently in the apple maggot fly *Rhagoletis* (see Jiggins and Bridle, 2004). Populations of this fly, infecting Hawthorn *Crataegus* spp., started to feed on apples after their introduction to America following European colonisation. This began a process in which the phenology of the apple feeding forms changed and now resultant changes in host identification and detection preferences have been detected (Feder *et al.*, 2003). This situation is entirely analogous to, for example, the evolution of the rainbow trout variant of *Gyrodactylus salaris* (see Sections 6.5 and 6.6), an evolutionary change which could not have taken place until after the introduction of rainbow trout into Europe in the 19th century. In *Rhagoletis*, it is argued that the recentness of the evolution of the apple feeding forms is such that sympatric evolution must

be involved (Jiggins and Bridle, 2004). Feder *et al.* (2003), however, have argued that the apple-feeding race of *Rhagoletis* derived from forms which were already reproductively isolated from other hawthorn-feeding forms by chromosomal inversions. This would push the date for the initial divergence much further back in time, and argues again for an allopatric split with reproductive isolation, which preceded the host switch. As Jiggins and Bridle (2004) point out, this does not minimise the importance of sympatric processes in the evolution of the host shift, but does indicate that allopatric processes are initially necessary to establish genetic differences between strains. The relevance of these speciation models to *Gyrodactylus* remains to be seen, but the analogy between *Rhagoletis* and the situation in *G. salaris*/*G. thymalli* appears strong (see Section 6.6), and Kearn (1996) for example, was convinced of the importance of host shifts as a means of gyrodactylid speciation. The role of chromosome inversions in *Gyrodactylus* is uninvestigated; if they occur, they could, as in *Rhagoletis*, lock large parts of the genome from recombination with other parasite genotypes. There have been no investigations of the existence of chromosome races or polyploids in *Gyrodactylus*, although the recent work of Ziętara *et al.* (2006) suggests the existence of potentially triploid races of *G. salaris.* The significance of this finding remains to be evaluated.

There is some evidence of co-evolution at the deeper levels of gyrodactylid speciation. This has been noted above (Section 6.5); the species of the sub-genus *Gyrodactylus* are mostly found on ostariophysans in Eurasia (not in America, where they have radiated onto other groups of fish, see Le Blanc *et al.*, 2006). This may represent a much older co-speciation event, of which only a few deep branches remain, while the terminal clades all represent fairly recent, host-switch driven speciation events. We now go on to discuss three possible speciation scenarios, of slightly different ages. These are: (i) relatively ancient speciation events on guppy hosts in the Caribbean (freshwater, tropical); (ii) more recent speciation events associated with European gobies (marine, temperate and subtropical); and (iii) the most recent events associated with Eurasian salmonids (cold-temperate, freshwater).

6.2. Evolution of Gyrodactylids on Guppies

Several papers (Harris and Lyles, 1992; Harris and Cable, 2000; Cable *et al*., 2005) have described the natural gyrodactylid fauna of poeciliid fishes in the Caribbean. Poeciliids are excellent models for micro-evolution with some of the most compelling evidence for natural and sexual selection in the wild being provided by the guppy, *Poecilia reticulata* (e.g. Endler, 1986; Magurran *et al*., 1992; Magurran, 2005). Guppies occur naturally in most freshwater streams in Trinidad (Houde, 1997), with marked phenotypic and genetic differences between upstream and downstream populations within a single river (Endler, 1980; Reznick and Endler, 1982; Shaw *et al*., 1994; Magurran *et al*., 1995). For almost two decades, predation pressure has been identified as the key factor driving guppy evolution (Endler, 1980; Reznick and Endler, 1982; Magurran *et al*., 1992, 1995; Reznick *et al*., 1997), although recent studies indicate that density-dependent regulation and resource availability may also have been important (Reznick *et al*., 2002). Surprisingly, selection pressures imposed by parasites have, until recently, been entirely ignored (Cable and van Oosterhout *et al*., 2003, unpublished; van Oosterhout *et al*., in press a, b).

Wild guppies sustain a range of fungal, protozoal, helminth and nematode infections and many fish carry multiple infections. The well-studied laboratory models *Gyrodactylus turnbulli* and G. *bullatarudis* (see Scott, 1982, 1985a, b; Scott and Anderson, 1984; Harris, 1986, 1988) are widespread and abundant parasites in natural populations of these fishes (Harris and Lyles, 1992; Cable and van Oosterhout, unpublished). The pathology of even moderate *Gyrodactylus* spp. infections, and their effects on behaviour, reproduction and survival of guppies, can be severe. For example, feeding response and activity of guppies (which are good indicators of health and alertness; Houde, 1997) are significantly reduced in guppies with only relatively small burdens of *G. turnbulli* (see van Oosterhout *et al*., 2003a). Heavily infected fish, with clamped fins and erratic swimming behaviour (Cable *et al*., 2002a), are ostracised by uninfected shoal mates (Cable and Griffiths, unpublished), and females discriminate against males infected with *G. turnbulli* which show reduced colour

pattern and courtship activity (Houde and Torio, 1992; Houde, 1997; López, 1998). Experimental infection with an isogenic culture of *G. turnbulli* furthermore showed that upland and lowland population guppies differed significantly in immunocompetence (van Oosterhout *et al.*, 2003a), with upland populations experiencing higher infection rates and mortality than lowland fish (van Oosterhout *et al.*, 2003). Furthermore, within guppy populations, the parasite-resistance of individual guppies is highly repeatable across infections (Cable and van Oosterhout, 2007).

We are becoming increasingly aware of the complex gyrodactylid fauna infecting poeciliid fishes and there is great potential for studying the interaction between poeciliid evolution and parasitism. The evolution of the poeciliids is partially known, but full molecular phylogenies are unavailable, and morphological phylogenies are uncertain (Poeser, 2003). Cable *et al.* (2005) described *G. pictae* from *Poecilia picta*, with a 5% difference in ITS sequence when compared with *G. turnbulli*. The respective hosts differ by 15% at the mitochondrial ND2 locus (Breden *et al.*, 1999). These differences are considerable, and suggest an ancient division between these two fish species and their parasites, which may relate to sea level changes in the Caribbean several million years BP. This system represents an excellent model which can provide a comparison with the relatively much more recent speciation events we observe in salmonid gyrodactylids.

6.3. Species Flocks on Gobies

A second example of gyrodactylid speciation on an evolving host group is illustrated by the gyrodactylids of gobies in the North East Atlantic, which clearly demonstrate that morphological similarity does not reflect relationship between forms. Sand gobies (*Pomatoschistus*, but also other inshore genera) represent a rapidly evolving species flock, the taxonomy of which is still under review. Gyrodactylids have been reported from two sand goby genera, *Pomatoschistus* and *Gobiusculus* (see Huyse *et al.*, 2004a, b). Significant events in the evolution of the hosts have included the Messinian

Salinity Crisis (~6 million years ago), which ended with the reopening of the Straights of Gibraltar and led to the separation of the *Pomatoschistus minutus* and *P. microps* clades, and the more recent Pleistocene glaciations that led to the evolution of the *P. minutus* species group within the Mediterranean. As ice retreated from the Last Glacial Maximum (LGM), ~20 000 years ago, gobies migrated North around the European coastline (Huyse, 2002; Huyse *et al.*, 2004b). Huyse *et al.* (2004b) regard sand gobies as being somewhat older than the most recent glaciation, and Gysels *et al.* (2004) make the assumption that *Pomatoschistus* spp. distributions have moved north and south in response to glaciations, including the LGM. Their molecular evidence suggests an expansion of *P. minutus* in the North Atlantic during the Eemian interglacial, 120 000 years BP (Gysels *et al.*, 2004). Both *P. minutus* and *P. microps*, which are not closely related and have been separated since the reopening of the Straights of Gibraltar, retreated southwards after the Eemian, but may have left refugial populations in the Southern North Sea area (Gysels *et al.*, 2004). This detailed and robust phylogeny of the host group, correlated with known geological events such as the Messinian Salinity Crisis and the LGM, allow testing for concordance in the pattern of evolution of their gyrodactylids. Furthermore, given Meinilä *et al.*'s (2004) estimates for mitochondrial evolution in gyrodactylids, these parasites should provide a useful fine-grained marker for evolutionary processes in gobies.

Pomatoschistus gobies are infected by *G. arcuatus*-like forms, noted but not described by Appleby (1996b). The morphometric divergence between stocks of *G.* cf *arcuatus* from different goby species suggested separate species (Geets *et al.*, 1999), which were described by Huyse *et al.* (2003) as *G. branchialis, G. arcuatoides, G. gondae* and *G. flavescensis*. Surprisingly, they are not closely related to *G. arcuatus* with a difference in ITS sequences of 13% (Huyse *et al.*, 2003). Unfortunately, *G. arcuatus*-like forms on Eurasian freshwater and marine fishes are poorly known, although they are common on gadids (Bychowsky and Polyansky, 1953; Malmberg, 1970), and it is not clear which is the sister group to the forms on gobies. The host preference of the four gyrodactylids varies. *G. gondae* occurs on *P. minutus* and the sibling *P. lozanoi* from the North Sea and from

Atlantic watersheds in Norway; *G. arcuatoides* infects only *P. pictus*; *G. branchialis* occurs on *P. microps* from the southern North Sea and Norway, and is closely related to *G. quadratidigitus* from the western Channel (Longshaw *et al*., 2003), while *G. flavescensis* infects *Gobiusculus flavescens* from Norway. Comparison of host and parasite molecular phylogenies (Huyse *et al*., 2003) could not distinguish between host switching and co-evolution for the *G. arcuatus*-like species infecting *Pomatoschistus minutus*, *P. lozanoi* and *P. norvegicus*, but these authors (Huyse *et al*., 2003) note that the evolution of these gyrodactylids can probably be related to the last ice age (i.e. less than 100 000 years BP). If this timing is correct, then the host switch of *G. ostendicus* to the more distantly related host *P. microps* (see Huyse and Malmberg, 2004) is accompanied by a remarkable change in morphology; this species appears more closely related to *G. harengi* based on hook shape, and only molecular analysis reveals the true affinities of this taxon. Similarly, Huyse *et al*. (2003) closely relate *G. anguillae* from the common eel *Anguilla anguilla* to *Gyrodactylus micropsi* from *Pomatoschistus microps*, a further major change in morphology following what seems to have been a fairly recent host shift. Host switching also appears to have been the main factor promoting speciation in the *G. rugiensis*/*G. micropsi* group from the fins of gobies (Huyse and Volckaert, 2002). More recently, Huyse and Volckaert (2005) have used a range of statistical approaches to test for co-evolution, as opposed to host switching, in this goby–gyrodactylid system. Although some of the methods used did generate evidence of co-speciation of host goby and gyrodactylid, particularly amongst the gill parasites, it is very difficult to distinguish genuine co-speciation from host shifts onto closely related hosts. When Huyse and Volckaert (2005) included some estimate of time from divergence in the analysis, it became apparent that all of the gyrodactylid sister species were probably much younger (related to Pleistocene events) than their sister host species, which had diverged in the Pliocene. This also rules out co-speciation and strongly suggests that infection of one host species predilects gyrodactylids to infect other closely related hosts by host shifting (Huyse and Volckaert, 2005). This is also the pattern that is observed in specificity studies (Section 5.3), that gyrodactylids normally infect a spectrum of more or less closely

related fish species although host shifts to unrelated fish species can also occur.

No experimental studies on the host preferences of these gyrodactylids of gobies have been undertaken, and no mitochondrial sequences are available. With evidence from both of these sources, we would be able to infer the evolutionary history of this group with greater confidence.

6.4. Refugia and Pleistocene Dispersals

The history of gyrodactylid speciation, within Eurasia and probably also North America, is closely tied to post-glacial history and the presence of isolated refugia for freshwater fish during the ice ages. The narrow host range of many gyrodactylids, and the molecular information now available, makes these highly attractive organisms for considering patterns of evolution of freshwater fishes in the post-glacial landscape, and can provide important corroboration of mitochondrial DNA evidence in studying evolution of the fishes themselves.

The precise age of gyrodactylid species is unclear, given the lack of a calibration for molecular divergence in the genus. However, several aspects of gyrodactylid evolutionary biology suggest that a major force responsible for their speciation has been the series of climatic upheavals known collectively as the ice ages. Over the past 800 000 years, Eurasia has alternated between phases of cold, dry climate (ice ages) in which ice sheets pushed south and sea level fell, and phases of warm moist climate (interglacials), in which ice retreated and rising sea levels subdivided the land mass. This pattern has never been precisely repeated, and so the location of ice masses and possible refugia has differed between glacial events. As ice sheets have extended south, flora and fauna are thought to have retreated in front of the ice, and to have survived in scattered refugia. During the LGM, three southern European refugia for terrestrial organisms are normally acknowledged: the Iberian Peninsula, Italy/Sicily, and the Balkans (Hewitt, 1999). The relative importance of each varied depending on taxon, but, based on mtDNA evidence, most terrestrial and some aquatic

organisms recolonised northern Europe from one or more of these three areas (Hewitt, 2004). This pattern of recolonisation is still continuing, as organisms with poor dispersive powers failed to colonise northern Europe before rising sea level cut dispersal routes. This is probably the historical reason for the absence of *G. salaris* from Norway and Britain.

Refugia for aquatic organisms are much more complex. Aquatic organisms can retreat southwards only so far before increasingly steep river gradients make habitat unsuitable. Reduction in sea level during glacial maxima increased the distance between header streams of different watersheds (the header streams of Rhine, Rhone and Danube would have been much further apart at the time of the LGM than today), and fundamentally changed the relationships between major watersheds and river systems (Figure 22). During the LGM, the English Channel River, emptying to the west of the European continental shelf somewhere off Brittany (Lericolais *et al*., 2003), received water from the Thames, the Seine and the Rhine. Finally, and perhaps most importantly, a series of large impounded glacial lakes existed along the edge of the ice sheet. These included the relatively small Lake Humber/Lake Pickering complex in what is today northern England (Clark *et al*., 2004), but also probably a moderately large lake in the southern North Sea, and a series of very large ice-dammed lakes along the margin of the ice in northern Russia and Siberia (Mangerud *et al*., 2001, 2004; Svendsen *et al*., 2003). These lakes, caused by ice-damming of rivers such as the Ob and Yenissei, were of such a size as to potentially have had impacts on global climate (Krinner *et al*., 2004; Mangerud *et al*., 2004). From an evolutionary perspective, these were large enough to sustain large, resident populations of many fish, including the anadromous salmonids, sticklebacks, cottids and perhaps gobies. The precise combination of fish within these lakes, and the consequences of genetic drift, would depend in part on their size, and the effective population size of each species within them. These lakes changed their conformation and connections during the Weichselian, from their inception some 90 000 years BP (Krinner *et al*., 2004; Mangerud *et al*., 2004) until the LGM ~20 000 years BP. During different glaciations, different combinations of glacial lakes probably arose (there is little evidence of events

Figure 22 Modern coastline (pale outline), and coastline 20 000 before present (BP) (dark outline) showing significant bodies of freshwater (pale blue). Note that most European watersheds drain into the Black Sea, which at this time was freshwater. The Volga system also connected with the Black Sea and the other European rivers for a short period ~18 000 years BP due to meltwater overflow into the Manych Pass. Consequently, there were opportunities for mixing and exchange of gyrodactylids throughout central and western Europe, with only the Channel River system (Rhine, Thames and northern French rivers) remaining distinct. This should be viewed as a dynamic evolving landscape; prior to this time (60 000 years BP) the rivers of western Siberia (Ob, Yennisei) drained into the Caspian; subsequent to the LGM, the Baltic became a freshwater lake, allowing interchange of fish and gyrodactylids between rivers draining into the Eastern Baltic.

prior to the Weichselian, because later ice sheets have erased traces of previous glacial episodes). During the LGM, for example, drainages of the Danube, Dniepr and Don were all interconnected, draining into the freshwater Black Sea (Mangerud *et al.*, 2004), and were linked via the Manych Pass to the Volga drainage into the Caspian Sea. At a previous phase in the last ice age (80 000 years BP), the Siberian rivers Yenissei and Ob were also prevented from draining north, and instead drained south to the Aral, Caspian and Black Seas (Mangerud *et al.*, 2004). This mixing of drainage patterns throughout

the last ice age probably accounts for the uniformity and richness of the Central European gyrodactylid fauna, with only the Channel River drainage basin remaining distinct (Figure 22). Ice lakes were freshwater or of very low salinity, and allowed mixing of fish stocks and harsh, possibly immunosuppressive environmental conditions, potentially provided ideal environments for host switching by gyrodactylids, with consequent speciation and evolution. Interestingly, the most closely related parasite species pairs infect different hosts (indicating a host switch, see Table 1), while similar species infecting the same host tend to be more distinct at a molecular level, suggesting evolution in different refugia, possibly during different glaciations (Zię̧tara and Lumme, 2002). This is best indicated by the gyrodactylids of the minnow, *Phoxinus phoxinus*. This cyprinid has a widespread distribution throughout Eurasia, and may reasonably be expected to have been restricted to several different ice lake refugia. Zię̧tara and Lumme (2002) noted that host switching triggers adaptation to the new host, and pointed to two phases in the evolution of *G. wageneri*-like species, one phase ~5 million years BP, the other ~1 million years BP. This latter, recent burst of host switching and speciation occurred within the period of glacial and interglacial warmings but we would question the confidence limits for this estimate, suggesting that in some cases, speciation and host switching has occurred as recently as the LGM, perhaps as little as 20–50 000 years BP. This is suggested very strongly by the biogeography of some molecular variants. Zię̧tara and Lumme (2002, 2003) identified distinctive molecular variants of several species, including *G. aphyae* and *G. macronychus*/*G. jussii* from opposite sides of the Fennoscandian watershed, from Oulu (draining to the Baltic) and from Oulanka (draining to the White Sea). During the Early, Middle and Late Weichselian glaciation (90 000–20 000 years BP), fishes from both the Baltic and White Seas were restricted to distinct ice-dammed lakes (Krinner *et al.*, 2004). During the preceding warm phase, the Eemian (~120 000 years BP), the two Seas were linked by a seaway through Lakes Ladoga and Onega, which would have led to the mixing of fish populations and subsequent geographical homogenisation of their gyrodactylids (Funder *et al.*, 2002). A slightly older event may have been the evolution of *G. limneus* and *G. phoxini* on minnows in

separate ice-dammed lakes, as these species do occur conspecifically (Malmberg, 1970), suggesting prior mixing during a warm phase. On the other hand, the evolution and shift in host specificity between *G. salaris* and *G. thymalli* appears to have occurred during the LGM and afterwards (see below). Within the large ice lakes (e.g. the Black or Caspian Seas 20 000 years BP and including the eastern Baltic today), conditions favoured the survival of freshwater fish alongside marine species. Within current day Greenland (where conditions probably approximate those within the ice-dammed lakes of the LGM), very low temperatures, and floating wedge-like layers of freshwater on salt at the heads of fjords, allows marine fish such as *Myoxocephalus* to penetrate into almost freshwater. It is under such conditions that the adaptation of marine gyrodactylids to freshwater probably occurred.

Glacial events influencing gyrodactylid speciation were not confined to the Baltic and northern Russia. One of only two gyrodactylids so far considered endemic in the British Isles is *G. rogatensis* from the bullhead *Cottus gobio* (see Harris, 1985). Elsewhere in Europe it is replaced by *G. cotti*, a more widespread species infecting a range of cottids, or by *G. hrabei*, described by Ergens (1957, 1971) as most closely related to marine forms infecting *Myoxocephalus* (e.g. Levinsen, 1881). *G. hrabei*-like forms infect *Cottus poecilopus* across much of its range (Ergens, 1961; Malmberg, 1972; Winger *et al*., 2005, unpublished). The occurrence of *G. rogatensis* is mirrored by molecular analyses of the host bullheads, which suggest that during the LGM either one group survived within the Channel River system and its tributaries, and has remained distinct within the UK and rivers as far east as the Rhine (Hänfling *et al*., 2002), or that this clade recolonised from southern Europe but has been subject to serial bottlenecking in the process (Volckaert *et al*., 2005). *G. rogatensis* appears to have switched to infect this clade within the catchment of the Channel River.

An interesting problem in recent gyrodactylid evolution concerns *G. wageneri*-forms infecting the three-spined stickleback, *Gasterosteus aculeatus*, and small cyprinids. The nine-spined stickleback, *Pungitius pungitius*, is infected by *Gyrodactylus pungitii*, which occurs throughout Scandinavia and is only distantly related to *G. gasterostei*, found on the three-spined stickleback. *G. gasterostei* occurs in northern

Germany (Gläser, 1974), but not in Scandinavia (Malmberg, 1970 failed to find this species), and probably evolved within the Channel River system, spreading eastwards into the Elbe system. The normal host is *Gasterosteus aculeatus*, and in Britain at least, this parasite is specific for this host (Cable *et al*., 2002b; Harris, unpublished). In the Czech Republic, however, *G. gasterostei* is considered a generalist, infecting primarily cyprinids (see Ergens, 1985a, b; Matejusová *et al*., 2001). Furthermore the three-spined stickleback is rare in central Europe (Ahnelt *et al*., 1995), and the molecular evidence of Ziętara and Lumme (2002) indicates that *G. gasterostei* from the stickleback and *G. aphyae* from the minnow are so closely related that the distance between *G. gasterostei* and one *G. aphyae* clade is less than that between the two *G. aphyae* clades. Clearly, *G. gasterostei* and *G. aphyae* are sister species, one the result of a host switch from the other. However, is the Central European *G. gasterostei* of Ergens (1985a, b) simply a misidentification, or is there a more complex situation, with this species infecting a different host in these central European river systems?

6.5. Paraphyly of *Gyrodactylus salaris*

The most complex situation discovered to date is the pathogenic *Gyrodactylus salaris*, and its close relatives, *G. teuchis*, *G. bohemicus* and *G. thymalli*:

(i) *G. teuchis* was described from rainbow trout (the type host) in France (Lautraite *et al*., 1999; Cunningham *et al*., 2001); other hosts include wild Atlantic salmon and brown trout. *G. teuchis* has ITS sequences which are distinct from *G. thymalli* and *G. salaris*, but morphological variation falls within the total range reported for *G. salaris* (see Mo, 1991a, b, c), and few qualitative differences are seen in the hamuli and ventral bar (Cunningham *et al*., 2001). There are small differences in the shape of the marginal hook sickle. In the original description, *G. teuchis* was thought to have evolved in an "Iberian refugium", but this species probably evolved in the Channel River watershed, as it is distributed along the Spanish and French Atlantic seaboards. It has been

reported from farmed rainbow trout in Scotland (Cunningham *et al*., 2001) and from wild brown trout in Denmark (Buchmann *et al*., 2000), but it is difficult to get an idea of the original range of this taxon as it is common on rainbow trout and may have been redistributed with this host. *G. teuchis* is not recorded in Norway and appears to be absent from wild salmon in the UK but this may be because salmon populations in southern rivers are so small, and parr generations do not overlap. Extermination of salmon from rivers around the North Sea in the 19th century also complicates any discussion of recreating the original range of *G. teuchis*.

(ii) *G. bohemicus* from rainbow trout (type host) and brook trout in a trout farm in South Bohemia, Czech Republic, was considered (Ergens, 1992a) most closely related to *G. thymalli* and *G. magnus*. Both hosts were introduced to Europe from America and the parasite has neither been recorded from brown trout in Central Europe nor from any host in North America. The identity of this taxon was a mystery until Lindenstrøm *et al.* (2003a) described the "*G. salaris* variant" (Gx), with slight morphological variation in the marginal hook and a predilection for infecting rainbow trout (Lindenstrøm *et al*., 2000). The ITS rDNA sequence of this rainbow trout variant differed by just three bases from that of *G. salaris sensu stricto,* and one of these bases exhibited intra- and inter-individual polymorphism (Lindenstrøm *et al*., 2003a, b). Lindenstrøm *et al.* (2003a, b) argued against the rainbow trout variant being *G. bohemicus*; however, perhaps this reverses the burden of proof, and it should be demonstrated that *G. bohemicus* described from a handful of specimens collected during faunistic work, is actually distinct from the Lindenstrøm rainbow trout variant. We propose that, until such evidence is forthcoming, *G. bohemicus* should be regarded as the first record of the Lindenstrøm *et al.* (2003a, b) "Gx" rainbow trout variant. Parsimony would suggest that this is safest course until positive evidence is found to discriminate *G. bohemicus* from the "Gx" variant.

(iii) *G. thymalli,* described from less than a dozen specimens from Slovakian grayling (Žitňan, 1960), was considered to be most

similar to *G. salaris* from salmon. *G. thymalli* can be distinguished from *G. salaris* and *G. teuchis*, being larger than either (Shinn *et al*., 2004), and yet the ribosomal sequences of the V4 and ITS regions of the rRNA gene array are identical to *G. salaris* (see Cunningham, 1997), suggesting that these taxa are more closely related than are any other *Gyrodactylus* species (Cunningham, 2002). The two forms were originally considered to have distinct IGS haplotypes, although they share many of the individual repeat units which make up the haplotypes (Sterud *et al.*, 2002). Recent research, however, shows that this interpretation is not correct (Hansen *et al*., 2005, 2006). The recently sequenced β-tubulin gene also shows differences between them (Collins *et al*., 2004a). These observations, in addition to major differences in pathogenicity and host specificity suggest that these are valid species (Sterud *et al*., 2002). *G. thymalli* from *Thymallus* spp. is widespread in Eurasia, including Kamchatka (Ergens, 1983), Poland (Hansen *et al.*, 2007), Slovakia (Žitňan, 1960; Hansen *et al.*, 2007), Britain (Denham and Longshaw, 1999; Hansen *et al.*, 2007), Norway (Hansen *et al*., 2003, 2006), Finland and the Kola Peninsula (Meinilä *et al*., 2004). The grayling is widespread in NW Europe and was certainly restricted to different ice lake refugia during the LGM (Koskinen *et al*., 2000, 2002; Weiss *et al*., 2002) giving rise to at least four major mtDNA lineages. There is however evidence of extensive mixing in contact zones between drainages (Gum *et al*., 2005) and, as with other salmonids, the distribution has been extensively modified by Man.

Recent work on *G. thymalli* has been based on samples from the Eastern Baltic, particularly Lake Kitka and the River Oulanka system (draining to the White Sea), the River Livojoki (Baltic drainage), and from Pjalma (Lake Onega) in Karelia (see Meinilä *et al*., 2004). Populations from the White Sea drainage had mitochondrial haplotypes belonging to quite different clades to those from the Baltic, again emphasising the importance of different glacial refugia. Meinilä *et al*. (2004) calibrated the divergence between White and Baltic Sea forms to be ~100 000 years, because, as noted above, an earlier

divergence would not have retained geographical separation following the Eemian interglacial. Meinilä *et al.* (2004) also analysed mitochondrial haplotypes collected by Hansen *et al.* (2003) from salmon, grayling and rainbow trout in Norway and Sweden, providing a unique opportunity to examine short-term evolution of *G. salaris* and *G. thymalli* in Scandinavia. These phylogenies (Hansen *et al.*, 2003; Meinilä *et al.*, 2004) may suggest that *G. salaris* on salmon is derived from *G. thymalli*. However, the data clearly show that *G. salaris* was introduced into Norway on several occasions, that it has been dispersed widely by anthropogenic means and that it is polyphyletic. Meinilä *et al.* (2004) consider *G. thymalli* to be a junior synonym of *G. salaris* and that all forms of this taxon from rainbow trout, salmon or grayling (named the *G. salaris* complex) should be referred to as the *G. salaris* cluster or *G. salaris sensu lato*. This mitochondrial evidence is not the full story, and ignores differences in nuclear sequencing data (Sterud *et al.*, 2002; Cunningham *et al.*, 2003; Collins *et al.*, 2004a; Hansen *et al.*, 2006), morphology (McHugh *et al.*, 2000; Shinn *et al.*, 2004) and host preference (Soleng and Bakke, 2001b; Bakke *et al.*, 2002; Sterud *et al.*, 2002) between the forms from grayling and salmon. It also oversimplifies the taxonomic situation. For example, Shulman *et al.* (2005) noted an apparently cold-adapted strain of *G. salaris* from the River Lhizma, in Karelia, while Olstad *et al.* (unpublished) have identified a parasite population from grayling which is morphologically intermediate between *G. salaris* and *G. thymalli.* The former originates from the apparent centre of diversity of *G. salaris* (see Meinilä *et al.*, 2004), but the latter, from Norway, is more problematical. This implies a very rapid host shift and morphological divergence if this clade has indeed originated from the *G. salaris* clades imported into Norway during the 1970s. A final reason for not changing the nomenclatural status quo is that in the case of *G. salaris*/*G. thymalli*, a pure DNA-based taxonomy may result in a species definition which fails to reflect the significant differences in host response and pathology and will scarcely be accepted by the fish management authorities involved in the recognition of such a highly pathogenic notifiable fish disease of European concern (Olson and Tkach, 2005).

There is also great confusion over the "rainbow trout variant" of *G. salaris* from Norway described by Mo (1991a) infecting Lierelva and Drammenselva salmon. *G. salaris sensu stricto* (i.e. the salmon infecting forms) is polyphyletic (Meinilä *et al.*, 2004), made up of a clade (clade I) which switched to salmon from grayling in North Fennoscandia and a clade (clade II) which switched to salmon and rainbow trout elsewhere, which Meinilä *et al.* (2004) consider genetically relatively invariant. This ignores the fact that clade II contains forms which can be distinguished morphologically (Mo, 1991a); the "Gx" form of Lindenstrøm *et al.* (2003a, b) and the variant of Jørgensen *et al.* (2006) are not identical to the "rainbow trout variant" of *G. salaris* described by Mo (1991a). More informative evidence about the nature of rainbow trout infecting forms is derived from the IGS data of Sterud *et al.* (2002) and Cunningham *et al.* (2003). This locus, in *G. salaris*, contains two repeat units, containing in turn a series of subrepeats. The pattern of repeats is fairly constrained in *G. salaris* from salmon, as would be expected if, as suggested by Meinilä *et al.* (2004), *G. salaris* is derived from such a restricted origin within clade III of *G. thymalli. G. thymalli* overall showed greater diversity in IGS repeat units than Norwegian *G. salaris* (see Cunningham *et al.*, 2003; Hansen *et al.*, 2006), but sampling of *G. thymalli* is relatively restricted geographically. Swedish populations of *G. salaris* contain more diversity of IGS haplotypes than Norwegian forms and rival the haplotype diversity seen with limited sampling of *G. thymalli* (see Hansen *et al.*, 2006). Parasites from rainbow trout reveal surprising diversity in IGS haplotypes, exceeding that of *G. salaris* from Norwegian salmon populations or *G. thymalli* from grayling. The Sterud *et al.* (2002) study showed that IGS haplotypes from the rainbow trout variant resembles in part repeat variants normally found in salmon-infecting *G. salaris* (repeat region 1), alongside variants normally found in grayling-infecting *G. thymalli* (repeat region 1). Given this diversity, it is impossible to reconcile the rainbow trout variant with the restricted mitochondrial diversity noted by Meinilä *et al.* (2004). The lack of stability in the IGS of the rainbow trout variant is intriguing. Cunningham *et al.* (2003) speculate that the greater diversity of IGS repeats may be due

to selection against many repeat types in either salmon-infecting or grayling-infecting forms. This again seems quite at odds with Meinilä *et al.*'s (2004) hypothesis that the rainbow trout variant is represented by a highly restricted subset of mitochondrial haplotypes, and therefore derived from a very restricted clade of *G. thymalli*. The IGS locus is under the constraint of concerted evolution in a similar way as the other ribosomal gene loci; it might be expected therefore that the variation within the rainbow trout variant will eventually stabilise in a relatively homogenous form. The lack of agreement between mtCOI (Hansen *et al.*, 2003; Meinilä *et al.*, 2004) and IGS phylogenies (Sterud *et al.*, 2002; Cunningham *et al.*, 2003; Hansen *et al.*, 2006) may not be intractable. Mitochondrial haplotypes spread through a population at different rates to nuclear genomic markers because of their maternal mode of transmission. This will be particularly marked in *Gyrodactylus*, with its predominantly parthenogenetic viviparous reproductive strategy. This may go some way to explaining the conflict over the status of the southern Norwegian populations of salmon-infecting *G. salaris*, one of which (River Lierelva) has been the principal strain used in experimental studies of the species (Bakke *et al.*, 2002) and is linked by mtCOI haplotype (Hansen *et al.*, 2003; Meinilä *et al.*, 2004) to the rainbow trout infecting forms. Cunningham *et al.* (2003), on the other hand, using IGS nuclear markers, place the Lierelva strain with the majority of other *G. salaris* strains from salmon. AFLP patterns, also based on nuclear markers (Cable *et al.*, unpublished) link Lierelva strain with the other salmon-infecting forms. There is however a history of introduction of the rainbow trout variant into the area immediately adjacent to the Lierelva/Drammenselva watercourses. The rainbow trout variant (Mo, 1987, 1991a) originally recovered from salmon in both rivers in 1987 might have originated from infected commercial hatcheries in Lake Tyrifjorden which imported salmonids (both rainbow trout and salmon) from Sweden (Mo, 1991a; B.O. Johnsen, personal communication). It has also been identified on escaped rainbow trout in Lake Tyrifjorden, from where it could have spread into both Drammenselva and Lierelva (probably via Holsfjorden; cf. Johnsen *et al.*, 1999). The same parasite was also found in eight rainbow trout hatcheries around Lake Tyrifjorden which may also

represent the origin of the river infections. There has therefore been ample opportunity for hybridisation and introgression of the rainbow trout mitochondrial haplotype into *G. salaris* populations in South East Norway.

Currently, we would have to conclude that the *G. salaris*/*G. thymalli* evolutionary scenario remains highly complex and further research is needed to understand the patchwork of strains of the parasite currently found in Scandinavia. An interesting recent discovery has been of a resident Arctic charr population in lake Pålsbufjorden (Buskerud, County, southern Norway, draining into the commercially important salmon river Numedalslågen), which supports a persistent *G. salaris* infection with a mitochondrial haplotype similar to that of Drammenselva parasites (Robertsen *et al.*, 2006, 2007b). This is the first observation of *G. salaris* maintaining a viable population on charr in the absence of any other susceptible salmonid species. This Arctic charr strain is of limited infectivity to salmon (Olstad *et al.*, 2005, 2007) and may have originated following another host shift from a rainbow trout variant. In Denmark, a low pathogenic strain of *G. salaris* to salmon is reported based on experimental laboratory tests but originally from rainbow trout which experienced high parasite intensities (Jørgensen *et al.*, 2006). All sequenced ITS clones of this Danish strain revealed only one single base substitution when compared to all other known species and strains of *Gyrodactylus* including Gx of Lindenstrøm *et al.* (2003a) and COI data demonstrated it to be closely related to one of the rainbow trout forms in Norway. Mitochondrial COI haplotypes cannot so far be linked with virulence. We must conclude that strains of the parasite were introduced into Norway from elsewhere in Fennoscandia, probably in the 1970s, and distributed widely by Man (Hansen *et al.*, 2003; Robertsen *et al.*, 2006, 2007a). This is clear from the very wide distribution of *G. salaris* clade I haplotypes throughout Norway and in the case of clade II haplotypes throughout Europe (Meinilä *et al.*, 2004; clade III according to Hansen *et al.*, 2003). It is a truism that the global threat to Atlantic salmon posed by strains of salmon-infecting *G. salaris* originating within Europe is not great. Further range extension will probably be by the rainbow trout form, which is not especially pathogenic to rainbow trout, and therefore easily

overlooked, but still pathogenic to salmon. Secondly, we can conclude that the primary evolutionary stimulus for the pathogenic *G. salaris* has been these host switches from grayling to salmon. A very recent origin for these different salmon-infecting clades cannot be ruled out. Numerous salmon, grayling, rainbow trout and charr infecting forms have since arisen, and have been distributed extensively by Man. Introgression of strains have probably been widespread, and the role of the rainbow trout in strain hybridisation and introgression may have been paramount.

6.6. Terminology of the *G. salaris* Species Complex

G. salaris and *G. thymalli* are almost identical at a molecular level and there is no support from mtDNA (CO1) sequences for monophyly of all *G. salaris* and *G. thymalli* haplotypes (Hansen *et al.*, 2003, unpublished data). These taxa therefore probably represent a case of incipient speciation with the sibling taxa representing either a semispecies (one or two polytypic species) or a superspecies (several sibling species; see Mallet, 2001), reproductively isolated by host preference. If we follow Meinilä *et al.* (2004), the relationship between *G. salaris* and *G. thymalli* is so close that they are merely host races of the same species, and both *G. thymalli* Žitňan, 1960 and probably *G. bohemicus* Ergens, 1992 should be treated as junior synonyms. Inclusion of the rainbow trout variant Gx with ITS differing to *G. salaris* at three positions (according to Lindenstrøm *et al.*, 2003a) and two other variants differing in one base substitution (Jørgensen *et al.*, 2006; Robertsen *et al.*, 2006, 2007a) to all other known species and strains of *Gyrodactylus* within *G. salaris* would make the latter polyphyletic as it appears to have evolved independently from different clades within *G. thymalli*. We appreciate that this is a difficult area, and that the names of these currently speciating forms must remain in flux. However, we propose to maintain *G. salaris*, *G. thymalli* and *G. bohemicus* as separate species until further clarification using new molecular markers, knowledge of the impact of different microenvironment factors on *Gyrodactylus* morphology and hybridisation experiments. It is most important that

practice is entirely transparent when depositing specimens or sequences (unfortunately not currently the case) so that future workers can interpret the early 21st century situation in the light of their own theoretical paradigms.

Part 3. Gyrodactylid synecology

7. EPIDEMIOLOGICAL MODELS: BRIDGING THE GAP BETWEEN MICRO- AND MACROPARASITES

The value of gyrodactylids as epidemiological models has been recognised for many years with some of the most elegant studies of host–parasite dynamics being conducted by Scott and co-workers in the 1980s using *G. turnbulli* (at the time called *G. bullatarudis*, see Harris, 1986; Richards and Chubb, 1995; Richards *et al*., 2000) on guppies (Scott, 1982, 1985a, b, 1987; Scott and Anderson, 1984; Scott and Nokes, 1984; Scott and Robinson, 1984; Harris, 1988, 1989; Leberg and Vrijenhoek, 1994; Richards and Chubb, 1996, 1998; Cable *et al*., 2002a). Gyrodactylid reproduction is characterised by a short generation time and completion of the life cycle *in situ* without the need for transmission. The period of infection is also short relative to host life span, due to the acquired host response (Section 8.1). Gyrodactylids bridge the gap between micro- and macroparasites and can potentially model other microparasites of animals and humans. A number of statistical and computer models have also been designed to risk assess the spread of gyrodactylid infections to new rivers (Anonymous, 1996; Paisley *et al*., 1999; Brun and Høgåsen, 2003; Høgåsen and Brun, 2003; Peeler and Thrush, 2004; Peeler *et al*., 2004; Jansen *et al*., 2005, 2007). Host age-structured population models have been used to assess the impact of disease-induced mortality at different stages of the life cycle of salmonids (de Clers, 1993). However, the full potential of gyrodactylid population models has yet to be realised due to the poorly known action of several micro- and macroenvironmental factors, and it remains impossible to predict the likely outcome of gyrodactylid introductions into novel watersheds. In particular, because individual fecundity is so low (normally less than

three daughters per worm), the statistical vagaries of survivorship and mortality make the outcomes of individual infections very difficult to predict. Gyrodactylids can be identified and counted long before their pathological effects are apparent, and so accurate, quantitative data can be collected during the entire infection of a single host (Jansen and Bakke, 1993a, b; van Oosterhout *et al*., 2003). The guppy–gyrodactylid interaction is particularly tractable, and is already a paradigm for studies of host–parasite evolution (López, 1998) because the ecology of both *G. turnbulli* (e.g. Scott and Anderson, 1984; Scott, 1985; Harris, 1988, 1989; Cable *et al*., 2002a) and guppies (Reznick and Endler, 1982; Magurran and Phillip, 2001; van Oosterhout *et al*., 2003) is so well understood. The wealth of information on this system provides an invaluable database with which to test the impact of various host variables on parasite biology (e.g. Cable and van Oosterhout, submitted for publication).

8. HOST IMMUNITY AND PARASITE PATHOGENICITY

8.1. Host Immune Responses

The subject of immunity of fish to monogenean ectoparasites has been reviewed in depth recently (Buchmann, 1999, 2000; Buchmann *et al*., 2001, 2003; Buchmann and Lindenstrøm, 2002) and will only be touched upon here. Understanding immune responses against gyrodactylids was long hampered by the misconceptions that fish and other poikilothermic lower vertebrates possess a primitive and unsophisticated immune system, and that ectoparasites washed by the external milieu would not be significantly affected by blood-borne immune responses. These misconceptions have now been corrected, but there are major differences between the immune response to gyrodactylids and that to other ectoparasitic helminths, indeed that to other ectoparasitic monogeneans. Furthermore, with over 400 *Gyrodactylus* species described, it would be surprising if there were not a range of response mechanisms operating against them.

The first study normally cited as identifying a response against gyrodactylids is that of Lester (1972), who noted shedding of mucoid

material by *Gasterosteus aculeatus* infected with *G. alexanderi*. Parasites became physically entangled within this insoluble slough and became detached from the surface of the fish. They survived dislodgement and were able to reattach to new hosts. Lester and Adams (1974a, b) went on to link this response to the immunological status of the hosts, but this non-specific, physical mechanism of elimination became a paradigm of gyrodactylid immunity for the next 25 years and more subtle involvement of the immune system was not seriously considered until the late 1990s. This was unfortunate because Schechmeister *et al*. (1962) had already shown that irradiated goldfish were far more susceptible to *Gyrodactylus* sp. than un-irradiated controls, suggesting strongly that an intact immune system is needed for a fully functional response against gyrodactylids.

Almost all gyrodactylid–vertebrate interactions which have been examined show evidence of parasite population limitation and decline. Apart from the *G. alexanderi*-stickleback system (Lester and Adams, 1974a), this has also been noted for *G. turnbulli* on guppies (Scott and Robinson, 1984; Madhavi and Anderson, 1985; Harris, 1988; van Oosterhout *et al*., 2003; Cable and van Oosterhout, 2007), *G. colemanensis* and *G. derjavini* on rainbow trout (Cusack, 1986; Buchmann and Uldal, 1997, respectively), *G. stellatus* on English sole (Kamiso and Olson, 1986), *G. katharineri* on carp (Gelnar, 1987a, b), *G. gobiensis* on gudgeon (Gelnar, 1987c), and *G. salaris* on rainbow trout, lake trout, brook trout and Arctic charr (Bakke *et al*., 1991b, 1992b, c, 1996). This phenomenon is also observed in other gyrodactylid genera, including *Gyrdicotylus gallieni* on clawed toads (Harris and Tinsley, 1987; Jackson and Tinsley, 1994). It has not been noted in *Isancistrum subulatae* on the squid *Alloteuthis subulata* (see Llewellyn, 1984). Typically, on an individual fish, gyrodactylid populations initially increase until the rate of population growth slows and declines, parasites are lost and eventually the infection is eliminated. A high proportion of fish may die as a result of infection. The macroenvironment may have a relatively minor effect on this process (but see Section 9) except to modify timing; responses have been noted in marine and freshwater fish, both tropical and cold water. The response can occur at low, potentially immunosuppressive temperatures (Bly and Clem, 1992) and has therefore been regarded as

distinct from a classical immune reaction. However, it shows inter-host heterogeneity (Bakke *et al.*, 2002; van Oosterhout *et al.*, 2003), and memory (Scott and Robinson, 1984; Scott, 1985a, b; Richards and Chubb, 1996; Bakke *et al.*, 2002; Cable and van Oosterhout, 2007), requires an intact immune system (Schechmeister *et al.*, 1962) and can be modulated by immunomodulatory hormones such as cortisol and testosterone (Buchmann, 1997a; Lindenstrøm and Buchmann, 1998; Harris *et al.*, 2000; Nielsen and Buchmann, 2003), all characteristics of an acquired immune response, usually mediated via antibodies. Although antibodies have been detected in natural infections of blood-feeding monogeneans such as *Heterobothrium okamotoi* (see Wang *et al.*, 1997), *Pseudodactylogyrus* (see Mazzanti *et al.*, 1999; Monni and Cognetti-Varriale, 2002) and *Discocotyle sagittata* (see Rubio-Godoy *et al.*, 2004), they have never been demonstrated in gyrodactylid infections. Buchmann (1998a, b, c) failed to find antibodies to *G. derjavini* in rainbow trout and we have similarly failed to find antibodies to *G. salaris* in either mucus or blood of infected salmon using ELISA (unpublished). Negative results are seldom published, but the absence of antibodies in these interactions is of considerable interest.

In the absence of specific antibody responses, inducible non-specific responses may play a part in limiting gyrodactylid population growth. Many species, including *G. derjavini* and *G. salaris*, are extraordinarily sensitive to host complement. Buchmann (1998a) noted binding of complement C3a to *G. derjavini* tegument and Harris *et al.* (1998) showed that living *G. salaris* are killed at physiologically relevant titres as low as 1:200, considerably lower than that needed to kill other parasites (Fishelson, 1989). Complement acts via the alternate (antibody-independent) pathway and pre-incubation in blood from fish exposed to high infections of *G. salaris* (and therefore most likely to possess antibodies against the parasite) failed to enhance killing, further suggesting that antibodies are not involved (Harris *et al.*, 1998). Killing is presumably so efficient because *G. salaris* in a freshwater environment are osmotically stressed when the integrity of the tegument is disrupted by membrane attack complexes (MACs). However, Moore *et al.* (1994) also found a complement-like factor very effective against *G. stellatus* from winter flounder, although in

this case the parasites were maintained in salt water. Buchmann (1998a, b, c) noted the response was not species-specific, an observation confirmed by Harris *et al.* (1998) who noted that salmon complement lysed *G. decorus* from roach, while trout complement lysed *G. salaris*. It would be wrong to suggest that complement is a general mechanism for killing gyrodactylids, particularly as we have noted greater resistance to complement in *G. turnbulli* from guppies (unpublished), and J.A. Jackson (personal communication) has indicated that *Gyrdicotylus* is also relatively resistant. However, we have noted a correlation between complement levels during the course of *G. salaris* infections on salmon and the susceptibility of the strain concerned, with resistant Neva salmon having a C3 complement titre in mucus some ~25% higher than the highly susceptible Alta strain (Bakke *et al.*, 2002). The level of complement, at least in the serum of resistant Neva fish, was also inducible in response to *G. salaris* infection, being some 20% higher 45 days after infection than in naïve fishes (Bakke *et al.*, 2002). These observations do suggest a role for complement in eliminating gyrodactylid infections of salmonids, but clearly there is much further work to be done to confirm this. In particular, salmonids express multiple C3 isoforms and have multiple genes encoding factor B (Sunyer, 2005). To date no functional studies of these different complement isoforms on gyrodactylids have been carried out, although differences in susceptibility to gyrodactylids could be related to complement diversity expressed by particular salmonids (Lindenstrøm, 2005).

As complement is so effective in killing gyrodactylids, a model based on alternate pathway activation can be suggested that does not require additional effectors to explain immunity. Alternative pathway complement activation occurs in response to carbohydrate moieties on the target surface and such molecules (particularly mannose-rich glycoproteins) occur on the gyrodactylid tegument (Buchmann, 2001). Gyrodactylids are always exposed to complement from host mucus and the accumulation of MACs on the tegument must limit their survival. Up to a point, complement-mediated tegument damage can be overcome by repair and this may partially explain the high density of secretory vesicles (Kritsky and Kruidenier, 1976; Bakke *et al.*, 2006) within the tegument. It may also explain the curious

phenomenon that detached gyrodactylids often display an inverted mortality curve, with higher age-specific mortality shortly after detachment. This is very clear in Harris *et al.* (1998; Figure 1), which shows percentage mortality of controls not exposed to complement and of parasites exposed to 1:1000 serum dilution, highest in the first 30 min after beginning the experiment. It is also apparent in *G. gasterostei* (see Cable *et al.*, 2002b), in which mortality increases in the first 10 h after detachment in parasites removed from sticklebacks, but not in the first 10 h of life of parasites born *in vitro*. This suggests mortality due to damage (from MACs or some other mechanism) present when the parasites are first detached from the fish, which is minimised after 10 h, either through denaturation of complement factors or because tegument turnover has removed them from the parasite surface. As an infection builds on a fish, complement titre and hence MAC damage may increase, either killing parasites directly or forcing them to migrate into the environment (Lester and Adams, 1974a, b). As time goes by this effect becomes more marked, until natality is less than mortality and emigration, and the parasite population begins to decline. On unsuitable hosts, the balance between the ability of the parasite to exploit the fish nutritionally and the rate at which complement complexes form is shifted in favour of complement attack, and the parasites fail to thrive.

Although this mechanism can explain observed patterns of gyrodactylid-host population dynamics, it is far from a complete explanation. In the first place, alternate-pathway activated complement is a non-specific, universal response whereas the immune response to gyrodactylids clearly is very specific, but much reduced in some fish strains which nevertheless do possess complement (Bakke *et al.*, 1999). Most importantly, a model based on complement activation fails to explain the evidence for modulation of the response by hormonal status, the evidence for memory, or the results now being obtained using microarray approaches. The response to gyrodactylids can be modulated by the physiological state of the host, particularly by its hormonal status. Testosterone suppresses the response to gyrodactylids (Buchmann, 1997a) and hormonal interventions which mimic stress have the same effect (Lindenstrøm and Buchmann, 1998; Harris *et al.*, 2000). Using hydrocortisone acetate implants,

Harris *et al.* (2000) found that immunosuppression made previously innately resistant brook trout, Arctic charr and brown trout relatively susceptible to *G. salaris*, suggesting that host specificity involves factors which can be modulated in cortisol-treated hosts. However, this treatment did not entirely suppress the response, although it is unlikely that hydrocortisone release from the implants had ceased. There may therefore be a second, late, mechanism, which only becomes effective after several weeks. Natural elevation of cortisol concentrations has been observed in fish infected with *G. derjavini* (see Lindenstrøm and Buchmann, 1998, 2000) and natural stressors have frequently been noted as triggering gyrodactylid population growth. This is perhaps most obvious in *Macrogyrodactylus polypteri* infections on *Polypterus* (see Khalil, 1970; Harris, unpublished observations). If true, then elevation of cortisol by parasite-induced stress could lead to responses against them becoming less efficient, leading to positive feedback and exponential parasite population growth.

A range of other mechanisms may be involved in immune responses to gyrodactylids. The tegumental carbohydrate moieties may be the target of other host defence molecules, particularly lectins (Buchmann, 2001; Jørndrup and Buchmann, 2005). Lysozyme was identified as a potential defence molecule in the early 1980s (Ingram, 1980), but Buchmann and Uldal (1997) were unable to find convincing evidence for its involvement in the response against *G. derjavini*. Similarly, C-reactive protein, pentraxins and low molecular weight anti-microbial peptides have all been suggested as protective against monogeneans including gyrodactylids, but as yet without evidence (Buchmann, 1999, 2000, 2001).

More recent understanding has come from work using microarray technology to dissect the signalling pathways involved in the early immune response to gyrodactylids. Lindenstrøm *et al.* (2004) showed increased transcription of the pro-inflammatory cytokine tumour necrosis factor (TNF)-α1, but not TNF-α2, 8 days post-infection. No increase was noted in secondary infections. Interleukin (IL)-8 transcription did not increase in either primary or secondary infections, but elevation of transforming growth factor (TGF)-β transcripts in secondary infections was dramatic; this transcript was hardly

detectable in uninfected fish or in primary infections, but was substantially elevated by 8 days post infection in secondary infections. The increase appears to have begun as early as 4 days post-secondary infection. Induced nitric oxide synthase (iNOS) was also strongly elevated in primary and secondary infections, while cyclo-oxygenase-2 (COX-2) showed significantly elevated transcription 8 days post-primary infection. In a similar study, Lindenstrøm *et al.* (2003b) demonstrated consistent elevation of two isoforms of IL-1β in primary infections, confirming earlier immunochemical results (Buchmann and Bresciani, 1998, 1999). Lindenstrøm *et al.* (2003b) suggest that IL-1β may activate mucus secretion, as the homologous molecule in mammals is a mucus secretagogue. If this were so, then activation of this cytokine would probably be central to the response. Lindenstrøm *et al.* (2006) have extended this work, suggesting that resistant salmon moderate their mucous cell proliferative response, rendering the microhabitat less suitable for *G. salaris*. Susceptible fish on the other hand are unable to limit their response to pro-inflammatory cytokines such as IL-1β, leading to hypersecretion of mucus and a more suitable host microenvironment. Matejusová *et al.* (2006) also record elevated expression of the myeloid leukaemia differentiation protein (Mcl-1) homologue and the opioid growth factor receptor protein (OGFr) homologue in susceptible salmon; both of which may be linked to epidermal regeneration processes, and in the case of Mcl-1 at least, may be induced by IL-1 produced early in the infection (Matejusová *et al.*, 2006). Later in the infection, a FIP-2-like gene (14 kDa interacting protein) is also induced in susceptible salmon (Collins *et al.*, 2007). The elevation of iNOS and COX-2 is interesting as they represent effector arms of the non-specific immune response, responsible for the release of NO and prostaglandins, respectively. It is difficult to see how such reagents could affect the external surface of a gyrodactylid monogenean, but such metabolites probably have major effects when ingested; Cable *et al.* (2002b) showed the ingestion of intact host cells and organelles, and the action of such metabolites within the gut is likely to be harmful.

Gyrodactylid infections lead to gross changes in epidermal histology which are clearly related to the immune response. Sterud *et al.*

(1998) showed that in *G. salaris* infections, mucous cell density declined in susceptible salmon of the Lierelva stock, but was not affected in resistant brook trout. Similar results were also noted in the case of *G. colemanensis* infections of salmonids (Wells and Cone, 1990). This could relate to differences in the dynamics of epithelial grazing by gyrodactylids, but the evidence above (Lindenstrøm *et al.*, 2003b, 2006; Collins *et al.*, 2007; Matejusová *et al.*, 2006), of specific changes in the expression of genes involved in mucous cell differentiation during infections suggest that this is a specific response. Appleby *et al.* (1997) observed no changes in mucous cell density in *G. salaris*-infected salmon of the relative tolerant Batnfjordelva stock but the epidermis was thicker and contained more cell layers than in uninfected fish. Following this, Buchmann and Uldal (1997) showed that the susceptibility of salmonids to *G. derjavini* was negatively correlated with mucous cell density (the most resistant hosts had the highest mucous cell density) and that parasites aggregated in sites with a poor supply of mucous cells, such as the cornea (Buchmann and Bresciani, 1998). Up to 30% of parasites may aggregate on corneas, which represent less than 1% of the total fish surface (Olafsdóttir *et al.*, 2003), suggesting a significant advantage to such aggregation. Mucous cell density can be manipulated by dexamethasone treatment in a manner correlated with the increased susceptibility of treated fish to *G. derjavini* (see Olafsdóttir and Buchmann, 2004). Heavily infected fish developed a higher density of mucous cells than sham-infected controls, suggesting that, while the parasites induce mucous cell production, dexamethasone inhibits their discharge so that the cells accumulate in the epidermis. In this case, however, it should be borne in mind that the host used, Atlantic salmon, is normally refractive to infection with *G. derjavini* and so this is a somewhat artificial situation. These findings are interesting in that they demonstrate the impact of gyrodactylids on the epithelial architecture of the fish, and that these changes are in some way involved in the host response. The parallel between the changes in epithelial goblet mucous cell density (e.g. Sterud *et al.*, 1998) and processes in the intestine during the mucus-mediated expulsion of gastro-intestinal (GI) nematodes (Ishikawa, 1994; Ishikawa *et al.*, 1995) has been noted previously (e.g. Sterud *et al.*, 1998). However,

similarities with the expulsion of GI nematodes go further. Cliffe *et al*. (2005) showed that changes in intestinal architecture and epithelial turnover have a major role in the elimination of *Trichuris muris*, an effect mediated via immunologically relevant cytokines. If a similar situation can be demonstrated in *Gyrodactylus*, there is potential for linking the ''physical'' explanations of expulsion (e.g. Lester, 1972) with the evidence for acquired immunity (see above), and the more subtle observations on signalling pathways of Lindenstrøm *et al*. (2004) and Matejusová *et al*. (2006).

8.2. The Heritability of Disease Resistance

Disease resistance in fish, as in most animals (May and Anderson, 1983; Chevassus and Dorson, 1990), is strongly heritable. Pathogens exert selective pressure locally (Lively and Dybdahl, 2000) and parasitism may be an important determinant of local adaptation in host metapopulations. Because disease resistance is physiologically costly, it will be relaxed if a pathogen disappears from a host population, and will not evolve or be sustained in the absence of a pathogen. Significant genetic variation has been found in several immunological parameters in Atlantic salmon (Fevolden *et al*., 1992; Fjalestad *et al*., 1996; see Bakke and Harris, 1998) and in overall resistance to a variety of diseases (see Bakke *et al*., 1999).

Closely related salmonids exhibit remarkable differences in susceptibility to infection with *G. salaris* and *G. derjavini* (see Section 8) ranging from hosts that serve as short-term transport vectors to those that are entirely susceptible to infection without any obvious immune response (Bakke, 1991; Buchmann and Uldal, 1997; Bakke *et al*., 2002). This range of response can even be demonstrated experimentally between individuals of the same strain (Bakke *et al*., 1991b, 1996, 1999, 2002; van Oosterhout *et al*., 2003; Cable and van Oosterhout, 2007). Genetically determined resistance was first demonstrated by Madhavi and Anderson (1985), who selected guppies for resistance to *G. turnbulli* over a 2-year period. By selecting from the most resistant and most susceptible fishes, they were able to breed susceptible and resistant lines and demonstrate heritability of resistance. F1 crosses

between susceptible and resistant fishes had intermediate resistance. Unfortunately, these experiments were not pursued. More recently, the heritability of resistance to *Gyrodactylus* within and between strains of Atlantic salmon subject to different breeding regimes has been re-examined for correlations with genetic markers associated with ectoparasitic resistance (Collins *et al.*, 2003, 2004b, 2005). The exact genetic basis of resistance is being investigated using a quantitiative trait loci (QTL) screening approach (Gilbey, 2004; Gilbey *et al.*, 2006). In F2 backcrosses of Baltic and Scottish salmon, 10 genomic regions were identified associated with heterogeneity in resistance to *G. salaris*. However, mapping these regions with QTLs could account for only 27% of the total heritability of resistance, suggesting that there are additional, as yet unidentified, regions of the genome that are involved in resistance. Sarginson *et al.* (2004) also reported on the differential expression of immune-related genes in a backcross between susceptible River Conon (Scottish) and resistant River Neva (Russian) salmon. This allowed the identification of 11 immune-related genes with significantly higher expression in susceptible fish.

Infections of hybrids can be a useful way to characterise the nature of genetic resistance. Bakke *et al.* (1999) used salmon X brown trout hybrids to test for susceptibility to two host-specific gyrodactylids, the salmon-specific *G. salaris* and the trout-specific *G. derjavini*. The patterns of susceptibility were complex, including susceptible hosts supporting exponential parasite population growth, responders that showed an increase followed by a decline in infection, and resistant fish on which infections failed to grow, but basically hybrids had susceptibility intermediate to that of their parents. While pure-bred *S. salar* included both highly susceptible and responding individuals which failed to eliminate *G. salaris* infection, pure-bred *S. trutta* were entirely resistant to this species. Pure-bred *S. trutta* ranged in susceptibility to *G. derjavini*; some possessed innate resistance while others were initially susceptible but then mounted a host response, usually eliminating the parasite. Pure-bred *S. salar*, on the other hand, were all susceptible to *G. derjavini*, but population growth rates were reduced and a host response frequently eliminated infections. The abundance of both gyrodactylids was lower on hybrids than on

their respective normal hosts, and a parental sire- and dam-influence on hybrid resistance was observed. When the sire was *S. salar*, the susceptibility of hybrids to *G. salaris* was similar to that of pure *S. trutta*; when the dam was *S. salar* innately resistant, intermediately susceptible and responding individuals were present. In the case of *G. derjavini*, when the sire was *S. trutta*, infections on hybrids were similar to those on pure *S. salar*; when the dam was *S. trutta*, an increased susceptibility was observed. This experiment has provided a fascinating insight into the processes of susceptibility and specificity, and should be followed up in other systems with shorter generation times which are easier to maintain in the laboratory.

8.3. Parasite Pathogenicity

Although gyrodactylids are potentially highly pathogenic (Bauer, 1988; Bakke *et al*., 2002; Jalali *et al*., 2005), little is known about resulting disease in fish populations (Cone and Odense, 1984; van Oosterhout *et al.*, in press b). At the individual level, feeding and attachment wounds destroy the osmotic integrity of the epidermis and encourage potential secondary infections (Snieszko and Bullock, 1968; Cone and Odense, 1984; Bakke *et al.*, 2006; see Figures 10, 13 and 23). *Saprolegnia* infections are occasionally associated with *G. salaris*-induced mortality in salmon parr (unpublished observations). However, Busch *et al*. (2003), who emphasised the pathogenicity of *G. derjavini* on rainbow trout found only weak evidence that virulence is enhanced by concomitant infection with the bacterium *Flavobacterium psychrophilum*. The extent to which different *Gyrodactylus* species are pathogenic is variable. Cone and Odense (1984) who studied the attachment site pathology of five species of *Gyrodactylus*, observed that only *G. salmonis* appeared to cause extensive fin damage due to the activity of the marginal hooks deeply buried into the host epidermis. They observed few bacteria in the wounds.

The impact of *G. salaris* is clearly dependent on host size but larger salmon parr are normally killed when infrapopulations attain thousands (Mo, 1992 observed living fish with up to 12 500 *G. salaris*). Up

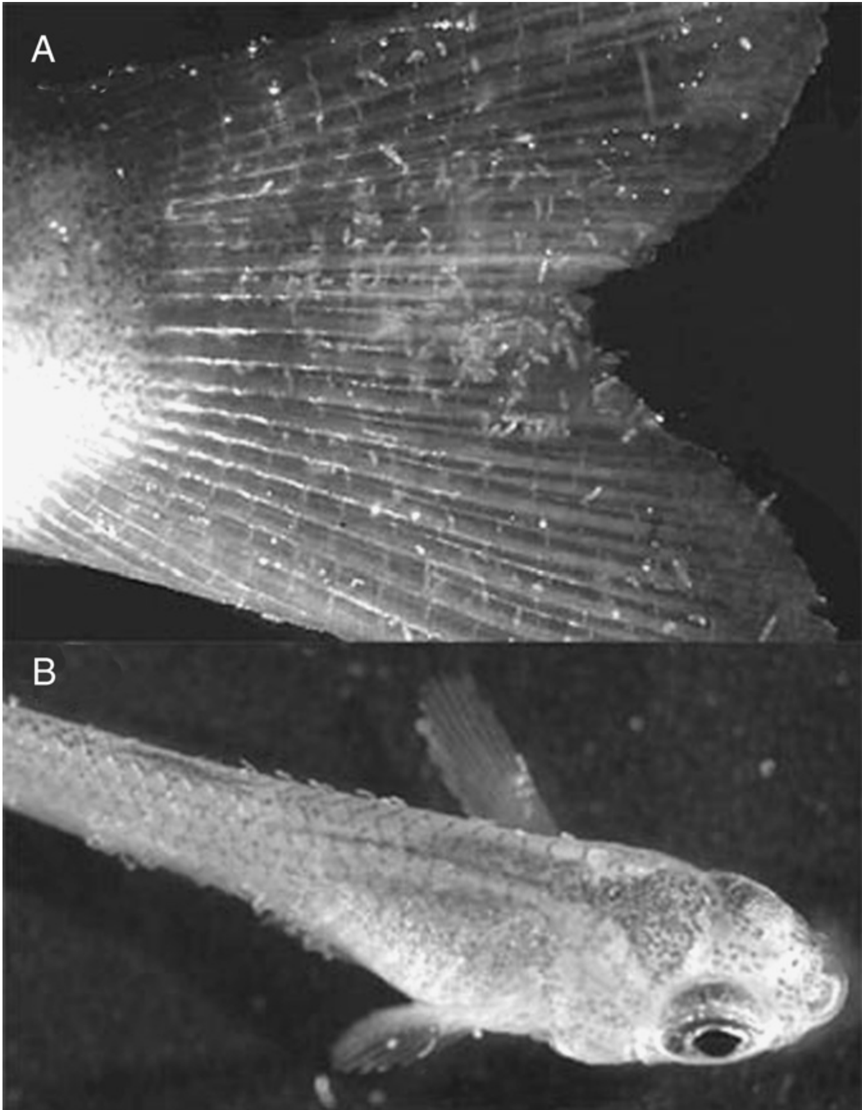


Figure 23 Infected (A) Atlantic salmon tail with *Gyrodactylus salaris* and (B) a guppy with a *G. turnbulli* infection. Small immature fish of both species can harbour several hundred worms.

to ~5000 *G. salaris* per fish did not cause obvious pathology except a thickening of the epidermis in Batnfordselva salmon (Appleby *et al.*, 1997; Bakke, personal observations), suggesting that disease tolerance, in addition to resistance, may occur in this strain. Overall, parasite-induced host death shows huge individual variability, and it is impossible to establish infection thresholds above which death is inevitable. *G. maculosi* may attain population sizes of up to 600 per gill chamber with no evidence of pathology (Cone and Roth, 1993), and *Isancistrum* may number thousands per squid without obvious mortality. By contrast, *G. bullatarudis* or *G. turnbulli* may kill guppies when only tens of parasites are present. Such differences can be apparent even on comparable hosts. *G. colemanensis* on *Salmo gairdneri* caused no pathogenicity, whereas *G. salmonis* on *Salvelinus fontinalis* was highly pathogenic (Cusack and Cone, 1986), possibly because of differences in attachment and feeding strategy (Cone and Odense, 1988; Section 2.3).

Models of gyrodactylid pathogenicity require consideration of parasite population growth rate, as host death becomes almost inevitable when the parasite population grows without check. The virulence of a gyrodactylid species or strain depends in the first place on population growth rate. For example, it is noted above (Section 7) that *G. thymalli* naturally has a lower population growth rate on grayling, than *G. salaris* does on salmon (Bakke *et al.*, 2002; Sterud *et al.*, 2002). Population growth rates for gyrodactylids infecting salmonids decline with age of infection and no correlation between growth rate and the intensity of infection was found (see Figure 9A and B in Bakke *et al.*, 2002). Only hosts supporting the highest initial parasite population growth rates are likely to suffer significant mortality and pathogenicity (e.g. Bakke *et al.*, 2002). There is a negative correlation between time to response in responding host strains and the proportion of innate resistant individuals of salmon (see Figure 13 in Bakke *et al.*, 2002), which also suggests a link between parasite population growth rate and possible pathogenicity. The model of pathogenicity/immunity put forward by Bakke *et al.* (2002; Section 8.1 above) suggested that gyrodactylid infections pose a stress on the host which weakens the immune response. If the response is weakened sufficiently by rapid initial population growth, the host is more

likely to succumb. Growth rate in turn is partly intrinsic to the parasite, but is also dependent on the closeness of the adaptation of the parasite strain to the host and on individual immune status. Local adaptation of parasites and hosts is therefore likely to be important in gyrodactylid infections (Lively and Dybdahl, 2000), tempered by macroenvironmental influences on host immune status.

8.4. Host Specificity and Immunity: Two Sides of the Same Coin?

A paradigm in gyrodactylid research is that host specificity and immunity are closely linked. This has grown from the important studies on salmonid immunity to *G. salaris* and *G. derjavini* and is perhaps inevitable; the simplest and most informative models are unnatural interactions (e.g. *G. salaris* on brook trout, Arctic charr or rainbow trout, *G. derjavini* on rainbow trout) where the boundary between immunity and host specificity becomes blurred. Experiments with immunosuppressants (Buchmann, 1997a, b; Lindenstrøm and Buchmann, 1998; Harris *et al.*, 2000), which rendered previously resistant host strains and species susceptible, also strongly link immunity with host specificity. Observations on immunity and specificity using these systems should be interpreted with caution. The natural host of *G. derjavini* is probably the brown trout, *Salmo trutta*, which has seldom been tested except by Bakke *et al.* (1999, 2002). The natural host of *G. salaris* is probably the Baltic race of Atlantic salmon but again only limited studies on this race has been undertaken (Bakke *et al.*, 1990, 2002, 2004a, b; Dalgaard *et al.*, 2003).

After successful transmission which relies on the parasite and host meeting in space and time, the physiological host specificity depends on the balance between the success of a gyrodactylid in exploiting the particular biochemical composition of the fish epidermis and the success of the fish at eliminating the parasite. Elimination may be via the host response, suggesting that host specificity is related to immunity, but the basic reason for failure is because parasites have been unable to feed properly or breed at a rate outstripping host-mediated loss. Quantifying specificity in gyrodactylids is difficult. Scott and

Anderson (1984) drew attention to the fact that gyrodactylids lie between traditional microparasites, for which a prevalence framework model is normally adequate, and macroparasite models. For macroparasites, which do not reproduce without transmission, specificity can be evaluated as establishment success (no. of adult parasites from a given inoculum) or reproductive output (no. of eggs/parasite). Other measures, such as worm size, or number of uterine eggs, give indirect estimates of host suitability. Gyrodactylid specificity can be estimated as the number of parasites recorded after a certain period of growth from a specific inoculum size, but this omits many subtleties of the infection process. Microparasite reproductive rate is normally estimated as Fisher's R_0, the number of secondary infections arising from a single primary infection (see e.g. Anderson, 1982). This has never been calculated for gyrodactylids, and the impacts of host behaviour or environment on this parameter have never been considered. Instead, gyrodactylid host specificity has usually been considered using the following parameters:

a) The percentage establishment of initial experimental infection;
b) Rate of initial parasite population increase on a host, calculated as:

$$r = \frac{\ln\ N_t - \ln\ N_0}{t}$$

where r is the rate of parasite population growth, N_t the population on day t, N_0 the population size on day 0, and t the time in days between census points (t_0–t_1) (e.g. Jansen and Bakke, 1991; Cable *et al.*, 2000);
c) The number of days before population growth becomes negative. Population counts give the impression of a sudden population crash, usually interpreted as activation of the host reaction (Section 8.1) leading to elimination of the parasites. Recalculation of published data for *G. salaris* indicates that the sudden overturn of the population (the "crash") follows a gradual slowing of population growth rate which begins early in the infection (Bakke *et al.*, 2002). Regression of population growth rate against infection age therefore allows estimation of the time needed for population growth to become negative.

d) The maximum time for which a host remains infected. The number of secondary infections generated is presumably proportional in some way to this period (see Soleng *et al.*, 1999a).

e) The total reproductive output of an infection. Knowing (b), (c) and (d) above, it is possible to calculate the total number of worms produced from a single worm infecting a single fish. Although there have been no attempts to accurately calculate R_0 for gyrodactylid infections on single hosts, it is felt that this is the measure which comes closest to R_0 when estimating the possible contribution of a single parasite infrapopulation to the overall epidemiology of gyrodactylid infections.

9. ENVIRONMENTAL INTERACTIONS

In the microenvironment, host susceptibility and resistance are the main factors which control gyrodactylid occurrence (Bakke *et al.*, 1991a, b), although host species and ecology, including seasonal population dynamics are also important (Bakke *et al.*, 2002; Malmberg and Malmberg, 1993; Soleng *et al.*, 1998, 1999a; Boeger *et al.*, 2005). Experimental studies with regular introduction of naïve hosts into closed, otherwise uniform aquarium systems by Scott and Anderson (1984) and Bakke *et al.* (1991b) have revealed oscillatory parasite population dynamics with cycles of host susceptibility and refractoriness. Macroenvironmental influences may modify these cycles. The most important macroenvironmental factor is probably temperature, followed by water chemistry (e.g. Soleng and Bakke, 1997; Soleng *et al.*, 1999b; Poléo *et al.*, 2004a, b) and water quality generally (see Bauer, 1968). However, biotic macroenvironmental factors, such as micropredation, may also influence gyrodactylid population dynamics.

9.1. Micropredation

Gyrodactylids are large enough to be visible to fish and other visual predators and, like other ectoparasites are vulnerable to micropredation, either by conspecific hosts or other interacting organisms.

This possibility was first suggested by Tyler (1963) who observed that *Gyrodactylus*-infected fish posed for and were cleaned by sticklebacks, and later by Lester and Adams (1974a). The transverse banding of the gut of *Macrogyrodactylus* species has been suggested as an antipredatory device, disrupting their outline for potential visual predators (Khalil, 1970). Harris (1982) found sticklebacks naturally support small numbers of the oligochaete worm *Chaetogaster*, known to feed on digenean cercariae (Khalil, 1961). However, he failed to find gyrodactylid remains within the gut of these worms, which fed instead on diatoms from the fish surface. However, on one occasion, gyrodactylid hooks were found in the faeces of a laboratory-maintained stickleback, suggesting that micropredation can occur. Several workers (Roubal and Quartararo, 1992; Whittington, 1996, 1998; Grutter, 2002; Grutter *et al*., 2002) have drawn attention to the potential importance of cleaner symbionts in removing monogeneans, and have described the cryptic adaptations of monogeneans to avoid this. The susceptibility of gyrodactylids to cleaner symbionts has now been demonstrated specifically between shrimps and fish. In laboratory experiments, the shrimps *Palaemon adspersus* and *P. elegans* removed more than half of a *Gyrodactylus* infrapopulation from plaice within 48 h (Ôstlund-Nilsson *et al*., 2005).

9.2. Temperature

Gyrodactylids have short life spans, and low fecundity (1–5 offsprings per worm), but still have the highest reproductive rates in the Monogenea (Bychowsky, 1961), approaching those of larger micro-organisms, with doubling times in tropical species such as *G. bullatarudis* or *G. turnbulli* of less than 24 h (Turnbull, 1956; Scott, 1982). Gyrodactylids are sensitive to environmental temperature because growth rates depend not upon fecundity but upon rate of embryo development. Reproductive rate at different temperatures has been examined in *G. gasterostei* (see Harris, 1982) and in *G. salaris* (see Jansen and Bakke, 1991). In the latter, generation time and time between successive births were negatively correlated with temperature; reproductive rate increased with increasing temperature. In *G. turnbulli* (see Scott and Nokes, 1984),

reproductive output also increased with temperature up to 27.5°C. This study is however difficult to compare with Scott (1982), because raw data on the timings of births was not presented, and reproductive rate is compounded of embryo developmental and survivorship. At 30°C, reproductive rate fell, because insufficient worms survived to give birth more than once. Scott and Nokes (1984) note that the relative timing of the births changes with temperature as well, so that the interval between subsequent births is temperature sensitive. Harris (1998) employed degree days to analyse reproduction in *G. gasterostei*, finding that the number of degree days needed for development declined slightly with increasing temperature. This may explain the relationship between the size of the hard parts of gyrodactylids and environmental temperature: as temperature increases the number of degree days needed for full development declines and, therefore, hooks do not attain the size they would reach at lower temperatures.

Environmental temperature also impacts upon gyrodactylid survival, low temperature extending life span both on (e.g. Lester and Adams, 1974a, b; Scott and Nokes, 1984; Jansen and Bakke, 1991; Andersen and Buchmann, 1998) and off the host (Olstad *et al.*, 2006). Cable *et al.* (2002b) showed that survival of detached *G. gasterostei* is temperature dependent with a maximum of 101 h at 4°C but only 67 h at 15°C. *G. salaris* (see Jansen and Bakke, 1991; Olstad *et al.*, 2005, 2006) had a maximum survival of 60 h at 3°C, declining to 27 h at 18°C. Temperature may also influence transmission, which in *G. salaris* on salmon is positively correlated with temperature (range 1.2–12.2°C) during single host-to-host contacts (Soleng *et al.*, 1999a). In *G. salaris*, although survival declined as temperature increased, this was more than compensated by the increase in reproductive rate (Jansen and Bakke, 1991). These changes in reproductive rate and survival have an impact on gyrodactylid population structure, with sub-optimal survival at high temperature characterised by a high proportion of asexual first births in the population. At lower temperatures, when survival is optimal, worms may survive long enough to reproduce sexually. This was noticed by Harris (1993), who showed that the proportion of new-born individuals in *G. gasterostei* populations could transiently rise to more than 60% of the total population, whereas the stable age structure would

predict ~40%. These transient events, leading to clonal reproduction in the parasite population, are probably triggered by short-term changes in weather.

Temperature may also influence the host immune response. Immunity is normally enhanced at high temperatures, as reaction rates of complex proteins such as the complement cascade increase. Gyrodactylid populations may therefore cycle faster on hosts at higher temperatures, and not reach such large sizes. However, it has never been critically established whether the failure of gyrodactylid populations to grow large at high temperatures is due to the host response, or simply to the reduction in survival attendant on high temperature. Gelnar (1991) noted that water temperature markedly affected the growth rate of laboratory populations of *G. gobiensis* on gudgeon. At 12°C, the intensity of *G. gobiensis* gradually increased, reaching a maximum significantly later and at a larger size than at 18°C; populations subsequently declined at both temperatures. A similar phenomenon was noted when temperature was gradually increased or decreased. A field study of *G. rhodei* on bitterling (*Rhodeus sericeus*) demonstrated that both prevalences and intensity of infection increased in autumn and winter months when water temperature decreased (Dávidová *et al*., 2005). By contrast, *G. katharineri* and *G. rutilensis,* parasitising carp fry and dace respectively, may be more thermophilic, as populations grew faster at higher water temperatures (Gelnar, 1987a, 1990). Anthony (1969) suggested that temperature may have different effects at different sites as skin parasites were more sensitive to temperature changes than gill parasites. However, a more likely explanation is that parasites in different sites were different species (see also Section 3.2).

Seasonal variations in gyrodactylid abundance are frequently attributed to environmental temperature (see review by Chubb, 1977). Such studies have generally formed part of Ph.D. theses and therefore focus on American and European north temperate species, mainly infecting small, experimentally convenient hosts such as sticklebacks. There are no tropical studies, and no studies outside America or northern Eurasia. Apart from the normal concerns over taxonomy, the biggest drawback to such studies is their restriction to a study period of 3 years or less. Gyrodactylid population dynamics are unstable (Scott and Anderson, 1984) and oscillate even when external

conditions are held constant (Scott and Anderson, 1984; Bakke *et al.*, 1991b). Longer studies are therefore needed to gain definitive insights into seasonal processes. There is also, in some studies, a danger that samples are taken too far apart. Because of their high reproductive rate and rapid temperature response, samples taken once per week or once per fortnight are necessary for accurate monitoring, and less intensive sampling reduces confidence in the results. For many north temperate freshwater gyrodactylids, between latitudes 30°N and 70°N, populations remain small through the winter, growing rapidly to form epidemics in spring, before almost disappearing in summer. There may then be a second peak of abundance as water temperature declines in autumn. This is seen in *G. katharineri* on carp in Slovakia (Hanzelová and Zitnan, 1982), for *G. aphyae*, *G. macronychus* and *G. magnificus* infecting minnows in the Kola Peninsula (Shulman, 1977), for *G. arcuatus* on sticklebacks in Poland (Morozinska-Gogol, 2002) and in America for "*G. elegans*" infecting *Notemigonus crysoleucas* in Illinois (Parker, 1965) and for species on *Fundulus* [principally *G.* (*Fundulotrema*) *prolongis*] in Connecticut (Barkman and James, 1979). The same pattern has also been seen in one study from a warmer environment; Rawson and Rogers (1973) noted a spring and autumn peak in abundance for *G. macrochiri* from both *Lepomis macrochirus* and *Micropterus salmioides* in Alabama, where summer water temperature can exceed 30°C. By contrast, Aydogdu (2006), working in Turkey, found *G. carassi* abundance directly correlated with temperature, with no evidence of high temperatures inhibiting parasite population growth. In some cases, for example, *G. stephanus* infecting *Fundulus* within the cold environment of Newfoundland, populations decline throughout the cold winter to a springtime low, and then increase slowly through summer to peak in late summer/early autumn (Dickinson and Threlfall, 1975). This might be an example of low temperature inhibiting gyrodactylid reproduction so much that populations fail to trigger a host immune response before temperatures start to fall again at the end of summer.

An interesting series of seasonal studies relate to the gyrodactylids of intertidal marine fishes. Srivastrava and James (1967) found that "*G. medius*" (not *G. medius* but an undescribed species; see Harris *et al.*, 2004) on rockling (*Onos mustela*) was most abundant in early spring,

declining sharply in July through the autumn period. A negative correlation between the occurrence of *Gyrodactylus pterygialis* and temperature was also observed by Hodneland and Nilsen (1994) in a Norwegian fiord. This decline occurred during the warmest part of the year, again suggesting that this gyrodactylid is either adversely affected directly by summer temperatures, or because the host immune system is more efficient at high temperature (Jansen and Bakke, 1993a). Appleby (1996a), working with gyrodactylids of *Pomatoschistus* in Norway, observed a similar pattern. In a similar study of gyrodactylids of cod, Appleby (1996b) noted some evidence of a bimodal distribution, but it was very clear that gyrodactylid abundance was greatest in the summer period. Similarly, Kamiso and Olson (1986) found that prevalence of *G. stellatus* infecting English sole was unimodal, reaching a maximum in June and declining to a minimum in October.

A final aspect of temperature and seasonality which can affect gyrodactylid epidemiology is the effect on the seasonal biology of the fish. Sticklebacks in southern England, have an annual life cycle, spawning in the summer following hatching. This places a major constraint on gyrodactylid biology, as parasites must transmit between generations during a few weeks in midsummer, giving an apparent midsummer drop in abundance (Chappell, 1969; Harris, 1982). Similarly, salmon in cold oligotrophic streams in North Norway may require 5 years before smoltification, leading to stratification of the parr population in the river and simple transmission between age groups. In southern England and France, on the other hand, smoltification occurs in the summer following hatching. If the river lacks a permanent parr population, it might be assumed that *G. salaris* would be unable to survive permanently. Similarly, in winter at low temperatures salmon parr remain deeper within the interstitial environment at the bottom of rivers, presenting different conditions for the parasite population and offering new opportunities for transmission compared to when these fish are feeding actively in the water column. In these ways, the basic reproductive rate of gyrodactylid infections may be modified in ways which cannot be predicted by a simple understanding of temperature relationships. Complications of this sort influence many of the seasonal studies which have been undertaken, but were not considered or explained by the authors. For example, Srivastrava

and James (1967) found an apparent midsummer decline in the abundance of gyrodactylids on rockling which correlated exactly with the migration of the new cohort of rockling onto the shore. If these fish were uninfected, this would depress gyrodactylid abundance significantly. Similarly, MacKenzie's (1970) study of *G. unicopula* on plaice is sometimes cited as an example of seasonal patterns in gyrodactylid abundance. However, MacKenzie (1970) followed a single cohort of hosts through their first 2 years of life; the steady increase in abundance through the first winter was probably due to epidemic spread of the parasite through a naïve host population, while the contradictory decline in the second winter may have been due to elimination from the now immunocompetent hosts. The most extreme example of the role of host biology perhaps is that of *G. katharineri* infecting *Varicorhinus steindachneri* in warm springs with a constant temperature (18–20°C). These parasites exhibited a seasonal cycle, despite the constancy of water temperature (Daniyarov, 1975). The confounding effects of temperature and seasonality on host behaviour and ecology may be very important in understanding gyrodactylid seasonal dynamics.

The seasonal dynamics of *G. salaris* on different salmon cohorts has been studied in detail in five Norwegian river systems: the Vefsna (Johnsen and Jensen, 1988) and Lakselva (Johnsen and Jensen, 1992; Johnsen *et al*., 2004) in North Norway, Lierelva in southeastern Norway, both naturally (Jansen and Bakke, 1993a) and in field experiments (Jansen and Bakke, 1993b), and Lærdalselva (Johnsen and Jensen, 1997) and Batnfjordelva in western Norway (Mo, 1992; Appleby and Mo, 1997). In addition, yearly surveillance data are collected (see Johnsen *et al*., 1999). These studies demonstrate a marked seasonality in occurrence, with a spring increase in parasite abundance, peaking in late summer or autumn, followed by a decline throughout winter and early spring when temperatures are close to 0°C. Both geographical and year-to-year differences occur, and the epidemiological mechanisms and external factors controlling the abundance of *G. salaris* remain largely unknown. In Batnfjordselva (Mo, 1992), peak abundance occurred in the late summer (July, August, September) followed by a decline through mid winter and early spring. In one year, there was a small peak in March and April,

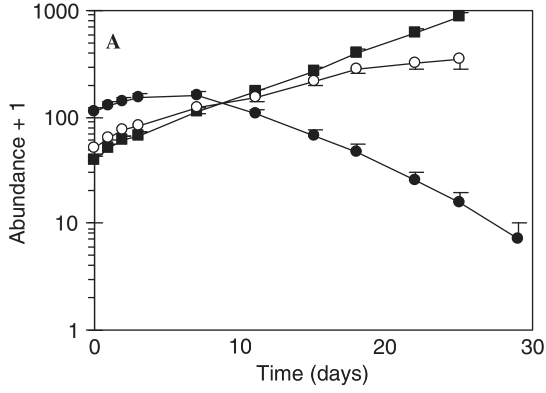
suggesting a bimodal pattern. Mo (1997) saw a similar unimodal pattern in *G. derjavini* on brown trout, peaking in July and August, and declining to a minimum in mid-winter. Transfer of *G. derjavini* onto salmon was most obvious during the midsummer peaks. Jansen and Bakke (1993a, b) on the other hand, working in the slightly warmer southeastern Lierelva river system, saw a pattern closer to the classic bimodal spring and autumn peak. The increase in spring was almost perfectly correlated with water temperature, but the decline in late summer was not related to temperature and may have been due to either immunity or parasite-induced host mortality. *G. salaris* is probably cold-adapted (Malmberg, 1973, 1988), but the discovery of large-scale interspecific genetic variation in this species (Hansen *et al.*, 2003; Meinilä *et al.*, 2004) raises questions about the potential of biological differences between *G. salaris* clades and haplotypes in relation to their temperature optima.

9.3. Salinity and Water Chemistry

Gyrodactylus species occur in freshwater, brackish and marine environments, but relatively few species are euryhaline. *G. arcuatus* is euryhaline (Malmberg, 1970), occurring in natural environments ranging from full seawater down to fairly acid soft water in montane lakes and streams. Molecular evidence suggests that all of these forms of *G. arcuatus* belong to a single panmictic species (Ziętara and Lumme, 2002, 2003, 2004; Cable and Harris, unpublished), and laboratory populations of this parasite derived from freshwater can tolerate seawater with minimal adaptation (Harris, unpublished). Apart from this, only *G. salaris* has been extensively tested for salinity tolerance (Soleng and Bakke, 1997; Soleng *et al.*, 1998). In this species, population growth increased at 5.0‰ salinity at 12°C, but at 7.5‰ salinity the population declined to extinction after 56 days, and at 20‰ salinity, survival was restricted to 12 h. Salinity tolerance was found to be significantly temperature dependent (Soleng *et al.*, 1998). These results suggest that *G. salaris* is a freshwater species, albeit able to survive high salinities for short periods. The salinity tolerance of *G. derjavini* was tested by Buchmann (1997b) who stressed that

G. derjavini seems less euryhaline than *G. salaris*. Parasites in 5‰ NaCl at 11°C survived only 4 days, in contrast to Soleng and Bakke (1997) who found that *G. salaris* survived more than a month (56 days) in 7.5‰ salinity at a comparable temperature. However, as Buchmann (1997b) used dilutions of sodium chloride, rather than of natural seawater, his results require confirmation.

Until recently, other parameters of water chemistry had not been studied. Anecdotally, many freshwater species are difficult to culture in soft water, and Malmberg (1970) alluded to the lack of gyrodactylids in water bodies stained with humic acids. This suggests that gyrodactylid diversity may be reduced in soft water. Following these observations, Soleng *et al.* (1999b) conducted the first experiments testing gyrodactylid survival in soft water and in dilute aluminium solutions (Figure 24). This metal is a frequent contaminant of acidified watercourses in northern Europe as it is leached out of soil and ground rocks. A combination of acidified water and aqueous aluminium (Al) had the most pronounced effects, eliminating *G. salaris* after 4 days at 202 μg Al/l without killing the salmon. The effect was dependent on Al concentration but relatively independent of pH, except at the lowest pH when the effect of Al was enhanced. Acidified water also impaired parasite survival in the absence of metal ions, and at pH 5.0, *G. salaris* was eliminated within 9 days (Figure 24). Similar results have been obtained with Al ions on the survival of *G. derjavini* from trout (Pettersen *et al.*, 2006). These results were confirmed by Poléo *et al.* (2004a) who went on to test the effect of other metal ions on survival. These are rarely found naturally, but are frequently a consequence of human pollution. They may however reach high concentrations in watercourses flowing through metalliferous substrates, such as the serpentine soils of South West England. Poléo *et al.* (2004a) found zinc (Zn) to be effective in controlling *G. salaris* without any apparent influence on salmon. Other heavy metals, including iron (Fe), copper (Cu) and manganese (Mn), had no effect, except at the highest Cu concentrations (Poléo *et al.*, 2004a). The mechanism behind the effect of aluminium on *G. salaris* is unknown, but could be direct (hypoxy-hypothesis) or indirect through the fish skin (repellent hypothesis) on the parasite (Soleng *et al.*, 1997). Gheorghiu *et al.* (2007), studying the effects of waterborne zinc on *G. turnbulli in vitro*



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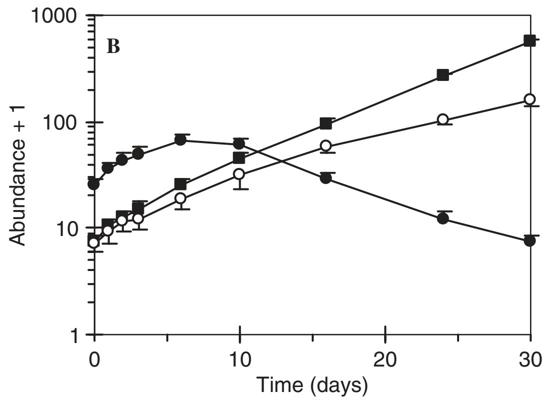
Time (days)



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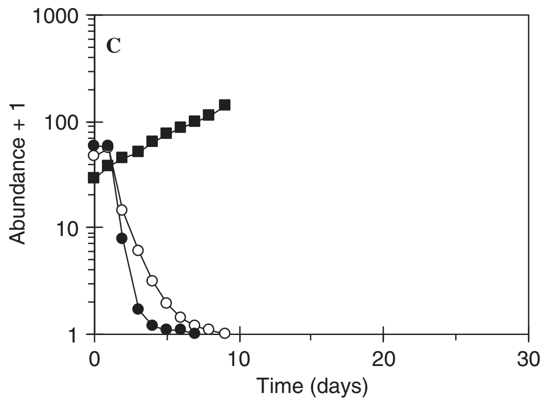

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and on isolated guppies, found that concentrations of 120 μg Zn/l are directly toxic to the parasite, and they hypothesised that the host response against *G. turnbulli* may be impaired by high Zn concentrations. The different ecology of the gyrodactylids needs to be considered in evaluating these studies. *G. salaris* infects a host which predominantly inhabits soft-water areas with high water flow, low temperature and minimal human-mediated pollution. *G. turnbulli* is a tropical species inhabiting montane streams, which may also be oligotrophic. The parasite does extend into lowland areas where pollution may be significant (Harris and Lyles, 1992; Cable *et al.*, unpublished). These studies emphasise the importance of a basic knowledge of environmental factors in order to understand population dynamics, range extension and dispersal of gyrodactylids, and have formed the basis of new control strategies. Al is now being used for large-scale control of *G. salaris* in the field (see Section 10.3 below).

9.4. Pollution and Water Quality

Pollutants may directly impact upon the population dynamics, distribution and dispersal of fish ectoparasites (e.g. Møller, 1985, 1987; Khan and Thulin, 1991; Koskivaara, 1992; Poulin, 1992; Overstreet, 1993). Lately, focus is shifting towards the study of chronic exposure to sublethal concentrations not previously considered to have an important effect on fish (Poulin, 1992; Dusek *et al.*, 1998). Because ectoparasites such as gyrodactylids are easily observed, they can be important indicators of host health, reflecting water quality. Monitoring gyrodactylid epidemiology in chronic toxicity tests and in

Figure 24 Course of infection of *Gyrodactylus salaris* on Atlantic salmon (*Salmo salar*) parr ($n = 15$ in each exposure tank at day 0) at 12°C. (A) (■) Untreated control water at pH 6.4; (○) acidified Al-poor water at pH 5.6; (●) acidified Al-enriched water at pH 5.6, 106 μg Al/l. (B) (■) Untreated control water at pH 6.4; (○) acidified Al-poor water at pH 5.2; (●) acidified Al-enriched water at pH 5.2, 93 μg Al/l. (C) (■) Untreated control water at pH 6.4; (○) acidified Al-poor water at pH 5.0; (●) acidified Al-enriched water at pH 5.0, 92 μg Al/l. Bars = standard error of the arithmetic mean. Note $\log_{10}$ scale. [Reproduced from Soleng *et al.* (1999b) with permission of Cambridge University Press.]

polluted areas is an area of potential interest which has so far been largely neglected (see Poulin, 1992a). The impact of environmental factors on gyrodactylid communities has been stressed by Malmberg (1957, 1964, 1970) who indicated that many factors including water chemistry, salinity, and water temperature could influence the occurrence and spread of gyrodactylids. Few studies have considered these interactions, but Koskivaara *et al.* (1991) demonstrated a correlation between gyrodactylid diversity on roach and water quality (see Koskivaara, 1992). The effect of liming strategies to reduce acidification in southern Norway and the West coast of Sweden (Alenäs, 1995), may have inadvertently influence both the potential for gyrodactylid colonisation or level of infection, directly or via interactions with aluminium and pH (Soleng *et al.*, 1999b; Poléo *et al.*, 2004a, b; Soleng *et al.*, 2005).

Part 4. Applied biology of the *Gyrodactylus salaris* epidemic

10. THE *GYRODACTYLUS SALARIS* EPIDEMIC IN NORWAY: IMPLICATIONS FOR OTHER COUNTRIES

10.1. History

The first gyrodactylid described from a Scandinavian salmonid was collected from the Hölle laboratory (now Hölleforsens Laxodling, Indalsälv, Sweden) in 1952, and later described (Malmberg, 1957) as *G. salaris* (syntype later redescribed by Ergens, 1983) (Malmberg *et al.*, 1994). In Norway, *G. salaris* was first observed in 1975, following heavy salmon mortality at the Akvaforsk fish hatchery in Sunndalsøra, Møre and Romsdal County (Bergsjö and Vassvik, 1977; Tanum, 1983; Malmberg, 1989). In this year, the parasite was recorded for the first time from wild salmon parr in the Lakselva and Ranaelva (Nordland County, North-Norway). A significant reduction of salmon parr (but not brown trout) density was noted over the next 3 years in Lakselva (Johnsen, 1978). The epidemic was originally thought to be related to environmental pollution, as agricultural and domestic pollutants entered the river and the river bed was overgrown

with algae (Johnsen, 1978), and it was assumed that *G. salaris* occurred naturally in Norway (Johnsen and Jensen, 2003). However, over the next 20 years evidence accumulated that *G. salaris* had been introduced, probably from Sweden (Heggberget and Johnsen, 1982; Tanum, 1983; Johnsen and Jensen, 1986, 1991; Mo, 1994) and that Norwegian salmon were especially susceptible to this invader.

The discovery of four new infected rivers including some in the mid- and west of Norway (cf. Heggberget and Johnsen, 1982), led to the establishment of a "*Gyrodactylus* committee" to assess the problem, and a surveillance program to track the spread of the parasite. These studies recognised *G. salaris* as a serious threat to Atlantic salmon in Norway, with dramatic declines in salmon parr density in infected rivers, followed after a few years by declines in catches of returning adults by sports fishermen (Johnsen and Jensen, 1991; Mo, 1994; Johnsen *et al*., 1999; Mo *et al*., 2004). The committee concluded in 1982 that *G. salaris* was non-indigenous, probably newly established, spread via hatcheries, restocking and subsequently fish migration in estuaries. Few infected parr survived to the smolt stage, implying that production of adult salmon in infected rivers was strongly threatened. Gyrodactylosis on salmon was declared a notifiable disease (Group B) in Norway in 1983, and recognised a significant fish disease by the Office International des Epizooties (OIE) (Anonymous, 2002a).

Johnsen and Jensen (1986) grouped the 26 rivers then known to be infected into 14 regions. They demonstrated a close correlation between the distribution of *G. salaris* and the stocking of fish from infected hatcheries. Between 1982 and 2003, 23 new river systems were identified as infected, at a rate of 0–3 new rivers per year (Jansen *et al*., 2005). Updated records of the parasite (Autumn 2006) and the actions taken in the rivers infected in Norway are summarised in Figure 25 (which is Plate 3.25 in the separate Colour Plate Section) and Table 2.

Brackish water dispersal has been most important in spreading the parasite (24 river systems), followed by restocking from known infected hatcheries (nine rivers). Dispersal from infected hatcheries into nearby watercourses with escaped salmonids probably accounted for seven river systems, while the origin of infection in four other river

Table 2 *Gyrodactylus salaris* infected rivers in Norway, the years of first observation and the years of extermination of the parasite by use of rotenone or aluminium* and the infection status per August 2006

Infected salmon rivers	Year of 1st observation	Year of chemical treatment	Status
Lakselva	1975	1990	Exterminated 1995
Ranaelva	1975	2003/2004	Uncertain status
Vefsna	1978		Infected
Skibotnelva	1979	1988, 1995	Infected
Røssåga	1980	2003/2004	Uncertain status
Bjerka	1980	2003/2004	Uncertain status
Drevja	1980		Infected
Fusta	1980		Infected
Steinkjervassdraget	1980	1993, 2001/2002, 2005, 2006/2007*	Uncertain status
Figga	1980	1993, 2001/2002, 2005, 2006/2007*	Uncertain status
Batnfjordelva	1980	1994, 2004*	Infected
Driva	1980		Infected
Usma	1980		Infected
Rauma/Istra	1980	1993	Infected
Henselva	1980	1993	Infected
Valldalselva	1980	1990	Exterminated 1994
Beiarelva	1981	1994	Exterminated 2001
Litledalselva	1981		Infected
Tafjordelva	1981	1987	Exterminated 1990
Norddalselva	1981	1990	Exterminated 1994
Eidsdalselva	1981	1990	Exterminated 1994
Vikja	1981	1981/1982	Exterminated 1986
Skorga	1982	1993	Infected
Aureelva	1984	1988	Exterminated 1992
Vikelva	1984	1988	Exterminated 1992
Måna	1985	1993	Exterminated 1989
Korsbrekkelva	1985	1986	Exterminated 1990
Bævra	1986	1989	Exterminated 1994
Drammenselva	1987		Infected
Lierelva	1987		Infected
Vulluelva	1988	1988	Exterminated 1997
Langsteinelva	1988	1988	Exterminated 1997
Sannaelva	1989	2004	Uncertain status
Bardalselva	1989	2004	Uncertain status
Storelva	1989	1991	Exterminated 1994
Innfjordelva	1991	1993	Infected
Hundåla	1992		Infected
Slettenelva	1993	2004	Uncertain status
Leirelva	1996	1996, 2004, 2006	Uncertain status
Lærdalselva	1996	1997, 2005/2006*	Uncertain status
Signaldalselva	2000		Infected
Lundselva	2001	2002, 2005	Uncertain status
Halsanelva	2002	2003	Infected
Hestdalselva	2002	2003	Infected
Sandeelva	2003		Infected
Ranelva	2006	2006	Uncertain status

systems is unknown. One river system was inadvertently infected via a fish transport (Jansen *et al*., 2005). Norwegian salmon authorities were quick to introduce active measures to combat the epidemic, favouring the ATPase inhibitor, rotenone to kill the fish, and hence the parasite, in infected rivers (Mehli and Dolmen, 1986). Subsequent discovery of the parasite in additional river systems led to an action plan for *G. salaris* eradication (Dolmen and Mehli, 1988), a plan later revised for 1995–1999. In addition, a comprehensive report by the Wild Salmon Committee, established in 1997 (Anonymous, 1999) highlighted reasons for the decline in the Norwegian wild salmon populations and suggested control measures. The Committee's conclusions gained wide political support from The Ministry of the Environment (St.meld. nr. 8, 1999–2000, "Regjeringens miljøvern-politikk og rikets miljøtilstand") which with the Committee plan (Anonymous, 1999) and previous drafts for action plans, formed the basis for the present action plan (Anonymous, 2000). In 2002, following direct commission from the Ministry of Environment, a plan for drastic action was developed by the Directorate for Nature Management (DN) and The Norwegian Animal Health Authority (SDT) which led to the official initiative for rotenone treatment of infected river courses (Anonymous, 2002b). This plan, based on the cost-benefit analysis of Krokan and Mørkved (1994), presented detailed information on disease prevention (e.g. disinfection of equipment, physical barriers for preventing fish migration and rotenone treatment) in each of the eight currently infected regions, with a priority list and a "cost-use" analysis. The priorities for action and research/developmental activities were assessed based on the threat of further disease spread within or outside of the infected region, the biological and economical value of the salmon stock in question, the infection status of other salmon rivers in the region, the possibility of eliminating the parasite from all rivers in the region, the local support of stakeholders for treatment, the probability of re-infection and the need for long-lived obstructions within rivers. Because several rivers have been re-infected shortly after treatment (Figure 25; Table 2) the methods for extermination of *G. salaris* have been revised and improved (Haukebø *et al*., 2000). Risk assessments for inter-river transmission or introduction into Norway (Anonymous, 1996;

Paisley *et al.*, 1999; Brun and Høgåsen, 2003; Høgåsen and Brun, 2003; Jansen *et al.*, 2005) and the North Calotte Region (Brørs, 2002) have also been undertaken. Recently (2001), a Gyromet project was established to develop a methodology using acidified aluminium to exterminate the parasite (see Section 9.3).

In the following account, we assess the current status of *G. salaris* within Europe, and then present an updated description of the distribution of *G. salaris* and the management initiatives to combat the parasite within Norway.

10.2. The Status of *G. salaris* within the EU

G. salaris has been recorded from eight EU member states, although records from wild fish in France and the Iberian Peninsula (Spain, Andorra and Portugal) should be considered doubtful due to confusion with *G. teuchis*. Early German records may also be doubtful. It is worth pointing out that only Norway, Sweden, Iceland, the Faeroes, the UK and Eire contain substantial stocks of salmon within the European Economic area, or contain significant wild populations. France and Spain and the Baltic States contain small, threatened populations of salmon, while plans to return salmon to the Meuse and Rhine would reintroduce the fish to central European states such as Germany, Belgium and Luxembourg. In most European states, the primary host of *G. salaris* is rainbow trout. Translocations of this host, either deliberate or as escapees, present the greatest threat of dissemination of *G. salaris.*

Sweden: Some clades of *G. salaris* from the Baltic-draining Indals älv (Malmberg, 1957) are native to Sweden, where the parasite is generally not a serious pathogen. *G. salaris* became well known in salmon and rainbow trout hatcheries from the mid-1970s onwards in both Baltic and Atlantic watersheds, and could survive for years on rainbow trout (Malmberg and Malmberg, 1987, 1991). No *G. salaris* epidemic is known from the infected Swedish Baltic river systems, Torne älv, Vindelälv and Mörrumsån (Malmberg, 2004). However, 11 rivers of the Swedish West coast, draining into the Kattegat and Skagerak and supporting populations of the East Atlantic salmon

race, are now infected. The parasite was first recorded in the Säveån (a tributary of the Göte älv) in 1989–1991, but it now seems to have disappeared from this river (Malmberg, 2004). Prior to this, there had been no evidence for parasite-induced mortality of salmon parr in Sweden (Malmberg, 1988), but subsequently, high parasite intensities (~1700 per fish) were observed combined with an average decrease in salmon parr density of 90% (Alenäs, 1998; Alenäs *et al*., 1998). On the Swedish West coast, salmon stocks that co-exist with *G. salaris* range from those without obvious fish mortality to those experiencing population decline (Malmberg, 1998). However, in spite of the fact that smolt and precocious males exhibit relatively high infections, especially in Högvadsån (River Åtran system), *G. salaris* is said not to cause problems in Swedish rivers (Malmberg, 2004). Epidemics on the Swedish West coast have not followed the same pattern as that in Norway, and the epidemiology of *G. salaris* in this area would repay closer investigation. The dispersal of *G. salaris* is probably also a result of the spread of infected rainbow trout, as escaped fish from farms in Lake Bullaren on the west coast have been found infected with *G. salaris* (see Malmberg, 2004). The introduction of the parasite to the Swedish West Coast has also proved to be more complicated than originally thought as Hansen *et al*. (2003) demonstrated the presence of different *G. salaris* clades in different rivers. Neither parasite pathogenicity nor host susceptibility can be assumed a priori, but must be experimentally tested.

Finland: *G. salaris* probably has a long history in Finland on salmon, as it shares the infected Torne älv along the border with Sweden and the Russian Neva stock has been heavily used for restocked purposes. After the first record of the parasite in Finland in 1984 (Rintamäki, 1989), the parasite has been found in ~40% of rainbow trout and salmon fish farms (Rimaila-Pärnänen and Wiklund, 1987) in northern Finland, including one farm in brackish water, one rainbow trout hatchery in Lake Inari (River Paatsjoki system) and others within the drainage area of Finnish rivers (Malmberg and Malmberg, 1991; Keränen *et al*., 1992; Aalto and Rahkonen, 1994; Koski and Malmberg, 1995; Koski, 1996; Rintamäki-Kinnunen and Valtonen, 1996). There have been no reports of *G. salaris* epidemics on wild salmon in Finland, which are supposedly resistant to the parasite

(Keränen *et al.*, 1992; Aalto and Rahkonen, 1994; Rintamäki-Kinnunen and Valtonen, 1996). However, *G. salaris* can cause serious problems in Finnish fish farms (Malmberg, 2004). *G. salaris* occurs in the Kierettijoki River which flows into the White Sea, and the Rivers Teno and Näätämöjoki, flowing to the Arctic Ocean, are at risk (Aalto and Rahakonen, 1994).

Denmark: Salmonids (salmon, brown trout and rainbow trout) from ponds and fish farms in Denmark, are generally infected with *G. salaris*, *G. derjavini*, *G. teuchis* and *G. truttae* (Malmberg, 1973; Buchmann and Bresciani, 1997; Buchmann *et al.*, 2000; Nielsen and Buchmann, 2001; Buchmann, 2005). *G. truttae* [described by Gläser (1974) from *Salmo trutta* (see Ergens, 1992b)] has not been recorded from Sweden, Finland or Norway but occurs frequently south of the Baltic (Poland, Denmark, Germany, Czech Republic, Slovakia and UK). *G. teuchis* also occurs in France and Scotland, and *G. derjavini* is widely distributed in Europe. Only *G. salaris* and *G. derjavini* were found on farmed rainbow trout in Denmark (Buchmann *et al.*, 2000). The situation under natural conditions in Denmark is complicated by the presence of *G. teuchis* on wild salmon and rainbow trout escapees, in addition to *G. salaris* variants which infect rainbow trout but exhibit low pathogenicity to salmon (Buchmann *et al.*, 2000; Lindenstrøm *et al.*, 2003a; Jørgensen *et al.*, 2006). No *G. salaris* epidemics on wild salmon have been reported in Denmark (Jørgensen *et al.*, 2006), possibly because the rainbow trout variants of *G. salaris* (see Lindenstrøm *et al.*, 2003a; Jørgensen *et al.*, 2006) do not readily reproduce on salmon, or it may be due to the general scarcity of wild salmon in Danish watersheds.

Italy: The recent molecular confirmation of *G. salaris* on rainbow trout in Northern Italy (H. Hansen and A.P. Shinn, personal communication) indicates that the parasite has probably now spread widely throughout Europe with the rainbow trout trade.

Germany: Lux (1990) recovered *G. salaris* for the first time in Germany and reported infections in 70% of rainbow trout farms (intensity <40 per fish). However, these observations should be considered doubtful pending molecular analysis, which is urgently needed.

UK, Northern Ireland and Eire: *G. salaris* was not observed in the UK by Shinn *et al.* (1995b) despite extensive screening of several

species of salmonid, and it has not been found subsequently as part of routine screening by fish health authorities. Although *G. thymalli* is present in England (Denham and Longshaw, 1989; Hansen *et al.*, 2007), *G. salaris*-like clades appears absent.

France: The first report of *G. salaris* in France was based on heavily infected farmed rainbow trout examined in Oulu, Finland, during an EU Workshop on *G. salaris* (see Johnston *et al.*, 1996). This record was subsequently assumed to be a misidentification due to confusion with *G. teuchis* (Lautraite *et al.*, 1999; Cunningham *et al.*, 2001). During their comprehensive sampling, Lautraite *et al.* (1999) did not find *G. salaris* in Brittany (10 sampling sites from seven main salmon rivers and three restocking farms) or the Western Pyrénées (eight sampling sites from six main salmon rivers and one restocking farm) which are the main French water basins harbouring wild Atlantic salmon populations. Colonisation by *G. salaris* would be expected if the parasite was present in France. *G. derjavini*, in contrast, was frequently recorded on salmon in the area (Lautraite *et al.*, 1999). However, the complete absence of *G. salaris* in rainbow trout hatcheries and farms in France awaits confirmation.

Portugal and Spain: If the records of *G. salaris* in France are incorrect (Lautraite *et al.*, 1999), Johnston *et al.*'s (1996) report of *G. salaris* in Portugal also needs to be confirmed. In Eiras' (1999) studies on rainbow trout and brown trout in several trout farms belonging to different hydrographic basins in Portugal, no *G. salaris* was observed. Johnston *et al.*'s (1996) record may represent *G. teuchis,* but this needs clarification.

The Czech Republic: *G. salaris* is probably absent from the Czech Republic. However, the discovery of *G. bohemicus* by Ergens (1992a) in a rainbow trout and brook trout farm, and the close resemblance of this species to both *G. thymalli* and to the rainbow trout variant of *G. salaris* (see Lindenstrøm *et al.*, 2003a, b) does suggest that further studies to establish absence would be worthwhile.

The status of G. salaris *in areas bordering the EU*: Outside the EU, *G. salaris* is abundant in the Kola Peninsula, Russian Federation, abutting northern Finland. There are however records of *G. salaris* from elsewhere in the Russian Federation, including from Black Sea drainages.

The Kola Peninsula: The first record of *G. salaris* in the River Keret (draining into the White Sea) dates from 1992 when it was probably introduced with stocked fish from the Ladoga-Onega region (Ieshko *et al*., 1995). According to Meinilä *et al*. (2004), infection has been traced to a helicopter-carried canvas bag used to transport salmon parr around Lake Onega and, in the same day, from another hatchery. An epidemic of *G. salaris* has been reported for this river, with a significant reduction in salmon parr density (see Johnsen *et al*., 1999).

Karelia: *G. salaris* has been observed on parr from Lake Ladoga (Ergens, 1983) and in rivers entering Lakes Onega and Ladoga (Ieshko *et al*., 1996). Prevalence and intensity of infection are reported to be low and no epidemics have been observed (Shulman *et al*., 2000, 2005). At least some Karelian populations appear to be cold-adapted (Shulman *et al*., 2005).

Ukraine and Georgia: *G. salaris* was reported on brown trout (*Salmo trutta fario*) in the River Seret, a tributary of the River Dnestr draining into the Black Sea (Malmberg, 1988), and was reported common on rainbow trout in two areas draining to the Black Sea, the River Pliva and the Jezero fish farm (Žitňan and Čankovič, 1970). Although outside the normal geographical range, these records are considered valid.

Bosnia Herzegovina: Žitňan and Čankovič (1970) recorded *G. salaris* from rainbow trout from the Adriatic coast (River Buna and the fish farm Blagaj) of Bosnia Herzegovina. Rainbow trout were widespread and *G. salaris* was common. Although far from the normal range of *G. salaris*, this record is normally considered valid.

10.3. The Current Status of *G. salaris* in Norway

Among the 24 identified *Gyrodactylus* species from freshwater fish in Norway (Sterud, 1999), only *G. salaris* has proved a serious threat to wild salmon (Bakke and Harris, 1998; Mo, 2004). Losses in 1984 were estimated at 520 tonnes (Johnsen and Jensen, 1986), equivalent to 25% of the total salmon catch (Egidius *et al*., 1991). In 14 rivers, the average density of salmon parr and adults was reduced by more than 85% (Johnsen *et al*., 1999; Johnsen and Jensen, 2003). The parasite

has exterminated salmon in six rivers and threatens populations in 34 other rivers. In some infected rivers (Skibotnelva, Røssåga and Vefsna), the relative abundance of salmon/trout hybrids has dramatically increased (by more than 50%), presumably because of the greater resistance of hybrids to *G. salaris* (see Bakke *et al.*, 1999; Johnsen *et al.*, 2004). Currently, annual loss due to *G. salaris* infections is estimated at 250–500 tonnes of salmon, approximately 15–20% of the natural smolt production (Anonymous, 1999). However, the economic cost of *G. salaris* is twofold: the loss in potential economic output and the direct cost of combating invasions. In Norway, the annual loss in lost fisheries, tourism etc. is calculated to be around USD 34 million per year, with a further expense of ~USD 23 million in surveillance and eradication. Since introduction 30 years ago, the parasite is estimated to have cost a total of USD 450–600 million (Directorate for Natural Resources, May 2002), without including indirect costs due to restrictions on the export of live salmonids within the EU, or the costs of surveillance and control in other countries within (e.g. Scotland) or outside (e.g. the Russian Federation) the EU. Without control measures, *G. salaris* would have reduced the Norwegian salmon fishery by at least 15% (Johnsen *et al.*, 1999; Johnsen and Jensen, 2003).

To date, 46 out of 379 salmon rivers and 39 farms (13 coastal salmon hatcheries/farms and 26 rainbow trout hatcheries/farms in Southern Norway) have been infected since in Norway since 1975 (Mo *et al.*, 2004; Mo and Nordheim, 2005, unpublished). Eradication using rotenone or acidified aluminium treatment has been attempted in 35 rivers, but of August 2006, 19 remain infected, 12 are under post-treatment surveillance, 15 have been confirmed clear of the parasite and in 8 rivers attempts have proved unsuccessful (Figure 25; Table 2). In 2000, a National surveillance programme was implemented for all uninfected salmon rivers by the Norwegian Animal Health Authority (now part of the Norwegian Food Safety Authority) responsible for sampling salmon in both rivers and fish farms. The spread of *G. salaris* is tracked by routine annual surveillance of 150 salmon rivers and biennial examination of a number of freshwater fish farms. Thirty salmon fingerlings, parr or smolt from each river have been sampled by electro-fishing each year. The fish are

killed, preserved in 96% ethanol and the skin and fins examined by the National Veterinary Institute (NVI, Harstad, North-Norway). This low number of salmon parr examined on a yearly basis makes an immediate record of an infection upon introduction very arbitrary. Additionally, there is no regular or fully satisfactory surveillance control after rotenone treatment and before the 5-year check if the parasite has been exterminated, preventing study of the origin and epidemic spread of *G. salaris* in newly infected rivers. The parasite has reappeared in several rotenone-treated rivers; in some (Skibotnelva, Steinkjervassdraget, Figga, Lundselva, Batnfjordelva, Leirelva and Lærdalselva) more than once. By the mid-1980s, the NVI (OIE reference laboratory for gyrodactylosis) extended surveillance to fish farms, especially rainbow trout farms. In 2004, salmon from 120 rivers (4509 individuals) were screened and *G. salaris* recovered after rotenone treatment in two rivers, the Leirelva in Nordland County (rotenone treated in 1996) and the Halsanelva (rotenone treated in 2003). *G. salaris* was not observed in the 34 fish farms examined in 2004 (Mo and Norheim, 2005). Late autumn 2006, Batnfjordelva as (one of three rivers treated with aluminium) was found reinfected.

Currently infected watercourses and fjords in Norway can be grouped into infection-regions, representing geographic localities where *G. salaris* has been recorded on wild salmon parr, and which are bounded by features limiting natural dispersal by drift or host migration. Originally, 14 infection-regions were identified but only eight (or nine; see Region 8 below) now remain. Official policy (Anonymous, 2002b) is to eradicate the parasite from infected rivers by proceeding from region to region depending on annual funding. Since 1990, *G. salaris* has been introduced to between one and three new regions: Lærdals Region (1996), Halsanelva and Hestdalselva, close to the Vefsna Region and Sandelva in Drammens Region. As of 2006, the eight regions (from north to south) are:

Region 1—Skibotn Region (Troms County): *G. salaris* first observed in 1979 in Skibotnelva. The region consists of two infected river courses, Skibotnvassdraget and Signaldalsvassdraget (and Balsfjordelva which shares an estuary with Signaldalselva), all with natural stocks of salmon and anadromous Arctic charr and trout. Skibotnvassdraget was rotenone-treated in 1988, but re-infected in

1992, re-treated with rotenone in 1995, and re-infected again in 1998. *G. salaris* was recorded for the first time in Signaldalselva in 2000.

Region 2—Rana Region (Nordland County): *G. salaris* was first observed in 1975 in Ranaelva. The region consists of six infected rivers, two large (Ranaelva and Røssåga) and four small, Busteråga (Slettenelva), Bjerka, Sannaelva and Bardalselva. All have natural stocks of salmon and anadromous trout, some also contain Arctic charr. Ranaelva, Røssåga and Bjerka were rotenone treated in 2003 and all rivers in 2004. These rivers will be declared parasite-free if there is no re-occurrence within the next 5 years after treatment.

Region 3—Vefsn Region (Nordland County): *G. salaris* was first observed in 1978 in the large River Vefsnavassdraget. The region also includes seven smaller infected rivers, the Fusta, Drevja, Hundåla, Leirelva, Ranelva, Halsanelva and Hestdalselva. Leirelva was rotenone treated in 1996 and declared parasite-free in 2003, but *G. salaris* re-appeared in 2004. The river was immediately rotenone treated again, and results of the treatment are awaited. However, in 2006 a new river was found infected in this region, Ranelva, close to Leirelva. Accordingly, both rivers were immediately rotenone treated and results of the treatment are awaited. Halsanelva and Hestdalselva were treated in 2003, but the parasite reappeared in Halsanelva in 2004 and in Hestdalselva August 2006. These two rivers may constitute a new infection-region as natural dispersal via the relatively long migration route through saltwater from the Vefsna should be considered unlikely.

Region 4—Beitstad Region (Nord-Trøndelag County): *G. salaris* was first observed in 1980 in the Steinkjervassdraget (Steinkjerelva, Byaelva and Ogna) and Figga. The parasite was first observed in the Lundselva, the third river of this region, in 2001. All river courses have natural stocks of salmon and anadromous trout. Steinkjervassdraget and Figga were rotenone treated in 1993, but the parasite returned in 1997 and the rivers were re-treated in 2001/2002. Re-infection was immediately noted and the rivers re-treated in 2005 to reduce the salmon migration from the rivers. However, despite Steinkjervassdraget being treated twice in spring and autumn 2005, *G. salaris* has again been recovered but upstream from the treated regions (see Hjettnes *et al.*, 2006 for an analysis of the epidemic situation). In August 2006, this river system and some adjacent minor

rivers were treated for the first time with aluminium sulphate based on the previous positive experiences with aluminium against *G. salaris* infections in Batnfjordelva (Region 5) and Lærdalselva (Region 6). This treatment with aluminium will be repeated in 2007. The success of this treatment in Steinkjersvassdraget is still to be seen.

Region 5—Sunndals Region (Møre and Romsdal County): The region consists of four infected river courses, one large (Driva) and three smaller (Usma, Batnfjordselva and Litledalselva), all with natural stocks of salmon and anadromous trout. *G. salaris* was first observed in 1975 in the Driva, Usma and Batnfjordelva. The latter was rotenone treated in 1994, but the parasite re-appeared in 2000. This river was selected for a pilot project for aluminium sulphate treatment (see Section 9.3; Table 2) in 2004. Late autumn 2006 the river was observed reinfected with *G. salaris*.

Region 6—Romsdals Region (Møre and Romsdal County): *G. salaris* was first observed in 1980 in Raumavassdraget and Hensvassdraget. This region consists of four infected river courses, the Raumavassdraget (Rauma and Istra), Innfjordelva, Hensvassdraget (Henselva) and Skorga, all rivers with natural stocks of salmon and anadromous trout. The rivers were rotenone treated in 1993 but the parasite re-occurred in Rauma in 1996, and from there has probably been spread to Innfjordelva (1999), Hensvassdraget (2000) and Skorga (2003).

Region 7—Lærdals Region (Sogn and Fjordane County): *G. salaris* was first observed in 1981 in the Vikja and in Lærdalselva in 1996. Vikja was successfully rotenone treated in 1981/1982. Lærdalsvassdraget was treated in 1997, but the parasite re-occurred in 1999. In 2005, a 24-km infected stretch of the river was treated with aluminium sulphate, a process which was repeated in 2006. Again, confirmation of the success of this trial has still to be announced.

Region 8—Drammens Region (Buskerud County): *G. salaris* was first observed in 1987 in the Drammensvassdraget and Lierelva, which both empty into Drammensfjorden. Both rivers have natural stocks of salmon and anadromous trout. Due to the complexity of this river and fjord system and of the fish fauna, there are no plans for rotenone treatment, but the rivers are restocked annually to maintain the salmon angling industry. In 2003 a new river, Sandeelva (Vesleelva; Vestfold County) was found infected with *G. salaris*, the

only other river with salmon in the area. However, this may represent a new infection-region, but infection from the nearby Drammens Region cannot be excluded.

Predicting host populations at risk of future invasion is problematic. However, three regions are deemed at high risk, as the parasite has previously occurred, or has been found in fish hatcheries but not on wild salmon:

Risk Region 1—Enaresjøen and Pasvikelva (Finland and Troms County, respectively). *G. salaris* was observed in Lake Enare in a rainbow trout hatchery and on escapees in the lake in 1990. The Norwegian Pasvikvassdraget is in the zone at risk;

Risk Region 2—Torneälv and Nord-Troms (Sweden/Finland and Troms County, respectively). Torne älv drains into the Baltic Sea and heavy restocking occurs within the Finnish catchment, where relative high infection levels have been observed on wild salmon parr. The Norwegian salmon rivers Målselva and Reisaelva are particularly at risk;

Risk Region 3—Iddefjorden (Sweden and Østfold County). Enningvassdraget drains several larger lakes in Sweden and Norway, including the Swedish Lake Bullaresjøen where *G. salaris* was observed in a rainbow trout hatchery and on escapees in the lake in 2001. Two Norwegian salmon rivers, Enningdalselva and Tista, are particularly at risk.

10.4. Control Measures

Eradication of a non-indigenous species is sometimes feasible particularly if it is detected early and resources can be applied quickly (Simberloff, 1997; Mack *et al.*, 2000). To eradicate an established invader, such as *G. salaris,* in natural areas is difficult, especially as it can exploit a range of commercially produced fish, and its reproductive rate and dispersal ability are high. Control measures to prevent the further spread vary in their efficacy and are dependent on the continued commitment and diligence of both salmon management authorities and relevant agencies (the fish traders, anglers and general public) to prevent re-introduction (Figure 26). In Norway, the methods considered and employed are designed to eradicate the parasite

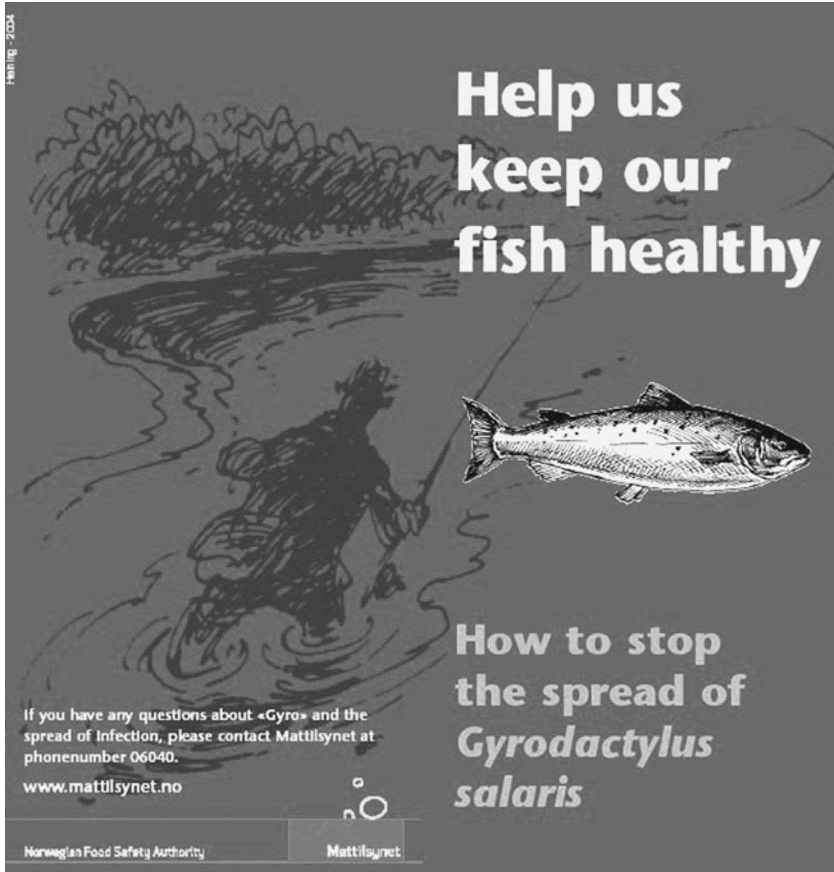

Figure 26 An example of the many information brochures from Norwegian authorities to protect from anthropochore spread of *Gyrodactylus salaris* to uninfected river systems. (Reproduced with permission from Norwegian Food Safety Authority.)

from the respective infection-regions. Hence, the re-occurrence of the parasite may be caused by unsuccessful rotenone treatment, or by new introduction from external regions. The time between eradication and reappearance of the parasite in the largest river in five of the infection-regions has been from 3 to 6 years (Hjeltnes *et al.*, 2006). The available methods for eradication of *G. salaris* include:

Biocides: Rotenone ($C_{23}H_{22}O_6$), an isoflavonoid from the roots of certain leguminous plants. It is insoluble in water and is normally

formulated as a liquid emulsion to allow rapid dispersion and efficacy as a piscicide. However, rotenone is non-specific and apart from being highly toxic to fish it also kills gill-breathing aquatic invertebrates. It has been widely used in Norway to eliminate *G. salaris*. Of the 46 *G. salaris* infected rivers, 28 were treated with rotenone between 1981 and 2003 (Guttvik *et al*., 2004). Different grades of rotenone are used, but CFT-Legumin^{TM}, which contains approximately 5.3% (w/w) of the active substance dissolved in a mixture of diethylene-glycol-monoethyl-ether and 1-methyl-2 pyrrolidone, is recognised as least dangerous for the environment at the concentrations used (25 mg/l) (1.0 ppb) during the first hours, and thereafter at 12.5 mg/l (0.5 ppb). This procedure may be repeated in infected rivers. The limit of rotenone tolerance of salmon parr is 0.2 ppb, the 24 h LC_{50} concentrations for rotenone active substance are between 2 and 10 ppb for salmonids that are among the most susceptible fish species. The lower limit for effective use of rotenone is set at 0.5 ppm (Guttvik *et al*., 2004). It is impossible to ensure 100% success with rotenone treatment (Haukebø *et al*., 2000), and after initial success exterminating the parasite in the River Vikja in 1981/1982, rotenone treatment has frequently failed (e.g. in Skibotnelva, Leirelva, Halsanelva, Steinkjersvassdraget, Figga, Rauma, Innfjordelva, Lærdalselva, Batnfjordelva). The failure is probably due to the complexity of effectively dosing all infected areas within a river system, but recolonisations cannot be excluded. After ~20 years experience, Haukebø *et al*. (2000) reviewed the procedures used and stressed the potential need for more extensive use of river obstructions, updated equipment, high-density rotenone compounds, prolonged periods of application, double treatments, increased focus on the land–water interface and better hydrological expertise as groundwater wells beneath the river beds have been a major problem (Brabrand and Koestler, 1999; Brabrand *et al*., 2005). There is also a concern of escape migration by salmon into groundwater wells or to river mouths or fjord basins, possibly triggered by the presence of rotenone or other substances in the river. After rotenone treatment, the river is artificially and naturally re-stocked with salmon of the local stock. Farms can be easily disinfected using antiparasitic drugs (Santamarina *et al*., 1991; Tojo *et al*., 1992, 1993a, b; Schmahl, 1993), although Norwegian

practice has been to enforce a dry fallow period to be certain of eradication.

Salmon and sea trout quickly recolonise treated rivers provided there is a sea population sufficient for recolonisation (Johnsen *et al.*, 1997; Lund, 1997). Wild salmon stocks preserved in gene banks are used for restocking purposes to supplement the contribution from the marine population and any surviving eggs of both salmon and sea trout buried in the gravel (Hartvigsen, 1997). The first generation salmon fry have a faster growth rate than normal, resulting in earlier smoltification and sea migration (Johnsen *et al.*, 1997). Although the effects on the genetic structure of fish populations are more pronounced in freshwater resident fish species than in anadromous or marine species, there are no empirical data on the genetic impact of rotenone on specific fish populations (Hindar, 1997).

The short-term impacts of rotenone on benthic macroinvertebrates have been studied by Arnekleiv (1997). The impact on estuarine fauna is largely unknown (Wingard and Swanson, 1992), but freshwater invertebrates are severely affected. In the River Steinkjerelva, treatment with rotenone led to an immediate catastrophic loss of invertebrates peaking $^1/_2$ to 2 h after treatment followed by a decline through the next hours (Arnekleiv, 1997). All common taxa were affected. Amongst the benthic fauna of the upper stream, a reduction of around 90% occurred shortly after rotenone treatment. However, some insect species survived in great numbers and recolonisation by benthic macroinvertebrates occurred rapidly, requiring 6 weeks to restore insect densities to previous levels. One year later, the common species of Ephemeroptera, Plecoptera and Trichoptera had all re-established (Arnekleiv, 1997). However, instability in species composition was observed and probably greatly underestimated due to limited sampling and lack of genetic studies to assess the effects on population structure. Generally, rotenone tolerance and the effects on invertebrates are little known; however, dramatic short turn effects are reported but differing between taxa and life stages (Binns, 1967; Morrison, 1977; Dolmen *et al.*, 1995; Arnekleiv, 1997). Rotenone treatment is reported to have had little long-term impact in Norwegian rivers (Mo, 1994) but criticism of its use is growing because of (i) several unsuccessful attempts to exterminate *G. salaris*; (ii) potential

deleterious founder-effects and genetic drift in restored fish populations; (iii) destruction of a stable host–parasite association based on resistant salmon which could form the basis for breeding natural resistance; (iv) the continuous threat of re-infection (Hessen, 1997); and (v) health concerns for humans. Rotenone formulations are placed in Class II (moderately hazardous) or III (slightly hazardous) of the WHO classification of pesticides. However, rotenone has been suspected of being carcinogenic (Gosalvez, 1983) and involved in the aetiology of human illnesses (see Tables in Ling, 2002), such as Parkinson's disease (Alam and Schmidt, 2002; Sherer *et al*., 2003). Following the EU Biocide Directive (Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998) rotenone must be removed from the market and its use ceased by 1 September 2006. The Norwegian Pollution Control Authority (SFT) in consultation with the Ministry of the Environment has applied to the EU-commission for exceptional permission to continue to use rotenone for *G. salaris* control until 2010. However, attention must focus on other strategies and the Norwegian authorities are seeking to identify alternative methods for *G. salaris* control. In Norway many substances have been assessed (Skjelstad and Simolin, 2001), but only aluminium or zinc show promise. However, most recently, Australian tea tree oil (TTO) has been tested on *Gyrodactylus* infected three-spined sticklebacks (Steverding *et al*., 2005). The results suggest that TTO, in combination with Tween 80 as an emulsifier, might be an effective and potentially environmentally friendly, treatment.

Parasitocides—metal ions: On-going development projects are currently investigating the partial replacement of rotenone by long-term treatment of rivers with aluminium sulphate with simultaneous acidification. The toxicity to *G. salaris* of aluminium and zinc ions in acidified water has been described in Section 9. Following these laboratory experiments (Soleng *et al*., 1999b; Poléo *et al*., 2004a), pilot studies have used aluminium sulphate to control *G. salaris* (see Poléo *et al*., 2004a, b) in Lærdalselva (2002, but full treatment in 2005 and 2006), Batnfjordselva (2003, but full treatment in 2004, see Hytterød *et al*., 2005) and for full treatment of Steinkjersvassdraget in 2006 and 2007. The application of Al is problematic as the concentration has to be balanced exactly to the specific water flow, chemistry and

temperature to kill parasites but not fish. So far, the full-scale treatment of Lærdalselva and Steinkjersvassdraget with Al to eradicate *G. salaris* appears promising but must await appraisal over the next 5–6 years. The macrobenthos community in Lærdalselva and Batnfjordelva was sampled extensively before and after Al treatment, but awaits full analysis.

Barrier methods: Mechanical methods of controlling non-indigenous organisms are sometimes effective. The closure of fish ladders and construction of artificial barriers in infected rivers can prevent spawning salmon re-colonising parts of the river course where the disease is present (Thorstad *et al*., 2001; Fjeldstad, 2004). Smoltification of salmon parr from above the barrier will then lead, over 2–5 years, to a total disappearance of salmon from the infected part of the river. This approach reduces the area containing infected fish, allowing more restricted and less expensive rotenone treatment. However, when barriers are close to the sea they are likely to be affected by flood, ice and debris, increasing cost and reducing effectiveness.

Selective breeding: Artificial breeding for resistance represents an apparently ideal solution to the problem of *G. salaris* susceptibility, and has been shown to be feasible as resistance has a strong genetic component (e.g. Bakke *et al*., 1990; Bakke and MacKenzie, 1993; Bakke *et al*., 1999; Cable *et al*., 2000; see Section 8.2). However, even once the practicalities are overcome to produce resistant fish for re-stocking, release of such genetically modified salmon into the environment will be controversial and politically sensitive. Each river system within the range of the Atlantic salmon is populated with a genetically unique fish stock, resulting from repeated bottlenecking and drift caused by the return of only a tiny number of breeding adults relative to the number of eggs laid (Mills, 1989). Conservation of this genetic diversity remains a priority for Norwegian conservation bodies (Anonymous, 1999) and therefore artificial selection for resistance has been ruled out except for complex river systems which are too complicated to be treated chemically (e.g. Drammenselva). Within Drammenselva, artificial mass selection for resistance to *G. salaris* of 50 family groups is in progress with both unexposed parr and the surviving exposed parr being cultured to maturity (Salte and Bentsen, 2004). Progeny from the most resistant fish will then be

tested for resistance and eventually used for restocking the river course. The main arguments against selection for disease resistance are that the heterogeneity of the salmon population will be reduced, escapees will impede the resistance development and artificial selection is time consuming and expensive. Because of the uniqueness of stocks, every infected river system will need its own breeding program. Perhaps most importantly, breeding for resistance will not eradicate the parasite and Norwegian rivers will continue to represent a focus for spread of the parasite. In addition, the evolutionary impact of increased host resistance to a particular parasite species is unknown. However, hopefully, such selective breeding experiments will increase our knowledge of the heterogeneity of Norwegian salmon resistance and of the heritability of resistance to gyrodactylids (Salte and Bentsen, 2004).

Integrated control measures: The governmental strategy in Norway aims for the complete extermination of *G. salaris* from all rivers, but there is still a need for "maintenance control" of the parasite (Schardt, 1997). As the drawbacks of individual control measures, such as rotenone, are realised (Sandodden *et al.*, 2004), integrated control strategies using barriers, aluminium and selective breeding appear increasingly attractive. The treatment of Lærdalselva in 2005 with Al also involved application of rotenone in small quantities where Al treatment was not feasible. The success of this combined treatment will not be fully assessed until 2010. River barriers or closure of salmon ladders are often used as a first step in reducing the area requiring chemical treatment. In Drammenselva, smolt or yearlings of the same stock are restocked in the river mouth and in uninfected tributaries of the river, respectively, to compensate for deaths caused by *G. salaris* and to maintain the number of fish returning to the river for angling. This has been a success in this river (Hansen, 1990, 1991) but poses the danger of reducing the evolutionary development of natural disease resistance.

Biological control: The introduction of natural enemies such as hyperparasitic bacteria or viruses as biological control agents to eliminate *G. salaris* have not been explored, although micro-organisms are frequently observed externally on gyrodactylids (Cone and Odense, 1984; Bakke *et al.*, 2006; Section 7 above).

10.5. Potential for Further Spread of *Gyrodactylus salaris*

Alterations in the distribution of the Earth's biota brought about by human transport and commerce (Mack *et al*., 2000) represent a major threat for the future biological diversity of the biosphere. The potential for further spread of *G. salaris* within Europe, either naturally or by Man, remains significant (Bakke and Harris, 1998). The parasite is not a problem of rainbow trout in aquaculture and it does not cause losses in natural salmon populations across most of its Scandinavian range (see Section 10.2). The perception of *G. salaris* as a pathogen declines further if the suggestion of Meinilä *et al*. (2004) to synonymise salmon- and grayling-infecting clades as *G. salaris* is followed; the parasite then occurs throughout the EU and neighbouring countries but causes problems only in Norway, and perhaps in some infected rivers at the Swedish west coast and the Kola Peninsula. However, experimentally other east Atlantic stocks of salmon, such as those from Scotland and Denmark seem highly susceptible to Norwegian *G. salaris* (see Bakke and MacKenzie, 1993; Dalgaard *et al*., 2003, 2004). Risk analyses show that the potential for spread of *G. salaris* via wild salmon is very sensitive to changes in salinity within estuaries (Paisley *et al*., 1999). However, Peeler and Thrush (2004) and Peeler *et al*. (2004) established that for England and Wales, the greatest risk of introduction was via the movement of rainbow trout. A recent risk analysis of watercourses in which *G. salaris* is already present from the Norwegian Veterinary Institute (Jansen *et al*., 2005) concluded that the major risk factors for the spread of *G. salaris* are in diminishing order: (i) dissemination by migrating fish in brackish water; (ii) spread from infected hatcheries and farms; (iii) restocking with infected fish; (iv) spread by equipment which has been in contact with infected fish; (v) spread by non-predicted human activity; (vi) spread of dislodged parasites with water; and (vii) natural spread by other host species. In this context we would stress the potential of wild Arctic charr to disseminate *G. salaris* amongst natural salmon populations (see Sections 5.3.2 and 6.5). This may also be the case for wild lake trout (*Salvelinus fontinalis*) and rainbow trout which are susceptible to *G. salaris* and

occasionally establish viable populations in Norway. Such populations may represent a reservoir for the spread of *G. salaris* and should be under surveillance (see Mo, 1988; Bakke *et al.*, 1992a, b, c; Hindar *et al.*, 1996; Knudsen *et al.*, 2004; Kristoffersen *et al.*, 2005; Robertsen *et al.*, 2006, 2007b; M. Eken, personal communication).

The trans-national spread of the parasite, particularly to highly sensitive islands as the UK, Ireland, the Faeroes and Iceland, or to the east Atlantic stocks of continental America, is most likely to occur with rainbow trout or other fish species. Recently (1 May 2004), regulations concerning salmon transportation within the EU were relaxed (EC decision 2004/453/EC) such that live salmonids could be moved to *G. salaris*-free areas, as long as the fish originated in coastal sites where the salinity does not drop below 25 parts per thousand. Peeler *et al.* (2006) concluded that this does not directly increase the risk of *G. salaris* introduction into new waterbodies, but that the risk is indirectly increased by the increased volume of salmonid transport. In general, the UK imports only salmonid eggs to ensure the exclusion of salmonid viruses such as Infectious Hepatic Necrosis and Viral Haemorrhagic Septicaemia (Scott, 2004). This should ensure the continued exclusion of *G. salaris* from the British Isles. However, the UK does also import salmonids for processing (Scott, 2004), it is conceivable that *G. salaris* could survive import in this way (see Section 3.3 for an appraisal of survival of *G. salaris* on dead fish) and remain attached to fish packaging materials long enough to gain access to a watershed containing live salmonids. The possibility that the parasite could survive for periods of days attached to eggs (Section 3.3) also raises the possibility of the spread of the disease with egg imports. Rationalisation in the international salmon industry has seen the recent closure of Scottish egg hatcheries with the proposal that eggs will be imported to farms from Scandinavia. Apart from the apparent breach of EU fish hygiene regulations in this proposal, this must represent an unacceptable level of risk for the importation of *G. salaris* into the UK. The illegal import of live fish (principally carp, wels and sturgeon) into the UK is also well known, and is thought to have been a route of origin for Spring Viraemia of Carp (SVC). Most imports do not come from countries where *G. salaris* is likely to be encountered, but clearly a route of entry into

the UK exists which is not easily controllable given existing legislation. The UK is used here to demonstrate that even with a highly developed legislative and monitoring framework to prevent the import of *G. salaris*, biosecurity is by no means assured. In the past 3 years, the import of sleeping disease (SD) syndrome and SVC have both highlighted failures in the established system. Given these failures in a country with a sophisticated and stringent system for assessing fish health, the continued spread of *G. salaris* within continental Europe, where transboundary controls on fish imports could never be as careful, is inevitable. As the parasite spreads within Europe and neighbouring states, the risk of import into the highly sensitive Atlantic Fringe countries increases.

11. CONCLUSIONS AND FUTURE RESEARCH AREAS

We have attempted to provide an overview of the biology of gyrodactylid monogeneans and to highlight the potential importance of this megadiverse group in studies of parasite evolution. The gyrodactylids are best known as viviparous fish parasites which are born already containing a developing daughter; here we highlight that this 19th century view of the group misses much that is biologically interesting and significant about them. In particular, we stress the importance of recognising that viviparity and progenesis are adaptations of only one group of gyrodactylids (albeit the most successful and currently the most species-rich part of the group). Studies on the egg-laying gyrodactylids are in their infancy, but are starting to live up to their early promise. Although Boeger *et al.* (2003) consider the radiation of viviparous gyrodactylids to have occurred recently, we feel that gyrodactylids are a primitive monogenean group, perhaps the remnant of an earlier diversification before the evolution of modern forms such as the capsalids, microbothrids and polyopisthocotyleans. A study of the egg-laying forms is the only way in which potentially plesiomorphic characters, shared with other primitive monogeneans can be identified, because progenesis has modified the viviparous genera so extensively. Further investigations, particularly in the tropics, may also highlight additional variants in gyrodactylid reproductive

biology, behaviour and ecology. We will also point to the general need for more zoogeographic, phylogeographic and population genetic studies which would provide methods to measure for example migration and transmission dynamics, the amount of inbreeding and the effective population size.

The genus *Gyrodactylus* in particular is megadiverse, with over 400 species described out of a potential total of 20 000 species. We have extensively reviewed the taxonomy and sub-generic phylogeny of the genus, and an important question which remains is whether the genus can be subdivided into more manageable natural units. We have identified three such units and have noted that many of the current generic descriptions do not correspond with natural divisions of the viviparous gyrodactylids. The three groups could be elevated to independent generic status. However, there remain many species which lie outside these three groupings and molecular sampling of the genus has been biased towards European species. More effort in sequencing additional gyrodactylids is essential, although such research may no longer appear novel. At present, inclusion of any new molecular sequence can lead to a dramatic re-drawing of phylogenetic relationships although the basic groupings may remain constant. We therefore conclude that it would be premature to fundamentally revise the genus *Gyrodactylus* to take account of molecular evidence, although this may become a feasible project within the next 5 years.

The primary means of evolution in the viviparous gyrodactylids has been host shifts, a phenomenon which has become very clear from molecular phylogenies of *Gyrodactylus*. In Eurasia, and possibly America, speciation and radiation of gyrodactylids has been greatly enhanced by the ice ages, creating and destroying a series of refugia within which host switches could occur. This phenomenon is potentially of particular interest to evolutionary biologists, but an understanding requires much more sophisticated analysis of patterns of host preference and of host specificity. This lack of knowledge is particularly apparent in relation to *G. salaris* in Norway; the precise relationships of the different forms which infect salmonids in Scandinavia, and which are actively evolving via a series of host shifts, remain obscure and elusive. There is a need to be very careful with nomenclature (which can be legally binding) in such a fluid situation,

and we would highlight the potential of this system for evolutionary biologists with an interest in the role of host shifts. The reasons why *G. salaris* is so damaging, when the congener *G. thymalli* and some *G. salaris* strains are not, remain obscure, and much additional research is needed on the role of gyrodactylids as potential biotic invaders. In particular, we need to identify potential future pathogens, particularly of salmonids, to predict their likely impact. This has lent additional urgency by the recent report (You *et al.*, 2006) that *G. brachymystacis* can establish pathogenic infections of rainbow trout in China, with the potential that this may also become a significant pest in aquaculture.

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Human Genetic Diversity and the Epidemiology of Parasitic and Other Transmissible Diseases

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ABSTRACT

This paper aims to review human genetic studies that are generally poorly known by parasitologists and scientists working on other pathogenic agents. The key proposals of this paper are as follows: (i) human susceptibility to transmissible diseases may often have a complex, multigenic background; (ii) recent discoveries indicate that major genomic rearrangements may be involved, possibly more so than DNA sequence; (iii) it is crucial to have a general population genetics framework of the human species based on neutral/historical markers to analyse reliably genetic susceptibility to infectious diseases; and (iv) the population level is a key factor. Ethnic diversity, a highly adaptive genetically driven phenotypic diversity, is possibly a valuable source for exploring human genetic susceptibility to transmissible diseases, since different populations have been exposed to drastically different geographic/climatic environments and different pathogens and vectors for tens of thousands of years. Studies dealing with human genetic susceptibility to transmissible diseases have mostly been based on the hypothesis that this factor is driven by only one or a few genes, and considered the individual more than the population level. Two different approaches have been developed for identifying the genes involved: (i) candidate genes and (ii) blind association studies (linkage analysis), screening the genome with a large number of high-resolution markers. Some loci involved in susceptibility to leishmaniosis, malaria and schistosomosis, for example, have already been identified. South American trypanosomosis (Chagas disease) is reviewed in detail to

show the methodological problems of this classical approach. Current knowledge on the general impact of transmissible diseases on human genetic diversity, mainly HLA polymorphism, and the hopes raised by recent major international programmes such as the Human Genome Project (HGP), Human Genome Diversity Project (HGDP), International Human Haplotype Map Project (Hap Map) and extended databases, networks and networks of networks will also be reviewed.

LIST OF ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
AIM	ancestry informative marker
bp	base pairs
CDCV	common disease/common variant hypothesis
CNV	copy-number variant
DNA	deoxyribonucleic acid
DTU	discrete typing unit
ELISA	enzyme-linked immunosorbent assay
Hap Map	International Human Haplotype Map Project
HAT	human African trypanosomiasis (formerly sleeping sickness)
HGDP	Human Genome Diversity Project
HGP	Human Genome Project
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
IS	insertion sequence
kb	kilobases
LCV	large-scale copy-number variation
MHC	major histocompatibility complex
MLEE	multilocus enzyme electrophoresis
MRV	multiple rare variant hypothesis
PCR	polymerase chain reaction
RAPD	rapid amplified polymorphic DNA
SNP (pronounced 'snip')	single-nucleotide polymorphism
STRP	short tandem repeat polymorphism

1. INTRODUCTION

1.1. A Call for an Integrated Approach

Human geneticists generally know nothing about the impact of a pathogen's genetic diversity on the transmission and severity of infectious disease; many may think that this impact is negligible, even though they do not have the slightest evidence for this belief. It is fair to acknowledge that most parasitologists and microbiologists have no information on the role played by human genetic variation in the transmission and severity of infectious diseases. It is now considered that this variation plays an important role. The host's and the pathogen's evolution can be considered as an arms race. The pathogen exerts a selective pressure on the host so that it can achieve its transmission cycle, and the host tends to eliminate the pathogen, or at least to control its populations, with its immune responses. The pathogen therefore has to escape these defence mechanisms. This co-evolutionary phenomenon (with a third player involved in vector-borne diseases) should be studied jointly as far as possible, in an integrated approach (Tibayrenc, 1998a, 2004). South American trypanosomosis (Chagas disease) could be an ideal model for this type of approach, both for experimental research and field studies (see Tibayrenc, 1998b, 2004). However, integrated studies remain the exception rather than the rule (Tibayrenc, 2002). It is hoped that specialists who work on pathogenic agents and are interested in integrative approaches may find the present review helpful. I have attempted to make the paper accessible to non-specialist readers who are not familiar with human genetics or, more generally, with genetics. A glossary of specialized terms has been provided on pp. 423–428, as has a list of abbreviations used (p. 379). The basic statistics used in this field of research are explained in Box 1 on p. 395.

1.2. Key Concepts

This article does not aim to be a comprehensive review of the state-of-the-art on human genetic susceptibility to infectious diseases. Nor is it

a catalogue of all those genes that have been suspected of playing a role in all known transmissible diseases. It deals rather with the manifestations of the constant co-evolution phenomena between our species and its pathogens that have proceeded through the entire evolutionary process of humankind. The article therefore considers not only those human genetic and genomic components that are involved in our susceptibility to infectious diseases, but also the general impact of the selective pressure exerted by pathogenic agents on the structure and diversity of our genome. Moreover, this article follows a few specific key concepts and working hypotheses. I stress that these concepts and working hypotheses are still highly debatable and are a reflection of my personal views (but not only mine), as follows.

(i) Even when examining a specific character (here susceptibility to infectious disease), it is misleading to focus exclusively on those genes that are assumed *a priori* to be more directly linked with this particular character (candidate genes) without considering the general genetic background of the individuals or the populations under survey. It is preferable to have a clear idea of the overall genetic diversity of our species over its whole geographical range and the evolutionary origin of this diversity. This strategy has been successfully applied to the study of pathogens (see, for example, Tibayrenc, 1995). Working within a comprehensive population genetics framework of the whole species in its entire ecogeographical range makes the research and study of relevant genes (those that are involved in the transmission and pathogenicity of infectious diseases) easier and more informative.

(ii) It is probable that genetic susceptibility to infectious diseases, like most phenotypic characteristics, generally involves multigene mechanisms rather than simple monogenic backgrounds.

(iii) Even if the whole sequence of all the genes involved were known, it would not necessarily be possible to understand the precise way they drive susceptibility to infectious diseases. Indeed, interaction between the many genes involved and between these genes and environmental factors could be very complex.

(iv) It is becoming apparent that major genomic rearrangements (macro-deletions and macro-duplications) play a major role in phenotypic expression (Check, 2005) in general (either normal or pathological) and in susceptibility to infectious diseases in particular. This has been verified in the case of HIV1/AIDS susceptibility, in which segmental duplications containing the gene *CCL3L1* could explain differences between people of different ancestry (African-Americans and Caucasian-Americans) (Gonzalez *et al.*, 2005).

(v) Gene-based susceptibility to infectious disease is manifested as a phenotype, and constitutes only a specific case of the general fact that very little is known about how genotypes lead to phenotypes (see below).

(vi) The overall functioning of the human genome has not yet been sufficiently grasped. This is currently true for any species. Indeed, in the present state-of-the-art, even knowing the entire sequence of a genome says little about the way this genome works to generate specific phenotypes. To illustrate this, the whole sequence of the human genome has now been completed (Craig Venter *et al.*, 2001) and the sequence of the chimpanzee genome is almost entirely known (Chimpanzee Sequencing and Analysis Consortium, 2005). However, the results of this huge effort are disappointing because the answer to why humans and chimps are phenotypically so different while their sequences are so similar remains a total mystery. Only 1.23% of base pairs are different between the two genomes. This includes all types of gene sequences, including coding sequences. The implications of this amazing result are clear: considerable and heritable phenotypic differences can be compatible with very minor sequence differences. This could be true for susceptibility to infectious disease and to the different clinical forms of a disease. Again, major genome rearrangements may play a considerable role in phenotypic differences between humans and chimpanzees.

(vii) When human genetic susceptibility to infectious disease is considered, the population level is as important, or more important, to consider than the individual level. Natural

selection acts on populations as well as, or more than, on individuals. Although the concept of race/ethnic diversity is controversial (Cho and Sankar, 2004), many scientists (see, for example, Jorde and Wooding, 2004) consider that it must be taken into account in biomedical research. As will become apparent, this is especially true in the case of transmissible diseases.

(viii) Geographic and climatic zones play a considerable role in the spread of infectious diseases, since they drive pathogen diversity and distribution of pathogen species. This effect may be exacerbated in the case of vector-borne diseases since geography and climate also have a strong impact on vector distribution. Naturally, geography and climate have had a profound impact on the genetic and genetically driven phenotypic diversity of our species.

(ix) The compound need to understand both the overall functioning of the whole genome and the relevance of population diversity is a significant challenge that must be met. Genomic analyses are time-consuming and costly. It is therefore necessary to keep a balance between using genomic analyses that have sufficient resolution and considering representative population samples. This is the very goal of the Human Genome Diversity Project (HGDP) and of the Hap Map project (see Sections 7.2 and 7.3, pp. 410 and 411), which is the nucleus of a new discipline: population genomics. A further step will consider gene expression as well, again at the double scale of the whole genome and representative population samples (population proteomics).

(x) Lastly, exploring the innate characteristics of the host (and the pathogen and, in vector-borne diseases, the vector) involved in the transmission and severity of infectious diseases makes it indispensable to elucidate the role played by the environment. It is generally considerable, and socioeconomic factors are a leading cause of epidemics. The spread of malaria, tuberculosis and AIDS occurs for many reasons other than genetics. They have not been named ‘the three diseases of poverty’ for nothing. However, genetics does matter. One has to evaluate

the respective impact of the innate and the environmental, not an easy task, since environmental causes may mimic genetic causes, and they interact a great deal.

The ten concepts summarized above lead to drastically different approaches from the classical approach relying on the search for a few genes at the individual level.

2. EXPLORING THE GENETIC BACKGROUND OF HUMAN GENETIC SUSCEPTIBILITY TO INFECTIOUS DISEASE

2.1. Classical Methodology

The basic working hypothesis underlying this research is that there are differences among individuals in terms of susceptibility to infectious disease, and that these differences have a genetic base. This hypothesis is explored by analysing the 'sibling (or sib) risk' (Δs): the statistical risk of one sibling exhibiting a given pathology when the other sibling does. In the case of infectious diseases, Δs is not inconsiderable, although lower than in the case of autoimmune diseases such as type I diabetes (Cooke and Hill, 2001). This is evidence for a genetic component (heritability) of susceptibility to infectious disease. Progress in genetics has provided efficient tools to look for the genes potentially involved in the susceptibility to infectious disease. Two main approaches can be classically distinguished: candidate genes and linkage studies.

In the candidate gene approach, the working hypothesis postulates that a given gene or a given set of genes is involved in susceptibility to a given disease. For example, a given gene or family of genes can be suspected because it is associated with biological processes that are usually involved in infectious processes. This is the case for those genes that are involved in immunological mechanisms. The genes that code for cytokine molecules are typical candidate genes. The same is true for the genes that pertain to the major histocompatibility complex (MHC), which are referred to as human leucocyte antigens (HLAs) in

the human species. These families of genes are therefore frequently studied when the putative genetic background of a given pathological disorder is explored. Another possibility is that the equivalent gene has been observed to play an important role in infectious processes in animal models. However, this lead could prove to be unrewarding: comparable gene sequences sometimes encode drastically different functions in different animal species (Blackwell, 1996).

When the linkage approach is considered, a specific region of the genome is identified through genome mapping with a broad set of microsatellite markers or other high-resolution markers such as single nucleotide polymorphisms (SNPs) and association analyses relying on twin sib pair/family/pedigree studies. Thanks to powerful new technologies, systematic screening is becoming easier and cheaper, and the number of microsatellite and SNP markers available is now high (see Section 7.3, p. 411). The exponential development of powerful bioinformatics tools is a great help in this kind of approach. The null hypothesis is a total lack of linkage disequilibrium, which is evidence for free recombination between the marker used and the pathological trait explored. If the recombination rate is significantly lower than 0.5, the linkage hypothesis is retained. This suggests that the region identified by the microsatellite mapping harbours genes that have something to do with the pathology considered. Even when such a region is delimited, one is far from the goal: such regions may contain hundreds of genes.

To look for this type of association, non-parametric tests have the advantage of not being dependent upon a working hypothesis involving genetic inheritance. However, they are less powerful than parametric tests. The more widely used parametric linkage test is the lod (logarithm of the odds) score (Morton, 1955):

$$\text{lod} = \log_{10}\left(\frac{\text{probability of data if disease and marker are linked}}{\text{probability of data if disease and marker recombine freely}}\right)$$

2.2. A Relatively Modest Harvest

A genetic component for susceptibility to infectious diseases has been postulated for many different diseases, including HIV (Dean *et al.*,

1996), tuberculosis (Alric *et al.*, 1999; Bellamy *et al.*, 2000), leprosy (Abel *et al.*, 1998), schistosomiasis (Dessein *et al.*, 1999), visceral leishmaniasis (Bucheton *et al.*, 2003) and malaria (Garcia *et al.*, 1998). Epidemiological surveys have provided evidence of familial aggregation of clinical phenotypes that are consistent with the existence of genetic factors involved in susceptibility to cutaneous leishmaniasis and visceral leishmaniasis in humans (Zijlstra *et al.*, 1994; Cabello *et al.*, 1995; Bucheton *et al.*, 2002). However, the links suggested in all the examples cited, although clear, are generally not very strong, and in many cases, the search for the genes that could be responsible for susceptibility to infectious diseases has been disappointing.

2.3. Problems Encountered

The gene identification methods described above are more successful when the following conditions are brought together: (i) when the pathological trait (phenotype) is clearly and precisely defined, which is the case, for example, for the different clinical forms of leprosy; (ii) when the genetic component of susceptibility is strong; (iii) when a limited number of genes is involved; and (iv) when precise hypotheses on the Mendelian inheritance of the involved genes are available. Needless to say, such favourable situations resemble planetary conjunctions and are probably the exception rather than the rule (Cooke and Hill, 2001). This may be why the search for susceptibility genes has been somewhat disappointing. The genetic control of infectious diseases (and of many other pathological processes) probably most often depends on a complex interaction between (i) many genes and (ii) these genes and environmental factors. Also, it increasingly appears that the genes themselves are not the only factor to be considered: the numbers of repeats and various rearrangements may play an important role (see Check, 2005). Lastly, as emphasized in Section 1 (p. 380), it is probably misleading to limit oneself to an approach exclusively based on individuals, as many studies investigating susceptibility to infectious diseases have done. Natural selection targets the population more than the individual. With infectious diseases,

interaction phenomena among members of a given group play a major role. The term 'herd immunity' (Anderson and May, 1990) refers to a group's overall level of immune defence. Let us consider the case where almost all members of a human population are vaccinated against a given infectious disease. The few individuals who are not vaccinated are protected by the other individuals because epidemics cannot spread within the group. Such group phenomena most probably play a major role in the evolution of genetic susceptibility to infectious diseases and are not sufficiently considered by the classical research on susceptibility genes. This is why it is so important to consider the population/ethnic level of analysis, because obviously different populations and ethnic groups have been subjected to different selective pressures and to different pathogens, transmitted by different vectors in the case of vector-borne diseases.

2.4. Chagas Disease: An Ideal Model on Paper for Integrated Genetic Epidemiology

Chagas disease (South American trypanosomosis) can illustrate the type of approach used in searching for genetic susceptibility to infectious diseases, and can reveal the limitations of some of these methods and explain the great hopes that are raised by the advent of powerful new technologies. This example is selected because I am especially familiar with it. However, I have chiefly studied it through the genetic variability of its causative agent, *Trypanosoma cruzi*, and to a lesser extent of its vectors, triatomine bugs. Let us also consider the third player, the host. As previously noted (Tibayrenc, 1998b), Chagas disease is a wonderful model for the study of co-evolution between pathogens, vectors and hosts, and the role played by the environment. It is therefore quite suitable for the integrated approach called for in Section 1. The three players—host, pathogen and vector—display impressive genetic diversity. Hosts and vectors involve many different species, one aspect of this diversity, so one of the players can vary while the other two are constant. This is all the more possible since Chagas disease is easy to maintain in complete experimental cycles. Chagas disease is widespread over vast geographic

areas and diversified ecosystems. Distributed from the southern United States to Argentina, Chagas disease exists in either sylvatic or domestic cycles.

Apart from the legendary *Escherichia coli*, *T. cruzi* (the parasite responsible for Chagas disease) is possibly the pathogen whose genetic variability has been the most thoroughly explored, both experimentally (Gaunt *et al.*, 2003) and by exhaustive analyses of the parasite's natural populations (for recent reviews, see Buscaglia and Di Noia, 2003; Tibayrenc, 2003; Campbell *et al.*, 2004). Both long-term clonal evolution (Tibayrenc *et al.*, 1986) and occasional bouts of genetic exchange (Gaunt *et al.*, 2003) have shaped *T. cruzi*'s genetic diversity. Although hybrid genotypes do exist and probably have a major epidemiological role (Campbell *et al.*, 2004), they have been stabilized by clonal propagation. One major outcome of all these studies is that *T. cruzi*'s natural multilocus genotypes are extremely stable on the scale of a human lifetime, and probably on an evolutionary scale. They therefore make easy-to-use units of analysis for all biomedical studies. Another major outcome is that the parasite's natural populations are subdivided into at least six clear-cut genetic subdivisions or discrete typing units (DTUs) separated from each other by great genetic distances (Tibayrenc, 1998a; Barnabé *et al.*, 2000). These DTUs are robust units of analysis for integrated genetic epidemiology. To make a long story short, although further active research in this field remains desirable, a very sharp and clear-cut picture of *T. cruzi*'s genetic diversity already exists, sufficient to construct an integrated genetic epidemiology of this disease (Tibayrenc, 1998b). The good news is that *T. cruzi* is extremely easy (although dangerous) to culture *in vitro*, which makes it excellent material for experiments.

As for the vector, the material for integrated approaches is also abundant. The vectors are 'true' bugs, heteropterous insects of the family Reduviidae, subfamily Triatominae. Three genera, *Triatoma*, *Rhodnius* and *Panstrongylus*, and dozens of species can transmit Chagas disease (Lent and Wygodzinsky, 1979). It is therefore easy to find a great deal of material to explore the role played by the vector in the transmission and severity of Chagas disease. Intra- and interspecific diversity of triatomine bugs has been widely explored by

morphometric analysis (Dujardin *et al.*, in press), population genetics based on molecular markers (Marcet *et al.*, 2006) and phylogenetic analysis (Bargues *et al.*, 2006). Chagas disease vectors are easy to use in experiments, since many species are easy to rear in the laboratory.

When mammalian hosts, and especially humans, are involved, the ideal situation existing on paper does not fulfil its promise. This is astonishing, since, as is the case for the pathogen and the vectors, this should provide a favourable scenario. The reservoir host of Chagas disease is very large, since all mammals, both domestic and wild, can be infected by *T. cruzi*. This gives a very broad range in which to compare the effects of the pathogen in many different host backgrounds. For exploring the role played by the host's genetics, many animal experimental models can be used. The most widely used are dogs (with their many breeds) and mice (with many different inbred lines). The mammalian hosts should therefore have been able to provide a rich harvest of putative candidate genes. This simply did not happen.

Considering the human host, Chagas disease affects highly diversified populations, of European, African, Amerindian and mixed ancestry, which should make it possible to explore in depth the role played by population/ethnic diversity (see below). Moreover, the clinical diversity of the disease provides many different clearly defined phenotypic categories (Gentillini, 1993), which could be explored by association studies. The disease starts with an acute phase, which can be either asymptomatic or symptomatic with a severe infectious syndrome. About 10% of the patients die at this stage. Survivors enter the indeterminate phase, in which the parasite hides in the host's cells, serological reactions (explored by ELISA and indirect immunofluorescence) are positive, and there are no symptoms. The majority of patients never exhibit any symptoms. Roughly 30% develop patent Chagas disease. In this last case, the main clinical forms are (i) cardiac (the majority of the cases, with severe cardiopathy leading to severe cardiac insufficiency); (ii) digestive (megacolon and megaoesophagus, approximately 3% of the cases); and (iii) cardiac and digestive. Some patients with symptoms have negative serological tests (Brenière *et al.*, 1984). Some patients have electrocardiographic abnormalities,

without clinical symptoms. Lastly, there are great differences in susceptibility to anti-chagasic drugs among patients (Toledo *et al*., 2003). There is therefore an abundant set of clearly defined clinical forms or phenotypic categories of Chagas disease. These different pathological phenotypes are not evenly distributed over the area of transmission. There are great differences among countries. In spite of these favourable conditions, knowledge of the possible role of human genetic diversity in the transmission of the disease and of its clinical forms is still in its infancy. Considerably more is known about leishmaniosis (Bucheton *et al*., 2003). Even the very cause of chronic Chagas disease remains under debate. It has been proposed that it could be a pure autoimmune disease (Kierzenbaum, 1985). However, it is now considered that the parasite is still present in the host's cells even in the chronic phase of the disease and causes a chronic inflammatory response. This is demonstrated by both PCR studies and classical xenodiagnosis. Obviously, in the search for genes of susceptibility, this debate is highly relevant.

A Brazilian study suggested a probable familial component in Chagas cardiopathy (Zicker *et al*., 1990). However, this was not confirmed in an Argentinian study (Morini *et al*., 1994). Many studies have looked for the possible role of HLA polymorphism in Chagas disease. Some of them reported significant associations (Fernandes-Mestre *et al*., 1998) while others did not (Fae *et al*., 2000). Large extended pedigrees have made it possible to demonstrate clear heritability of some serological characteristics. The heritability of *T. cruzi* seropositivity in Brazil is 0.556, which is high (Williams-Blangero *et al*., 1997), while the levels of immunoglobulins A and G in Brazil also have a heritability of 0.33 (Barbossa *et al*., 1981). These results fit personal observations made in some areas of Bolivia, where 100% of the triatomine bugs collected (all *Triatoma infestans*) were infected with *T. cruzi*, while 50% of the children were still seronegative at the age of 10 years. The presence of vectors in all houses was massive, and surely all the children had been bitten hundreds or thousands of times since they were born. However, only some of them had become infected.

These are the only robust results gathered in the search for genetic components of susceptibility to Chagas disease. To my knowledge, a

specific ethnic component has not been sought (see below). However, such an investigation could be informative. Some populations have lived for a very long time in areas that are free of Chagas disease. This is the case for the Aymara Indians of the Bolivian altiplano, which is at an altitude of 4000 m, while triatomine bugs are not found above an altitude of about 3500 m. Consequently, the Aymaras may have not been exposed to Chagas disease for thousands of years. It is only very recently (in the 20th century) that they have started colonizing lowland areas for economic reasons.

Our knowledge of human genetic susceptibility to Chagas disease and to its clinical forms therefore remains quite limited. The candidate gene approach (HLA polymorphism) has proved to be disappointing, as for many other diseases. Specialists in this field see its future in the use of extended pedigrees and genome-wide scans (see Section 7.3, p. 411) rather than in the search for candidate genes (Williams-Blangero *et al*., 2003). I agree, but I consider that study of the pathogen and vector components is indispensable, using the integrated approach called for above.

3. HUMAN DIVERSITY REVEALED BY NEUTRAL/HISTORICAL GENETIC MARKERS

As noted above, rather than studying genes responsible for susceptibility (or those assumed to be so) in an isolated manner (the classical approach, see above), it is wiser first to build a general population genetics framework of the species under consideration (Tibayrenc, 2004). Neutral genes and neutral polymorphisms are the best choice for designing this overall picture because they are considered to be passive markers of the time elapsed. In other words, the extent of differences in neutral genes between two populations or species are considered to be proportional to the time elapsed since these two populations or species shared common ancestors (historical markers).

By definition, a neutral polymorphism is not influenced by natural selection. Although it is extremely difficult to strictly verify this assertion, many genetic traits are classically considered as neutral. For

example, in the genetic code, synonymous mutations have no effect on the amino acid encoded. Non-coding sequences (introns, spacer DNA sequences) are considered neutral. This is supported by the fact that their molecular clock is generally faster than the molecular clock of coding sequences, which are prone to natural selective pressure. The use of genes that are highly selected for the study of population genetics could lead to very misleading results (see the discussion of HLA alleles in primates, Section 6, pp. 406).

Another requirement for establishing a reliable population genetic framework of any species is to use a sufficiently large set of genes. Sufficient does not mean thousands. It is interesting to note that the phylogenetic tree shown in Figure 1 was designed after study of the polymorphism of only 29 different genes. It gives the same general picture as the trees shown in Figure 2 (Plate 4.2 in the Colour Plate Section), based on a much broader range of markers. However, the use of two or three genes only could be grossly misleading.

Two factors could lower the correlation between how long two populations have been separated and the genetic distances by which they differ: (i) when the populations are not strictly separated and exchange migrants (the case for human populations), the genetic distances between them are underestimated, since migrants tend to homogenize the gene pools of the populations compared; and (ii) when founder effects interfere, which increases genetic distances. Absolute datings ('these populations split apart 123 351 years ago on a Friday morning') should therefore be considered with caution, which is not always done. Relative datings are more reliable. In Figure 1, it can safely be inferred that Iranians and Europeans shared more recent common ancestors than did Africans and Chinese.

By its nature, neutral polymorphism has a limited predictive power on adaptive polymorphism (see also Section 4). On the other hand, it permits construction of a general population genetic framework for the species under study, reveals the degree of genetic similarity between populations and their rates of migration and genetic exchange, and provides much other relevant information. As noted earlier, it is therefore convenient to have this general picture drawn before studying specific genes (for example, in the human species, those genes that are specifically involved in the transmission and severity of infectious

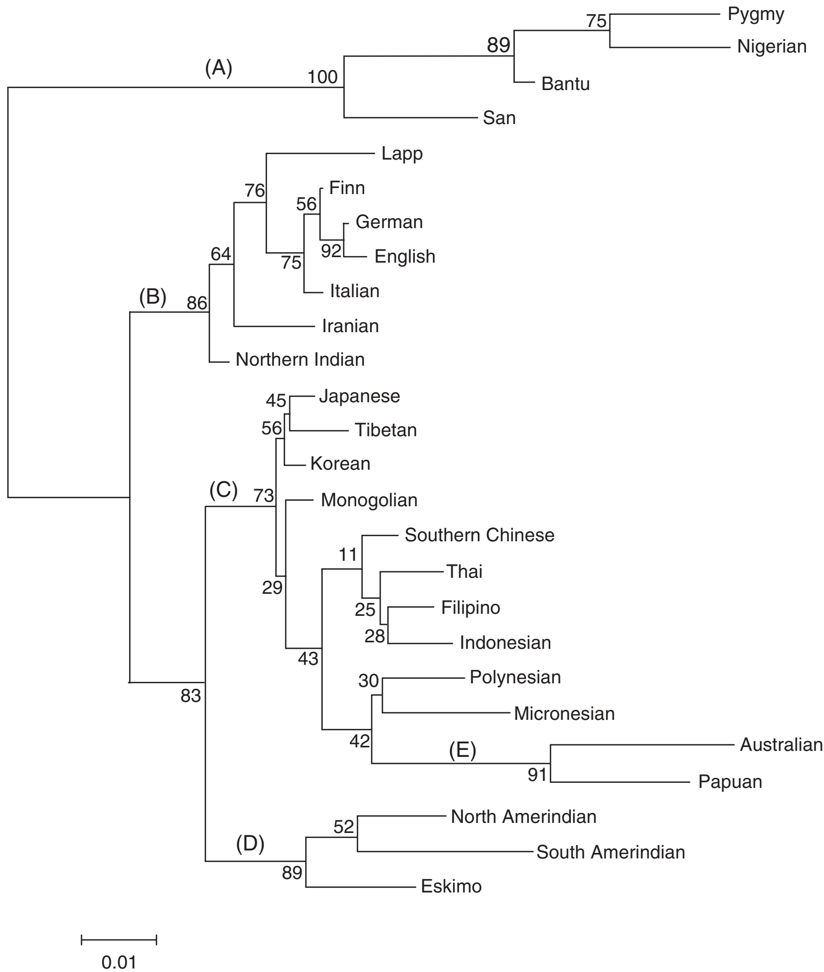


Figure 1 Phylogenetic tree of the main human ethnic groups designed from isoenzyme markers (after Nei and Roychoudhury, 1993). Subdivisions match the ethnic groups classically distinguished by physical anthropology (Valois, 1971) and are supported by strong bootstrap values, although they do not correspond to phylogenetic subdivisions. Scale bar indicates genetic distance (Nei, 1972).

diseases). Specific markers, isoenzymes, have played a major role in elucidating the population structure of humans and a countless number of other organisms, including plants, animals and pathogenic agents.

3.1. The Isoenzyme Revolution

In the late 1970s, a revolution occurred in population genetics. Until then, the theoretical basis of this discipline had remained very speculative, because direct analysis of genetic polymorphism was not available. The isoenzyme technique provided a direct measure of genetic polymorphism for the first time. Admittedly, this polymorphism dealt with a special category of genes, those that code for enzymes. However, provided that a sufficient number of enzyme loci were considered (multilocus enzyme electrophoresis or MLEE), it was thought that the polymorphism revealed was representative of the entire genetic variability of the organism. Our group's long experience shows that this is a very robust assertion. When pathogens are considered, population genetics and phylogenetic analyses designed after MLEE studies fit those relying on more sophisticated molecular markers extremely well (Tibayrenc, 1995; Tibayrenc and Ayala, 2002).

Isoenzymes were very fashionable in the 1970s and 1980s. An incredible number of papers on nearly the entire range of living organisms was published. Indeed, MLEE is the perfect example of a generalist genetic marker, i.e., a marker usable for any kind of organism (Tibayrenc, 1998a). This wide use of isoenzymes for a number of species, including organisms that can be crossed in the laboratory such as fruit flies, makes the Mendelian inheritance of this polymorphism fully understood. It is considered neutral and therefore is a time marker.

MLEE has been widely applied to the analysis of human genetic variability (Nei and Roychoudhury, 1993) and has made it possible to describe what is now considered to be a classical pattern of this variability (see Section 3.2 below).

3.2. A Classical Pattern of Human Genetic Variability

MLEE made it possible to gather the main results, which were confirmed and refined later by more modern technologies such as microsatellites, *Alu* insertion sequences, or SNPs.

(i) When historical markers are used, the main human ethnic groups are separated by genetic distances that are comparable

to the distances observed between local populations of *Drosophila*. Moreover, approximately 85% of human diversity is found within populations (for example, Europeans) and only 15% is due to differences between populations (for example, between Europeans and Africans) (Nei and Roychoudhury, 1974, 1993; Nei, 1978). Differences between populations can be measured by the F_{ST} component of the F statistics (Box 1).

(ii) However, when phylogenetic trees are designed with MLEE and other, more modern markers, they reflect the ethnic subdivisions classically used in physical anthropology (Valois, 1971) quite well (Figures 1 and 2 [Plate 4.2]). It is interesting to note that the main subdivisions seen on this tree are supported by high bootstrap values. This results from the presence of certain alleles that have much higher frequencies in some ethnic groups than in others (domestic alleles). This property (the presence of markers that are specific to a given group, or at least have a much higher frequency in that group) has been widely used with more modern techniques to elaborate sets of markers capable of identifying ethnic groups (ancestry informative markers or AIMs) (Pfaff *et al.*, 2001). DNAprint Genomics markets kits that can identify the ancestry of any individual and even his or her

Box 1 F-statistics

A method was proposed by Wright (1951) for describing genetic population structure with three F-statistics, F_{IS}, F_{IT} and F_{ST}, whose relationships are $(1–F_{IT}) = (1– F_{ST})(1– F_{IS})$, where F_{IS} can be defined as the correlation between homologous alleles in individuals in the local population and F_{IT} as the allelic correlation in the total population. They are also called fixation indices (F_I) and describe departures from Hardy–Weinberg expectations within local populations vs. the total population: $F_I = (h_o/h_e)$, where h_o is observed heterozygosity and h_e is expected heterozygosity. $F_I = 0$ corresponds to Hardy–Weinberg equilibrium; $F_I > 0$ indicates a deficit of heterozygotes, due to either inbreeding ($F_{IS} > 0$) or inbreeding and population subdivision ($F_{IT} > 0$).

F_{ST} is a commonly used measure of population subdivision (if $F_{ST} = 0$, $F_{IT} = F_{IS}$) and can be interpreted as the variance of allelic frequencies among populations.

degree of admixture with a high rate of reliability (http://www.dnaprint.com/welcome/home/index.php). AIMs have been used in forensic medicine and are also used to compare the genetic susceptibility of different ethnic groups to given pathological traits (see Section 5, p. 400).

The high bootstrap values observed in the tree in Figure 1 show that a very cautious interpretation of the results of these statistics is indispensable. Many scientists consider that high bootstrap indices are a strong indication of the robustness of phylogenetic lines. The subdivisions shown in Figures 1 and 2 are by no means phylogenetic lines, but only geographical populations of the same species that have been isolated by geographical distance for a relatively short time: it is considered that all present human populations shared common ancestors in Africa roughly 100 000 years ago (Solignac, 1998). This is the 'Out of Africa' theory, which is considered the most parsimonious one by most geneticists (Torroni *et al*., 2006).

It is remarkable that analyses based on a wide range of modern molecular markers have fully confirmed the results obtained by the pioneering MLEE studies of the 1970s (see Figure 1) (i) by showing that most of the genetic diversity observed is within rather than between populations and (ii) by clearly identifying the ethnic groups described by classical anthropology (Valois, 1971) (Figure 2 [Plate 4.2]).

4. GENETICALLY DRIVEN PHENOTYPIC DIVERSITY

4.1. The Major Gap between Genotype and Phenotype

It is hard for non-specialists to believe, but knowledge in genetics at the dawn of the 21st century is both immense and in its infancy. Anyone looking at a photograph of the author of this review (see http://www.th.ird.fr) will note that he does not have a thick head of hair. Now, nobody, not even the finest geneticist, knows the first thing about the genetic background of balding. This is true for most phenotypic traits that give rise to the appearance of human beings (and other living creatures): the shape of the nose, the eyes, the colour of the skin, size, weight, etc. We have only hypotheses, we know that

balding has something to do with gender, since women are rarely affected. Testosterone obviously plays a role, but many very masculine men do not become bald, and the contrary is true for many less masculine men. To make a long story short, we do not know anything about the genetic mechanisms of balding. However, this characteristic has an obvious familial and ethnic component, which suggests that it is genetically driven. The same holds true for those characteristics that are not visually apparent: blood pressure, cardiac rhythm, number of red blood cells, etc., which obviously have strong implications for survival and fitness and whose genetic background is largely hypothetical. To me, this makes any theory on evolution presently very speculative in essence, since the target and basic material of natural selection is the phenotype, and the way it is driven by the genotype remains hypothetical.

4.2. Phenotypic Traits that have an Obvious Genetic Basis

Many morphological traits have strong heritability, even if they are also influenced by environmental factors. This is the case for size, body shape, skin colour, hair texture, etc. Everybody has been struck by the facial resemblance among members of the same family, which can skip generations. Three members of the Bourbon royal family of France, Louis XIV, Louis XV (his son) and Louis XVI (the unfortunate grandson who was guillotined) exhibited a striking facial resemblance through the famous Bourbon nose, which was apparently a dominant characteristic.

When phenotype is taken in a broad sense (any observable characteristic apart from the genome itself), this remains true. Most phenotypic traits have strong heritability, and this seems to be the case for susceptibility to infectious diseases (see Section 2, p. 384).

It is interesting to note that genetically driven phenotypic variability among populations can be much higher than the genetic differences among them as measured by neutral/historical markers. When the F_{ST} component (see Box 1) of these historical markers is as low as 0.15, the same component measured for skin colour among

human populations ranges from 0.6 to 0.9 (Harpending, 2002). It is true also for face morphology: the degree of variability between different human populations is far higher than the same character for two different ape species, chimpanzee (*Pan troglodytes*) and bonobo (*Pan paniscus*) (Sarich and Miele, 2004). From these facts it can be said that genetically driven phenotypic differences among populations and ethnic groups are far stronger than the genetic differences revealed by historical markers (Figures 1 and 2), although they roughly follow the same distribution pattern, which corresponds to the ethnic groups classically described. Susceptibility to infectious diseases is also a phenotype that has a certain genetic component, as demonstrated by heritability studies and the sib risk (Section 2.1, p. 384) (Cooke and Hill, 2001).

The working hypothesis that comes to mind is that the genetic background of this phenotype (susceptibility to infectious diseases) may also follow, to some extent, the pattern recorded for morphological phenotypes. In other words, this character, explored in the present review, has some significant connection with populations and ethnic groups. For this reason, it is useful to discuss below the scientific relevance of the concepts of race and ethnic group, the object of intense current debate (Wolf, 2005).

4.3. The Probable Major Role of Genomic Rearrangements in Phenotypic Expression

Possibly a major revolution in genetics is taking place today, comparable to the discoveries of exons, introns and transposons in the 1980s and, even, the genetic code in the 1950s. The Human Genome Project (HGP) (see Section 7.1, p. 407) and all sequencing projects are more or less founded on the hope that deciphering the entire DNA sequence of a given organism will provide a nearly complete understanding of how it works. Scientists may have promised too much here. Countless times I have read that the sequencing of *Plasmodium falciparum* will make it possible to eradicate malaria. Now we are possibly discovering that the sequence itself is not that important, or at least is not the only character to take into account. It is important to underline that, when one

considers only the sequence, humans are strikingly alike. Two randomly selected individuals have an identical sequence for 99.9% of their genome (Feuk *et al*., 2006). It is unexpected that these tiny sequence differences would account for genetically driven phenotypic differences among individuals and populations.

The unexpected discovery of the last few years is that genomic rearrangements are highly variable among individuals and populations. By comparing individual genomes, it has been found that, in some individuals, large segments are duplicated, while in other individuals portions are lacking or are inverted. This polymorphism, which has been called structural variation, is seen in individuals with no pathology. It is also referred to as large-scale copy-number variation (LCV) (Lafrate *et al*., 2004) or large-scale copy-number polymorphism (Sebat *et al*., 2004). The term 'copy-number variant' refers to either LCVs or smaller DNA segments that exist in variable copy numbers among individuals. LCV involves the gain or loss of DNA segments that can be as long as several hundred kilobases. Lafrate *et al*. (2004) have found an average of 12.4 LCVs per individual in 39 healthy individuals taken as controls and 16 individuals with previously identified chromosomal imbalances. The LCVs were randomly distributed throughout the genome. Many contained complete genes, which shows that they are not restricted to introns or intergenic regions. The number of CNVs of 50 kb or more is estimated at 100 per individual. There is also a significant number of intermediate-sized CNVs (8–40 kb) and many smaller structural variants (<8 kb) (Feuk *et al*., 2006). It is important to underline that this copy-number variation is seen in non-pathological individuals.

My belief is that these discoveries mark the entry of a new era in the field of genetics and will revolutionize our views on the links between genotype and phenotype, both at the individual and the population levels.

Studies are just beginning, and it is too early to know the relevance of LCVs and CNVs on the expression of normal vs. pathological phenotypes. Some data suggest that structural polymorphism might play a role in determining fitness, with strong implications for natural selection and evolution. A particular 900 kb inversion variation in European individuals is an example. This polymorphism seems to be positively selected in

the population of Iceland. Carrier women are statistically more fertile (Stefansson *et al*., 2005). Gene enrichment as a consequence of structural polymorphism could also be involved in general defence responses, including resistance to bacteria (Feuk *et al*., 2006). It is important to explore to what extent the frequency of these LCVs differs among populations and ethnic groups (see Section 5.1, p. 400). This is still poorly known. However, the case of segmental duplications containing the gene *CCL3L1* shows that (i) this kind of polymorphism may have drastic phenotypic effects (here resistance to a major transmissible disease) and (ii) may be differently distributed among different human populations (see Section 5.2, p. 403) (Gonzalez *et al*., 2005). Moreover, a well-documented study investigating 71 ethnically diversified individuals (23 African-Americans, 24 European-Americans and 24 Han Chinese) and 100 deletion polymorphisms has shown that African-Americans showed more diversity for this kind of polymorphism, as is the case for SNP polymorphism (Hinds *et al*., 2006).

Structural variation rather than sequence variation is possibly responsible for the important phenotypic differences between humans and apes. As we have seen (Section 1.2 (iv), p. 382), the sequences of our genome and the chimpanzee's genome are extremely similar (Chimpanzee Sequencing and Analysis Consortium, 2005). Recently, no fewer than 651 structural rearrangement differences have been described between us and our closest cousins (Newman *et al*., 2005). The genes contained in these structural rearrangements could play a major role in the expression of different phenotypes between humans and apes.

The importance of this discovery has led to setting up a specialized database aiming to put all the information relevant to genomic variants in a single place (see Section 7.4, p. 412).

5. ARE RACES AND ETHNIC GROUPS BIOLOGICALLY MEANINGFUL AND MEDICALLY RELEVANT?

5.1. The Biological Nature of Ethnic Groups/Races

The biological relevance of races and ethnic groups is a highly ideologically loaded debate. Since most Western societies are now

multiracial communities, the political implications of the debate are weighty, and it is hard to consider human populations with the same cold, objective eye as populations of newts or carabic beetles. If we try to do so, human ethnic groups fit rather well into the classical definition of subspecies or geographical races. As we have said above, population trees designed with historical markers closely follow classically identified ethnic groups (Figures 1 and 2), which supports the view that human ethnic groups can be considered as phenotypically differentiated geographical populations of the human species. Moreover, there are differences between ethnic groups at the level of structural genomics. The genome appears to be composed of linkage disequilibrium blocks separated by 'hot-spot' recombination blocks. The level of linkage disequilibrium is lower in Africans than in other populations, which suggests that non-African populations are subsets of Africans (Cavalli-Sforza and Feldman, 2003).

Phenotypic differentiation among ethnic groups, particularly skin colour, is probably adaptive to environmental factors (mostly climatic), although some authors (Harpending, 2002) postulate that they are mainly the result of sexual selection. Anybody having experienced long-term stays in tropical or equatorial areas can testify that people of African origin are better adapted to such climates than Europeans. French Guiana has not been called the 'white people's grave' for nothing. Indian populations of the Bolivian Altiplano are very well adapted to high altitudes (the Altiplano is 4000 m above sea level). Studies at the French–Bolivian Institute of High-Altitude Biology (IBBA) (http://saludpublica.bvsp.org.bo/ibba/) have shown that the original ethnic group of the Altiplano, the Aymara Indians, are the best adapted. They have a powerful respiratory capacity (barrel-like chest) and make red blood cells very easily. The Quechuas, who are more recent immigrants (they are the descendants of the Incas and came to the Altiplano area in the 15th century), are less well adapted. Bolivians of direct Spanish (or other European) ancestry are even more poorly adapted. Certain individuals suffer from certain pathologies (polyglobulia) even if they were born in La Paz, and have to live in lowland areas. Crossbred people are half-way between Indians and Caucasians in altitude adaptation. The least well adapted are of course the 'gringos', the Caucasians who were born

outside the country. It is remarkable that the Aymara became so well adapted in only a few thousand years. This illustrates the very strong phenotypic adaptability of our species to environmental characteristics. It is logical to predict that this property involves genetic susceptibility to infectious diseases as well (see below). If the Aymaras are well adapted to altitude, the other side of the coin is that they do not enjoy living in lowland areas. However, many are obliged to for economical reasons. Experience at the IBBA has shown that they had greater susceptibility to transmissible diseases than native inhabitants of these lowland regions.

As for the biological validity of ethnic groups, it can be noted that in the special issue of *Nature Genetics* published on this topic in 2004 (Cho and Sankar, 2004; Jorde and Wooding, 2004; Keita *et al.*, 2004; Rotini, 2004; Tishkoff and Kidd, 2004), the opinions of the different contributors were quite disparate. This shows that there is no consensus among specialists on this point. To a large extent, it is a war of words and a battle of definitions. In any case, it can be noted that a phylogenetic definition of subspecies or geographical race is not logical for any species, since by definition subspecies do not constitute distinct clades. They are still capable of interbreeding. It is therefore not unexpected that the existence of different human ethnic groups is not fully corroborated by strict phylogenetic criteria (Keita *et al.*, 2004). This would be the case for any geographical race /subspecies in any species. Today the validity of the concept of subspecies/geographical race is debated by many, not only for our own species. This is really a matter of appreciation. Any reasonably trained naturalist knows that subspecies do exist. They result from recurrent, obvious observations and delight collectors of carabic or cetonic beetles. It is interesting to note that while many authors reject the biological validity of human ethnic subdivisions (Cho and Sankar, 2004) and do not accept their status as geographical races, other species receive totally different treatment with rather comparable genetic data. For very long, the zebu, which is completely interfertile when crossed with the European ox, has been considered a distinct species (*Bos indicus*). It is only recently that it has lost that status. It is nevertheless considered a full subspecies (*Bos taurus indicus*). The polar bear is considered a fully distinct species (*Ursus maritimus*), different from the brown bear (*Ursus arctos*). However, the two 'species' produce fertile

offspring, and are genetically very close, according to both nuclear and mitochondrial genes (Yu *et al.*, 2004; Fulton and Strobeck, 2006). When phylogenies are designed using mitochondrial genes, polar bears appear as a subset of brown bears. Brown bears from Alaska are genetically more closely related to polar bears than to other geographical populations of brown bears (Avise, 2004).

This debate on the validity of the subspecies concept is a never-ending story (as is the current debate on the species concept; see Avise, 2004) and could fill an entire treatise. Apart from this debate, for the purposes of this article, the important point to be explored is the medical relevance of population/ethnic diversity in the specific case of transmissible diseases.

5.2. Population and Ethnic Diversity with Regard to Transmission and Severity of Infectious Diseases

The selective pressure caused by infectious diseases exhibits considerable differences among geographical locations, ecological areas, climatic zones and continents. Pathogens are not equally distributed; for example, malaria exerts its selective pressure mainly in tropical areas and almost totally spares northern countries. As noted in Section 1 (p. 380) this should be even more closely examined in the case of vector-borne diseases, since geography and climate strongly drive vector distribution as well, and in most instances there is high vectorial specificity for any given pathogen. For example, human African trypanosomiasis (HAT; formerly called sleeping sickness) is strictly transmitted by tsetse flies. These Diptera of the genus *Glossina* are found only in subtropical Africa. The selective pressure of HAT is therefore limited to that part of the world. Some authors have noted that the northern limit of tsetse fly distribution roughly follows the southern limit of the penetration of Islam in Africa; perhaps Arab invaders were stopped by HAT.

Different human populations would therefore be expected to exhibit different patterns of susceptibility/resistance to different infectious diseases. Moreover, the way our genome has been shaped by the selective pressure exerted by transmissible diseases would also be expected to be different in different ethnic groups. The data presented below support these expectations.

It is a classical notion that native Amerindian populations were decimated by the pathogens brought by Europeans. Tuberculosis is a classical example (Poulet, 1994). This concept has even been put to practical use in the first attempt at biological warfare by the British army in Canada during the French–British wars. Clothes of patients who had died from smallpox were given to the Indians who had collaborated with the French army. The Indians proved to be extremely susceptible to the smallpox virus.

The differences in AIDS epidemiology between Africa on the one hand, and North America and western Europe on the other, have obvious socioeconomic, environmental and behavioural explanations. However, it is now clear that genetic factors also have an effect (Dean *et al*., 1996; Samson *et al*., 1996), which makes Africans more susceptible to AIDS than Europeans. The proposed explanation is allelic frequency differences for a deletion in the CCR-5 chemokine receptor gene. This deletion, which seems to have a protective role, is much more frequent in Caucasians than in Africans. At the genomic level, as already noted, segmental duplications containing the gene *CCL3L1* are able to explain differences between people of different ethnic backgrounds (African-Americans and European-Americans) (Gonzalez *et al*., 2005).

Another viral disease, hepatitis C, also has an unequal distribution of genetic susceptibility among ethnic groups (Thio *et al*., 2001). Furthermore, a parasitic disease, visceral leishmaniosis, shows strong ethnic differences in the ratio of asymptomatic to symptomatic infections (Ibrahim *et al*., 1999; Bucheton *et al*., 2002).

Turning to the impact of infectious diseases on our genome's structure, a classical example of differential ethnic impact is the case of malaria in Africans and African-Americans (Alison, 1954; Labie, 1994; Miller, 1994). Balanced polymorphism, due to the selective pressure of malaria, has been fairly well demonstrated for haemoglobin S (sickle cell anaemia). The homozygous genotype S/S is 100% lethal in Africa, whereas the heterozygous individuals (S/A) have a selective advantage compared with children (A/A) who do not have the mutant allele genotype. Heterozygous individuals (S/A) are resistant to *P. falciparum*, the causative agent of the most malignant form of malaria. This persistence of a deleterious gene due to selective pressure exerted by a disease is a case of balanced polymorphism.

Another illustrative case of balanced polymorphism is Melanesian ovalocytosis (Tanner *et al*., 1991), which is caused by a mutation in erythrocyte band 3. The existence of the balanced polymorphisms involved in sickle cell anaemia and Melanesian ovalocytosis suggests a high mortality rate from malaria in Papua New Guinea and Africa. Interestingly, the trait persists in African-Americans, although they have been spared the selective pressure of *P. falciparum* malaria for more than one century.

Although their genetic relationships with malaria are less clear, it has been proposed (Miller, 1994) that the high frequency of other pathological traits in African-Americans and Africans are attributable to a selective advantage they confer in malaria-endemic areas. This could be the case for high blood pressure and iron overload with cirrhosis. It is worth noting that although African-Americans have undergone great genetic admixture from Caucasian populations, they still exhibit very specific genetic polymorphisms linked to malaria selective pressure. Statistical genetic differences between African-Americans and Caucasian-Americans are also reflected in their HLA polymorphism (see Section 6, p. 406), which is considered to be caused by the selective pressure of infectious diseases.

It is probable that major transmissible diseases other than malaria have played and still play a major selective role in sub-Saharan Africa. HAT and schistosomiasis are possible examples. Today, the HIV epidemic is most probably is having a drastic selective impact on African populations.

The few examples cited above support the view that ethnic diversity is a character to be taken into account in the study of the relationships between human genetic diversity and infectious diseases. Differences are not limited to the broad groups previously referred to as *grand'races* (Valois, 1971) (Caucasians, Africans, Asians) and seem to act also, if not more, at a finer level. This is conceivable since different selective pressures caused by pathogens and vectors may act at very subtle eco-geographical levels. An illustration is the differential susceptibility to malaria among West African ethnic groups. The Fulani ethnic group exhibits a high level of resistance to malaria compared to other groups, although all live in the same area (Modiano *et al*., 1999).

Differences between ethnic groups, even at a subtle level, are important to take into account for research, surveillance and control of

infectious diseases, and easier identification of susceptible individuals. Tools are available to survey these differences even in the case of populations that have undergone admixture (Tang, 2006). Ancestry informative markers (Pfaff *et al.*, 2001) are able to detect the degree of admixture in individuals and populations with a high degree of reliability. For this reason, they are used in forensic medicine.

5.3. Ethnic Diversity: A Gold Mine for Medical Research?

My conviction is that the ethnic diversity of our species, the result of long-term strong selective pressure in drastically different environments, will prove to be an immense source of discoveries in the field of genetic susceptibility to infectious diseases, their clinical forms and the drugs and vaccines used to combat them. This could be true for many diseases, not only transmissible diseases (Tang, 2006). In this perspective, some recent proposals (Wolf, 2005) aiming to outlaw the making of any scientific reference to the concept of race, are most uncalled for. Racism is highly reprehensible. Scientific debates on the biological nature and medical interest of ethnic groups, are not.

Lastly, one must keep in mind that in the same population there can be considerable individual differences in genetic susceptibility to infectious diseases (Abel and Dessein, 1997). Both population and individual levels must therefore be considered in attempts to study and control infectious diseases.

6. OUR GENETIC INHERITANCE HAS BEEN SCULPTED BY INFECTIOUS DISEASES

Haldane (1949) was the first to propose that infectious diseases have been the most drastic selective pressure acting on the evolution of the human species over the last 5000 years. This selective pressure would have been exerted mainly by tuberculosis in Europe and malaria in Africa (Miller, 1994). It is certain that massive epidemics such as the black plague, or more recently Spanish influenza, which killed dozens of millions of individuals, had drastic demographic effects. But endemic

diseases such as tuberculosis and malaria, which act continuously over thousands of years, probably have a stronger selective effect.

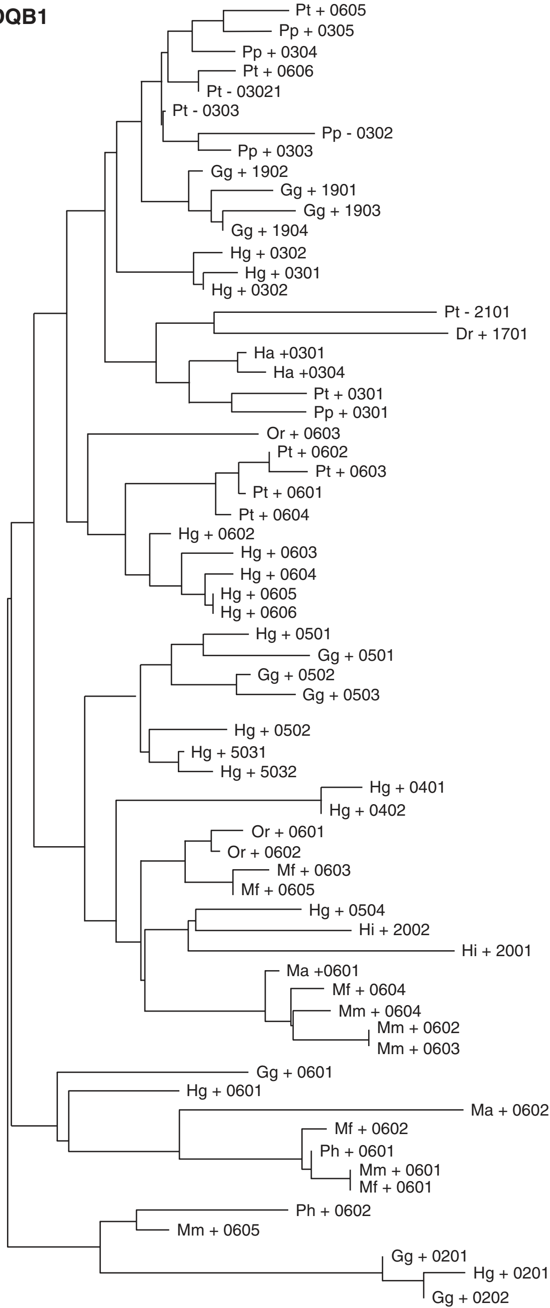
In fact, it is probable that the selective impact of infectious diseases has been operating for much longer than 5000 years and has acted through the whole evolutionary process of our species. However, epidemics spread more easily in large human populations grouped in large cities than in the scattered small populations which existed before the agricultural era. Selective pressure has therefore probably been stronger since the Neolithic era.

A solid indication of the impact of infectious diseases on human evolution is the very strange molecular evolution pattern of the HLA system. Some HLA human alleles have a very ancient coalescence time, before the phylogenetic divergence among Catarrhinians (Old World monkeys), and are genetically more closely related to some *Macaca* alleles than to other human HLA alleles (see Figure 3). The explanation suggested by Ayala and Escalante (1996) is balanced selection by infectious agents, mainly malaria. Logically, HLA polymorphism is very different between different human populations, for example between African-Americans and Americans of European ancestry, since the close ancestors of the first group underwent the selective pressure of malaria. This fact has practical medical consequences, since HLA typing in African-Americans lacked precision before the advent of sequence typing and led to a higher rate of organ transplant failures in this group. It is most probable that the HLA system is not the only part of our genome that has been drastically affected by the selective pressure exerted by transmissible diseases.

7. MAJOR INTERNATIONAL PROGRAMMES THAT WILL BOOST UNDERSTANDING OF HUMAN GENETIC DIVERSITY

7.1. The Human Genome Project

The HGP can be considered as the equivalent for molecular biology and genomics of the Apollo programme to conquer the moon. When the required technology (automatic gene sequencing) became


DQB1
Pt + 0605
Pp + 0305
Pp + 0304
Pt + 0606
Pt - 03021
Pt - 0303
Pp - 0302
Pp + 0303
Gg + 1902
Gg + 1901
Gg + 1903
Gg + 1904
Hg + 0302
Hg + 0301
Hg + 0302
Pt - 2101
Dr + 1701
Ha +0301
Ha +0304
Pt + 0301
Pp + 0301
Or + 0603
Pt + 0602
Pt + 0603
Pt + 0601
Pt + 0604
Hg + 0602
Hg + 0603
Hg + 0604
Hg + 0605
Hg + 0606
Hg + 0501
Gg + 0501
Gg + 0502
Gg + 0503
Hg + 0502
Hg + 5031
Hg + 5032
Hg + 0401
Hg + 0402
Or + 0601
Or + 0602
Mf + 0603
Mf + 0605
Hg + 0504
Hi + 2002
Hi + 2001
Ma +0601
Mf + 0604
Mm + 0604
Mm + 0602
Mm + 0603
Gg + 0601
Hg + 0601
Ma + 0602
Mf + 0602
Ph + 0601
Mm + 0601
Mf + 0601
Ph + 0602
Mm + 0605
Gg + 0201
Hg + 0201
Gg + 0202

available, two rival programmes, one monitored by a private company (Celera Genomics; http://www.celera.com/) and one that relied on an international consortium of public institutes coordinated by the National Institutes of Health in Bethesda, Maryland, USA (http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml), entered a race lasting several years and published the entire sequence of the human genome almost neck and neck, although Celera Genomics had a small advantage in time (Craig Venter *et al*., 2001). The results were somewhat surprising, since our species, which is considered (probably with a high dose of anthropocentrism) as the most sophisticated product of evolution, possesses only roughly 25 000 genes. This is a hard lesson for those speculative scientists who have professed for decades, without the slightest evidence, the dogma of a minimum number of 100 000 genes for humans.

The HGP has proved to be an invaluable source of information and has already led to the discovery of many genes of interest involved in pathological processes (Gary, 2001). However, it is probable that knowing the whole sequence of the genome will not lead automatically to a thorough understanding of how genes drive the expression of phenotypes (see Section 1, p. 380). Inferring the contrary is possibly akin to thinking that knowledge of the alphabet makes it possible thoroughly to appreciate the genius of Shakespeare. Another limitation of the HGP is that it gives a dim idea of the entire picture of genetic diversity of our species, since only very few individuals, all of Caucasian origin, were used as donors. This is why the

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Figure 3 Phylogenetic tree based on 64 HLA (see the Glossary on p. 423) *DQB1* alleles (exon 2) of 10 species of primates: Hominoidea: Hs = *Homo sapiens*, Pp = *Pan paniscus* (bonobo), Pt = *Pan troglodytes* (chimpanzee), Gg = *Gorilla gorilla* (gorilla), Or = *Pongo pygmaeus* (orang-utang), Hl = *Hylobates lar* (gibbon); Cercopithecoidea: Ma = *Macaca arctoides*, Mf = *Macaca fascicularis*, Mm = *Macaca mulatta* (rhesus macaque), Ph = *Papio hamadryas* (hamadryas baboon). The numbers after each species designation refer to the alleles. The tree was designed according to the neighbour-joining method (Saitou and Nei, 1987). Human alleles are scattered throughout the tree. Some of them appear to be phylogenetically closer to *Gorilla* or *Macaca* alleles than to other human alleles (after Ayala and Escalante, 1996).

two programmes discussed in Sections 7.2 and 7.3 (pp. 410, 411) were initiated.

7.2. The Human Genome Diversity Project

After the start of the HGP, it appeared that, with only one individual donor being used for each part of the genome to be sequenced, one important feature would be missed: the genetic polymorphism of our species. This was all the more worrying as the individuals selected for the HGP were all Caucasians. Today European populations exhibit strong genetic homogeneity, and much of the overall genetic variability of our species is found in African populations, at least when historical markers are considered (Solignac, 1998). It was soon proposed (Cavalli-Sforza *et al.*, 1991) that a complementary programme to the HGP be designed, surveying a representative sample of human genetic polymorphism through the analysis of diversified gene sequences. The project, named the Human Genome Diversity Project (HGDP; http://www.stanford.edu/group/morrinst/hgdp.html), focused on those human populations that had remained genetically isolated for a very long time ('native people'), since they are considered to convey more precise information than urban populations, which have been subject to much admixture. The project generated great opposition from many organizations representing indigenous peoples, mainly the World Council of Indigenous Peoples (WCIP) (Kahn, 1994a). The scientists involved in the HGDP maladroitly claimed that it was urgent to analyse the genetic diversity of indigenous people because some of them were threatened with extinction. Although the concern of these organizations about what they termed genetic colonialism was quite understandable, from a scientific and medical point of view having a clear idea of human gene sequence polymorphism remains highly desirable. While the HGDP was in trouble, other valuable initiatives were launched. An African-American Diversity Project has been designed (Kahn, 1994b). The specific HLA polymorphism seen in this group is one of its particular interests (see above). The European Union has launched a diversity programme in Europe through a network of laboratories stretching from

Barcelona to Budapest (Kahn, 1994a). Lastly, a Chinese HGDP has already yielded valuable results (Chu *et al.*, 1998). Fortunately, the HGDP has now been restarted on a happier basis (Cann *et al.*, 2002). A total of 1064 lymphoblastoid cell lines have been collected at the Jean Dausset Foundation in Paris (http://www.cephb.fr/), corresponding to 51 populations from all continents. These have already been genotyped with 404 microsatellite markers distributed throughout the genome. However, this sample, although it is already broad, is considered insufficient by some authors, who propose the survey of up to 10 000 individuals (Cavalli-Sforza and Feldman, 2003). Moreover, from the medical point of view, the information garnered will be drastically limited by the fact that the data on the samples concern only the gender, population and geographical origin of the subjects. For ethical reasons, no information will be issued on possible pathological traits. It will therefore be impossible to establish any links between given pathologies (including infectious diseases) and genetic polymorphisms. Limiting information derived from the data relating to the samples is ethically estimable. However, it is highly desirable to complement the HGDP with more specialized programmes, including thorough collection of relevant clinical and biomedical information. This will be beneficial not only for medical purposes, but also for basic understanding of human evolution, since a dominant characteristic is its long-term co-evolution with pathogens.

7.3. The Hap Map Project

'Hap Map' stands for International Human Haplotype Map Project (http://www.hapmap.org/). This huge programme is handled by an international consortium (International Hap Map Consortium, 2003) whose goal is to determine the genotypes of 10^6 or more sequence variants (SNPs), their frequencies and the degree of association between them. The goal was to make sure that one SNP would be assayed for every 5000 bases of sequence. This detailed mapping should provide a high-resolution tool to look for associations between the variants and many pathological traits. Four different populations have been studied: Africans (unfortunately represented by

only one ethnic group, the Yoruba from Nigeria), Han Chinese, Japanese and Caucasian-Americans. This sample, although better than the sample used for the HGP (which included only a few Caucasian individuals) is highly biased. Han Chinese and Japanese are ethnically very closely related and therefore should not be separately sampled. The genetic diversity of Africans, as already noted (Solignac, 1998), is considerable and will not be correctly represented by a single ethnic group. Lastly, many ethnic groups are lacking, including Australian natives, Amerindians, Indians, Middle-East populations and Pacific Islanders. The sample used for the HGDP is much better (see Section 7.2, p. 410). The use of the imperfect Hap Map sample will have limitations when looking for those diseases whose genetic background follows the so-called common disease/common variant (CDCV) hypothesis, in which the genetic factors involved are evenly distributed among populations and are 'relatively' frequent (about one in ten million). If the opposite hypothesis prevails (the multiple rare variant [MRV] hypothesis), the Hap Map project will lack resolution (Rotini, 2004). Unfortunately, available data do suggest that genetic traits related to susceptibility to infectious diseases are population-linked and therefore follow the MRV pattern. It can therefore be feared that the Hap Map project will be of limited help in this specific biomedical field of research. However, the first results of this programme have provided many researchers with a mine of valuable data and have contributed to the discovery that genomic rearrangements (either deletions or duplications) are much more common in our genome than expected, and could play a major role in phenotypic expression (Couzin, 2006).

7.4. Consortia, Databases, Networks and Networks of Networks

The blossoming of new, diversified technologies and the increasing number of teams surveying human genetic/genomic variation and its medical implications run the risk of leading to cacophony and anarchy. Fortunately, many international initiatives are being taken to try to coordinate these disparate efforts. The Internet makes it

possible to connect all the teams working worldwide on a given topic. The main goals of these many endeavours are to standardize methods and technological approaches, to avoid redundancies and to allow the dissemination of negative results, which are sometimes difficult to publish in the classical scientific literature. Among the many initiatives that have been born in recent years, a few illustrative examples are cited below.

As we have seen, the discovery of the importance of genomic rearrangements (copy-number variant, large-scale copy-number variation) has stimulated a great deal of research, and has shown that the polymorphism of the human genome does not limit itself to sequence and SNPs. The multi-entry, very user-friendly Database of Genomic Variants (http://www.projects.tcag.ca/variation/) collects all the relevant information in this field (Flintoft, 2005). It is constantly updated with new data obtained by the research teams that handle the database and from surveys of the published literature. It is possible to enter the database either by a gene of interest or by a whole chromosome. In the last case, all identified genomic variants and their location on the chromosome are presented. Interestingly, the population component of the variants is taken into account. The database provides information on the frequency of each variant and the ethnic origin of the individuals in whom it was identified.

Another database for genomic variation is being planned, the HUGOBase (Integrated Human Genome Variation database) (Anonymous, 2006). An internet site is not yet available.

An important initiative has been launched specifically to explore the links between pathologies and human variation at the genomic level. It is a network of networks, the HuGENet™ or Human Genome Epidemiology Network (http://www.cdc.gov/genomics/hugenet/default.htm). It is composed of 23 specialized networks (Ioannidis *et al.*, 2005) that cover a broad range of pathologies, from cardiovascular disease to brain tumours and assisted reproduction. It is distressing that infectious pathologies are not sufficiently represented; a very specialized network covers HIV only. I have proposed to the coordinators of HuGENet™ that they should set up a general network of infectious diseases that will cover all transmissible pathologies, including those of viral, bacterial, parasitic and fungal origin. This

new network will be based on the integrated approach that is described in the present paper and will take into account the co-evolution phenomena between host, pathogen and vector. The proposed name of the network is Infection, Genetics and Evolution (inspired by the journal of the same name).

8. CONCLUSION

At least when historical markers are considered, our species has limited genetic variability because we are a fairly young species: our common mothers and fathers lived only 100 000 years ago (Solignac, 1998). At the same time, we have relatively high genetically driven phenotypic polymorphism resulting from strong selective pressures acting differently on separate geographical populations (Tibayrenc, 2004). Although direct evidence is still limited, strong circumstantial evidence clearly shows that susceptibility to infectious diseases has a genetic component acting both at the individual and population/ethnic levels. This makes pharmacogenetics (Cressey and Lallemant, in press) a field that has a promising future: the administration of a given drug should be adapted to the genetic make-up of individuals and populations. When the population level is considered, it could be more informative to use a finer level (for example, Fulani vs. Mossi; see Section 5.2, p. 403) rather than the major subdivisions classically distinguished by physical anthropology (Valois, 1971). However, when analysing multiethnic populations from a medical point of view, it would be misleading to neglect the ethnic component, at least for research purposes.

The exponential development of new powerful technologies and the cross between genomics and bioinformatics is probably generating a new scientific revolution that can be compared only to the discovery of the double helix. It is apparent that our genetically driven phenotypic variability, both normal and pathological, is not explainable by sequence variation only. Major genomic rearrangements seem to play a major role. Since populations are the target of natural selection as well as individuals, it is all the more desirable to develop the new field of population genomics. More than ever, we are

in the dark age to understanding susceptibility of infectious diseases but, fortunately, in the golden age of genetics (Tibayrenc, 2001).

Lastly, the indisputable impact of this genetic component of human susceptibility to infectious diseases, and its co-evolutionary interactions with the genetic diversity of vectors and pathogens, should not lead to the neglect of another major component acting on the transmission and severity of these human scourges: the crucial role of the environment, chiefly manifested as socioeconomic factors.

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Glossary

Allele. One of two or more different molecular form of a given gene.

Allelic frequency. The ratio of the number of a given allele to the total number of alleles present in the population under survey. For example, in a fruit fly population (fruit flies are diploid and sexual), a given gene is surveyed, of which two alleles, *a* and *b*, are present. Among 100 individuals, there are 36 a/a homozygous genotypes, 16 b/b homozygous genotypes and 48 a/b heterozygous genotypes. Since the organism is diploid, in 100 individuals there are 200 alleles. In an a/a homozygous individual, there are two *a* alleles. In a b/b homozygous individual, there are two *b* alleles. In an a/b heterozygous individual, there is only one *a* allele and only one *b* allele. The total number of *a* alleles is hence twice the number of a/a individuals (36×2) plus the number of a/b individuals ($72 + 48 = 120$). The frequency of this allele is $120/200 = 0.6$. Similarly, the total number of *b* alleles is twice the number of b/b individuals (16×2) plus the number of a/b individuals $= 80$. The frequency of this allele is $80/200 = 0.4$. Since there are only two alleles, this last figure could have been calculated by subtracting the frequency of the *a* allele from 1 ($1-0.6 = 0.4$).

Allopatry. The state of living in different geographic locations (cf. sympatry).

Alu insertion sequence. Dispersed repeated DNA sequences in the human genome, consisting of roughly 300 base pairs in approximately $3-5 \times 10^5$ copies, constituting roughly 5% of the human genome. These sequences are easily transposable. They are specifically cleaved by the restriction enzyme *Alu* I. (See also insertion sequence.)

Ancestry informative marker (AIM) (Pfaff *et al.*, 2001). AIMs are the subset of genetic markers that differ greatly in allele frequency across the populations of the world. Most polymorphisms are shared among all populations, and for most loci the most common allele is the same in each population. An AIM is a unique combination of genetic markers that occurs mostly in particular founder population sets but may also be found at varying levels across all or some of the populations found in different parts of the world (definition communicated by DNAprint Genomics from their website [see p. 396], with the permission of the company).

Balanced polymorphism (*balanced selection*). Genetic polymorphism that persists in a population because the individuals heterozygous for the alleles concerned have a higher degree of fitness than either homozygous individual. The classical example is sickle-cell anaemia, in which heterozygous individuals are protected against malaria, whereas homozygous individuals are not and have a fatal form of the disease.

Bioinformatics. The technology of informatics as applied to biological research, more specifically to genomic and phylogenetic analysis. Bioinformatics is the management and analysis of data using advanced computing techniques. Bioinformatics is particularly important as an adjunct to genomics research, because of the large amount of complex data this research generates. It is also widely used in the analysis of genomic expression (postgenomics, proteomics).

Bootstrap analysis (Felsenstein, 1985). In phylogenetic analysis, generation of pseudo-replicate data sets by randomly sampling the original character matrix to create new matrices that are the same size as the original. The frequency with which a given branch is reproduced by this randomization procedure is recorded as the bootstrap proportion. These proportions can be used as an evaluation of the robustness of individual branches in the tree. A bootstrap value of 95 for a given branch means that this branch has been found by the procedure 95 times out of 100 or 950 times out of 1000. This statistic must be cautiously interpreted (see Figure 1).

Clade. Evolutionary lineage defined by cladistic analysis. A clade is monophyletic (it has only one ancestor) and is genetically isolated (it evolves independently) from other clades.

Cladistic analysis. A specific method of phylogenetic analysis designed by the German entomologist Willy Hennig (1966). It is based on the polarization of characters, that are

separated into ancestral (plesiomorphic) and derived (apomorphic) characters. Only those apomorphic characters that are shared by all members of a given clade (synapomorphic character) are considered to be phylogenetically informative. For example, feathers are specific to the clade 'birds' (which is a class) and are possessed by all birds. They are synapomorphic characteristics of that clade. Cladistic analysis was chiefly designed for morphological characteristics and its use for molecular data is still a matter of controversy (Tibayrenc, 1998a).

Coalescence time. Time elapsed between the common ancestral copy (one gene in one individual) and two or more copies of a given gene at the present time.

Copy-number variant. DNA sequence $\geqslant 1$ kb, present with a variable copy number by comparison with a reference genome. Includes insertions, deletions and duplications (Feuk *et al.*, 2006).

Cytokine. An intercellular messenger protein (lymphokine, monokine, interleukin, interferon, tumour necrosis factor), which is released by lymphocytes and macrophages. Cytokines favour communication among cells of the immune system and between immune system cells and cells belonging to other tissue categories. They play an important role in defence against infectious agents.

Discrete typing unit (DTU) (Tibayrenc, 1998a). Sets of stocks that are genetically closer to each other than to any other stock, are stable in space and time, and are identifiable by common genetic, molecular or immunological markers called tags. DTUs are reliable units of analysis for applied studies in which the genetic diversity of the pathogen is taken into account and are an ideal target for molecular epidemiology surveys.

Ethnic group. A group whose common identity is based on racial and/or cultural association.

Exon. The DNA sequence of a gene that codes for proteins (cf. intron).

Fitness. The relative ability of an organism to survive and to transmit its genes to the next generation.

Founder effect. When a small subsample of a larger population settles as a distinct population, its genetic diversity might be only a small part of the genetic diversity of the original population. For example, the present population of French Canadians was founded by only a few thousand French people, most originating from a limited number of French regions. Their original genetic diversity (and the diversity of their last names) was therefore lower than that of the entire French population. For this reason, some genetic traits that are rare in the general French population were by chance over-represented in the original founder population. This explains why some pathological traits (e.g., amyotrophic lateral sclerosis) are more frequent in Québécois than in the present-day French population.

F-*statistics*. See Box 1 (p. 395).

Gene. A DNA sequence coding for a given polypeptide. More broadly, any given DNA sequence.

Gene sequence (*genetic sequence*, *DNA sequence*). Can be compared to a series of letters corresponding to the primary structure of a real or hypothetical DNA molecule or strand. The possible letters are A, C, G and T, which correspond to the four nucleotide subunits of a DNA strand (adenine, cytosine, guanine and thymine). This coded sequence corresponds to the basic genetic information. A DNA sequence may code for proteins. In this case, it directly monitors the succession of amino acids that constitute the primary structure of the protein. Many DNA sequences have no known coding function.

Genetic distance. Various statistical measures inferred from genetic data, estimating the genetic dissimilarities among individuals or populations. Genetic distances can be based on the percentage of band mismatches on gels (the case for markers such as MLEE or RAPD) or allelic frequency differences or the percentage of sequence divergence. Two very popular indices of genetic distance have been proposed by Jaccard (1908) and Nei (1972).

Genomics. Molecular analysis of the whole genome of a species instead of particular genes.

Genotype. Genetic constitution of a given organism (cf. phenotype).

Geographical race. Term equivalent to subspecies.

Heritability. Proportion of variation in a trait among individuals in a population that can be attributed to genetic effects. The heritability of most common diseases is considered to be 40%.

Heterozygote. An individual of a diploid organism in whom both copies of a given gene have a different molecular structure: this individual harbours two different alleles of the same gene (cf. homozygote).

Homozygote. An individual of a diploid organism in whom both copies of a given gene have an identical molecular structure.

Human leucocyte antigen (HLA). Cell surface protein detected by blood testing that exhibits considerable diversity among individuals and determines an individual's leucocyte type. Ten numbers constitute this system: two A numbers, two B numbers, two C numbers, two DR numbers and two DQ numbers. One number at each genetic locus (A, B, C, DR and DQ) comes from the mother, the other one is given by the father. HLAs are also called histocompatibility antigens or tissue antigens because organ recipients and donors should have compatible HLA genotypes, otherwise the transplanted organ is recognized as non-self (foreign) and is eliminated. This set of genes is involved in the presentation of antigenic peptides to the immune system and plays a major role not only in tissue compatibility but also in infectious processes (cf. major histocompatibility complex).

Insertion sequence (IS). A small bacterial transposable element, approximately 1000 bases long, with short inverted repeated sequences at its ends.

Intron. A gene region that is not translated into a protein sequence. In coding genes, introns are interspersed with coding regions called exons.

Isoenzymes. Different electrophoretic variants of a given enzyme, reflecting the variability of the gene that codes for this enzyme in the population under survey. Differences in migration among different isoenzymes of the same enzyme are due to differences in their overall electric charge, which in turn is a result of the individual electric charge of each amino acid that composes the enzyme. Electrophoretic differences therefore reflect differences in the amino acid sequence and hence the upstream gene sequence.

Large-scale copy-number variation. Copy-number variant up to several hundred bp in length (Lafrate *et al*., 2004). Also called large-scale copy-number polymorphism (Sebat *et al*., 2004).

Linkage disequilibrium. Non-random association of genotypes occurring at different loci. See recombination.

Locus. The physical location of a given gene on the chromosome. By extension, in the genetic 'jargon', the gene itself.

Major histocompatibility complex (MHC). A complex of genes found in mammals that function especially in determining the histocompatibility antigens found on cell surfaces. MHC class I molecules on the surface of a cell infected by a virus or another pathogenic agent present foreign antigens to CD8 cytotoxic T lymphocytes. MHC class II molecules are found on the antigen-presenting cells of the immune system and display the antigen which activates CD4 cells [cf. human leucocyte antigens (HLA)].

Microarray. Also called a gene chip or a DNA chip. Microarrays are composed of large numbers of molecules (generally, but not always, DNA) distributed in rows in a very small space. When DNA molecules are used, they act as probes for the DNA of the organism under study, to see whether the genes present on the test sample (the one plotted on the chip) are also present on the sample under study. Microarrays can also enable the study of gene expression by providing a 'snapshot' of all the genes that are expressed in a given cell at a particular time (transcriptome). Microarrays are powerful tools for screening complex samples (many genes of a given organism, or complex samples composed of several organisms).

Microsatellite. A short DNA sequence, usually 1–4 bp long, that is repeated in a row along the DNA molecule. In humans, as in many other species, there is great variation from one person to another (widely used in forensic applications for individual identification) and among different populations in the number of repeats. Numbers of repeats for a given locus define microsatellite alleles. There are hundreds of places in human DNA and in most other species that contain microsatellites. Microsatellites are fast-evolving markers, with a high resolution level, and are found in many different organisms.

Molecular clock. In its strict, original sense (more correctly called the DNA clock hypothesis), the concept that the rate of nucleotide substitutions in DNA remains constant or at least is a function of time. In a broader sense, simply the speed of evolution of that part of the genome that codes for the variability of a given genetic marker. This rate is driven by the rate of substitution/mutation and is influenced by natural selection; it can be regular or irregular.

Multilocus enzyme electrophoresis (MLEE). Isoenzyme analysis based on the analysis of a broad range of enzyme systems. Each enzyme system corresponds to one or several genetic loci. MLEE has been widely used in population genetics for virtually any kind of living organism and has provided the first reliable picture of the general genetic diversity of human populations (Nei and Roychoudhury, 1974; see Figure 1).

Natural selection. Process first described by Charles Darwin that favours certain genotypes to the detriment of others over generations because they are better adapted to survive and have therefore more abundant offspring (they have a higher fitness). It is entirely driven by the interaction of an organism with the environment.

Neutral gene. (neutral polymorphism). A gene or genetic polymorphism that does not undergo natural selection.

Non-parametric test. Statistical test for which a distribution curve cannot be designed, either because the parameters of the equation are unknown or because the statistical model cannot be represented by any equation. A non-parametric statistical test is typically simpler, and its results are more robust, than those of parametric statistics. However, all things being equal, it has lower resolution.

Panmixia (adjective *panmictic*). A situation in which gene exchanges occur randomly in the population under survey.

Phenotype (*phenotypic*). All observable characteristics of a given individual or a given population distinct from the genome. The phenotype is not limited to morphological characteristics and includes, for example, physiological quantities (blood pressure, muscular strength, etc.) or biochemical quantities (level of cholesterol, etc.). The phenotype is produced by the interaction between the genotype and the environment. The part that is driven by the action of the genotype constitutes the heritability of the character under study.

Phylogenetics. A branch of genetics that aims at reconstructing the evolutionary past and relationships of taxa or of separate evolutionary lines.

Polymerase chain reaction (PCR). Technique that copies the complementary strands of a target DNA chain through a series of cycles until the necessary quantity is obtained. PCR uses synthesized primers whose nucleotide sequences are complementary to the DNA flanking the target region. The DNA is heated to separate the complementary strands, then cooled to allow the primers to bind to the flanking sequences. The enzyme *Taq* DNA polymerase is added and the reaction is allowed to pass through the required number of replication cycles. PCR is widely used in molecular diagnosis of infectious diseases, but is also the basis for many molecular techniques used in human genetics, such as the study of microsatellites. This technique makes it possible to work on very small quantities of biological material (hair, small skin cuts, a few drops of blood). Since DNA molecules are extremely stable, PCR reactions can be carried out on dry samples or on tissues conserved in alcohol, or even on fossils.

Proteomics. The study of the expression, function and interaction of all the proteins expressed by the genome of a given organism; it is widely used to explore pathogenic processes (by

comparing gene expression in healthy and sick individuals). Proteomics explores gene expression at the scale of the entire genome and is therefore the logical complement of genomics.

Race. Population characterized by a set of common genetic features (Gentillini, 1993); the biological validity of the concept of race is presently the object of many debates (see below) (cf. ethnic group, subspecies and geographical race).

Recombination. Reassortment of genotypes occurring at different loci through the processes of meiosis and crossing over. Random recombination leads to a total lack of linkage disequilibrium. Free recombination results in the expected probability of a given multilocus genotype being the product of the observed probabilities of the single genotype composing it. For example, in a panmictic human population, if the observed frequency of the AB blood group is 0.5 and the observed frequency of the Rh(+) blood group is 0.5, the expected frequency of the individuals who are both AB and Rh(+) is $0.5 \times 0.5 = 0.25$, because ABO and rhesus (Rh) blood groups are coded by distinct genes on separate genomic regions, which explains why they recombine randomly. Inhibition of recombination leads to linkage disequilibrium (i.e., the predictions of expected probabilities for multilocus genotypes are no longer met). As an example, if the observed frequency of the individuals who are AB and Rh (+) was statistically significantly higher than 0.25, this would be evidence that the two loci are linked together (i.e., not transmitted independently); if this frequency was 0.5, it would indicate total linkage between AB and Rh (i.e., the two characteristics are transmitted as a single unit).

Restriction fragment length polymorphism. Variability in the DNA of a given organism evidenced by the use of bacterial restriction endonucleases. The endonuclease cuts the DNA at specific restriction sites characterized by given sequences and the polymorphism of the DNA fragments thus obtained can be visualized on gels, either directly by ethidium bromide staining or by Southern blot hybridization with specific probes.

Sexual selection. Selection which promotes traits (e.g., the peacock's tail, the cock's crest, the lion's mane) that increase success in mating, sometimes to the detriment of the overall fitness of the organism.

Short tandem repeat polymorphism (STRP). Synonymous with microsatellite.

Sickle cell anaemia. A generally fatal form of haemolytic anaemia observed in individuals who are homozygous for the autosomal, codominant gene H^s. The erythrocytes of these individuals contain an abnormal haemoglobin, Hb^s. These erythrocytes undergo a reversible shape alteration when the oxygen concentration in the plasma falls slightly and develop a sickle-like form. These pathological red cells have a shortened lifetime. Approximately 0.2% of African-American newborn babies suffer from sickle cell anaemia. Heterozygous individuals have some protection against malaria, which explains why this pathological trait has been maintained. It is a typical case of balanced polymorphism.

Single-nucleotide polymorphism (SNP, pronounced 'snip'). Polymorphism of one-letter variations in the DNA sequence. SNPs contribute to differences among individuals and populations. Most of them have no effect, others cause subtle differences in countless features, such as appearance, while some affect the risk for certain diseases. SNPs are widely used as high-resolution population markers and are the basic tool used in the so-called Hap Map project (see below). SNPs can be detected by (i) restriction fragment length polymorphism, (ii) sequencing, (iii) denaturating high-performance liquid chromatography, (iv) mass spectrometry and (v) array-based resequencing/microarrays. SNPs constitute approximately 90% of all human genetic variations, and SNPs with a minor allele frequency $\geqslant 1\%$ occur every 100–300 bases throughout the human genome, on average. It is important to note that there are allelic frequency variations among different human populations, with the result that a SNP that is common in one geographical or ethnic group may be much rarer in another (see ancestry informative marker).

Spacer DNA. In eukaryotic and some viral genomes, untranscribed DNA segments that flank functional genetic regions or cistrons.

Subspecies (also called geographical race). A geographical population of the same species that exhibits distinct genetically-based phenotypic traits, making it possible to distinguish most individuals of one population from most individuals of another population. Subspecies are given trinomials in the Linnean nomenclature (e.g., *Triturus marmoratus pygmaeus*, the dwarf subspecies of the marbled newt in Spain). Since they pertain to the same biological species, different subspecies of the same species are potentially interfertile and have fertile offspring when they have overlapping geographical distributions.

Sympatry. Living in the same geographic location (cf. allopatry).

Synonymous mutation. Mutation that results in a change in the DNA but not in any protein due to the redundancy of the genetic code.

Transposon. A short DNA sequence that has the property of moving from one chromosomal location to another.

Xenodiagnosis. A classical and efficient (although highly unpleasant) method of diagnosis for South American trypanosomiasis (Chagas disease). Triatomine bugs reared in the laboratory and fed on chickens (so that they are not infected with *Trypanosoma cruzi*) are allowed to feed on patient suspected of having Chagas disease. Three weeks later, the faeces of the bugs are checked for the presence of trypanosomes. Xenodiagnosis can work with a chronic case when circulating forms of the parasite are very rare; the triatomine bugs act as biological incubators for the parasite.

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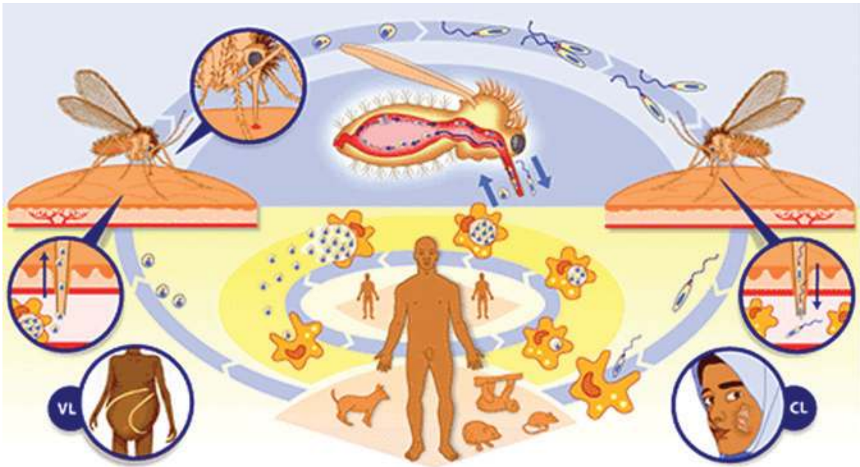


Plate 1.1 Life cycle of *Leishmania donovani*. During a blood meal, an infected phlebotomine sand fly releases metacyclic promastigotes into vertebrate hosts such as humans. The parasites are then phagocytosed by host macrophages and change into amastigotes. After amastigote multiplication and rupture of the macrophage, the amastigotes invade neighbouring macrophages. The life cycle is complete when a phlebotomine female is infected while feeding on the blood of an infected host. The amastigotes released in the sand fly's intestine change into procyclic promastigotes before migrating towards the pharynx and the proboscis (WHO, http://www.who.int/tdr/diseases/leish/leish.htm). CL, cutaneous leishmaniasis; VL, visceral leishmaniasis.

Plate 1.2 Female *Phlebotomus papatasi*, a vector of *Leishmania*. Phlebotomine sand flies are small, with a body length seldom exceeding 3 mm. Their colour ranges from almost white to almost black. Three features make them easy to recognize: when at rest, they characteristically hold their wings at an angle above the abdomen; they are hairy; and, when coming to feed, they typically hop around on the host before settling down to bite. Unlike mosquitoes, their attack is silent (Killick-Kendrick, 1999) (WHO, http://www.who.int/tropical_diseases/databases/imagelib.pl?imageid=9106001).

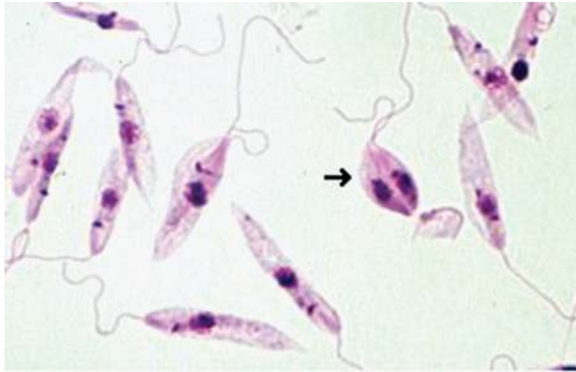


Plate 1.3 Promastigotes of *Leishmania tropica* (from culture); note multiplication by longitudinal binary fission (arrow) (http://www.msu.edu/course/zol/316/lsppscope.htm; photo by Brian E. Keas, reproduced with permission).

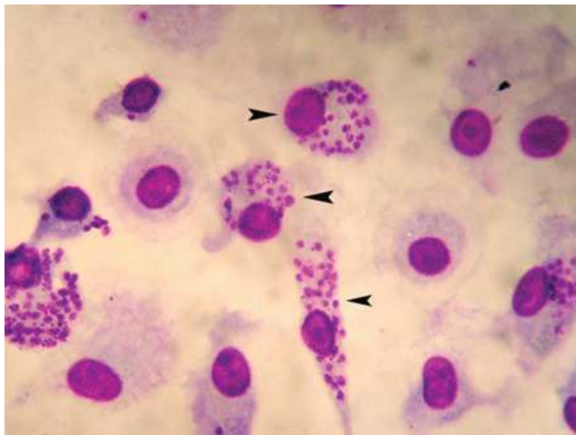
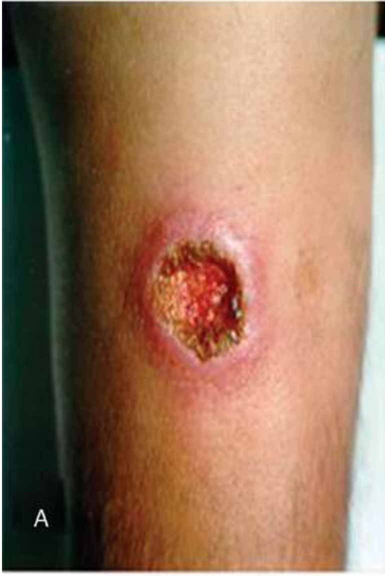
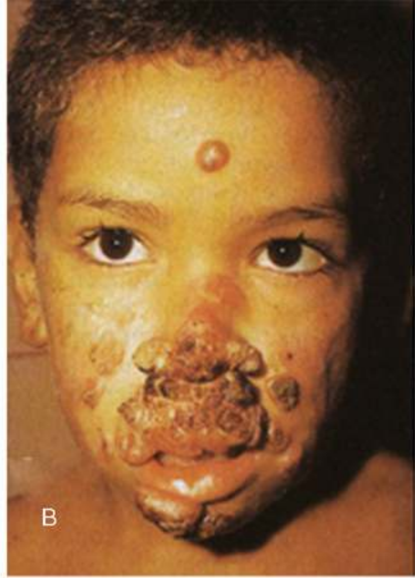


Plate 1.4 Amastigotes of *Leishmania donovani* within macrophages in culture (arrows); (photo by Bruno Bucheton, reproduced with permission; all rights reserved).





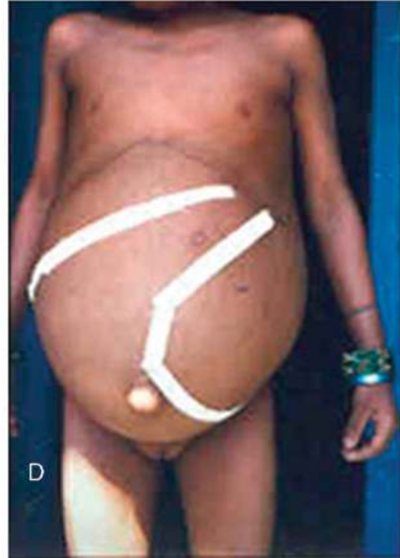


Plate 1.6 Pathology of leishmaniasis. (A) Cutaneous leishmaniasis (photo by Richard D. Ward, University of Keele, UK, reproduced with permission; http://www.keele.ac.uk/depts/aep/staff/rdwres.htm). (B) Mucocutaneous leishmaniasis, resulting in total disfigurement of the face (photo by Wolfgang Bommer, University Hospital Goettingen, Germany, reproduced with permission). (C) Diffuse cutaneous leishmaniasis (photo by Philippe Desjeux, World Health Organization, reproduced with permission; http://www.who.int/leishmaniasis/en/). (D) Visceral leishmaniasis (photo by Philippe Desjeux, World Health Organization, reproduced with permission; http://www.who.int/leishmaniasis/disease_epidemiology/en/index.html).

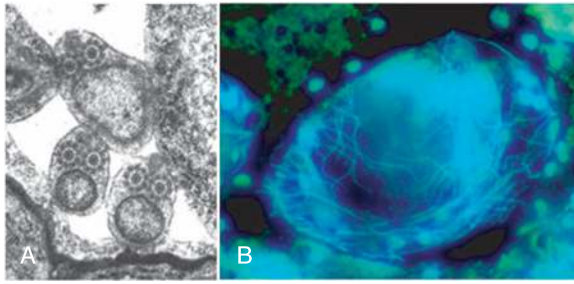


Plate 3.17 (A) Transmission electron micrograph of spermatozoans and of (B) fluorescence microscopy image of inseminated sperm within the ECFR of a bis-Benzimide-stained (see Harris *et al*., 1997) *Gyrodactylus gasterostei* specimen.

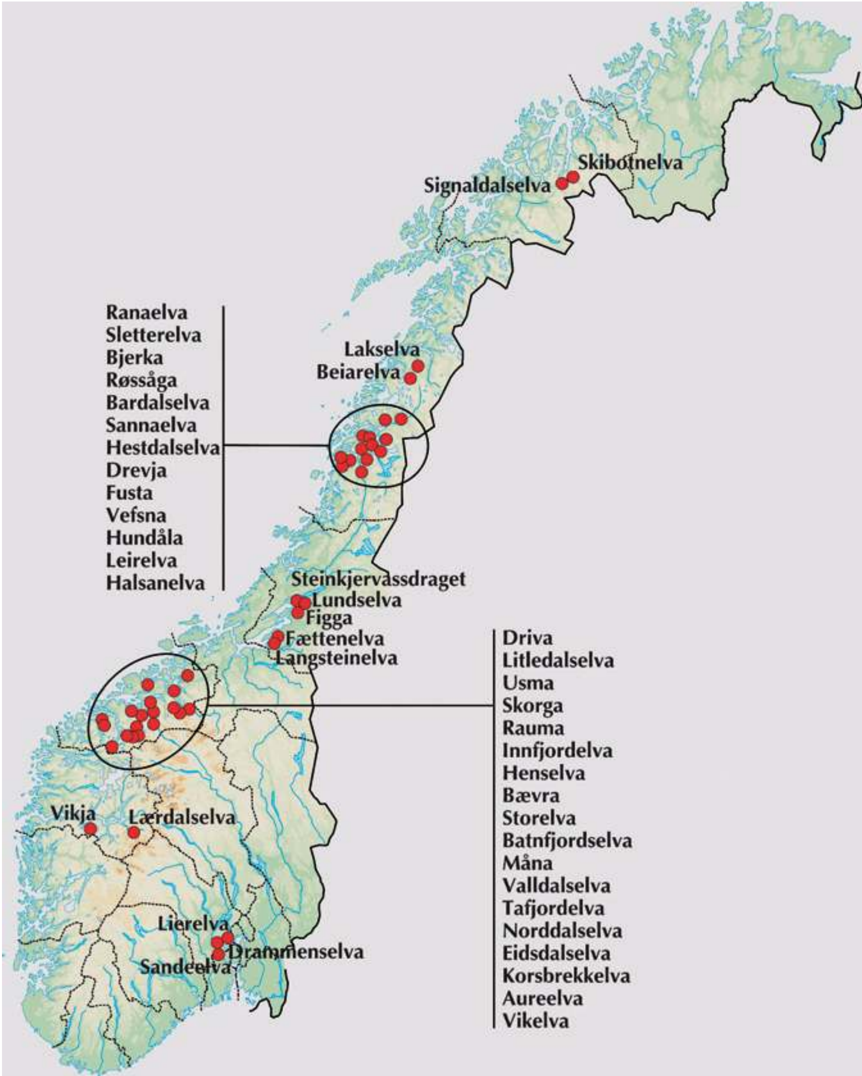


Plate 3.25 The distribution of *Gyrodactylus salaris* on Atlantic salmon in Norwegian river systems per August 2005 since the first observation of the species in mid-1970s. In 2006, one new river is infected, Ranelva, *Region 3—Vefsn Region* (Nordland County); see text. (Reproduced with permission of the Directorate for Natural Resources, Trondheim, Norway.)

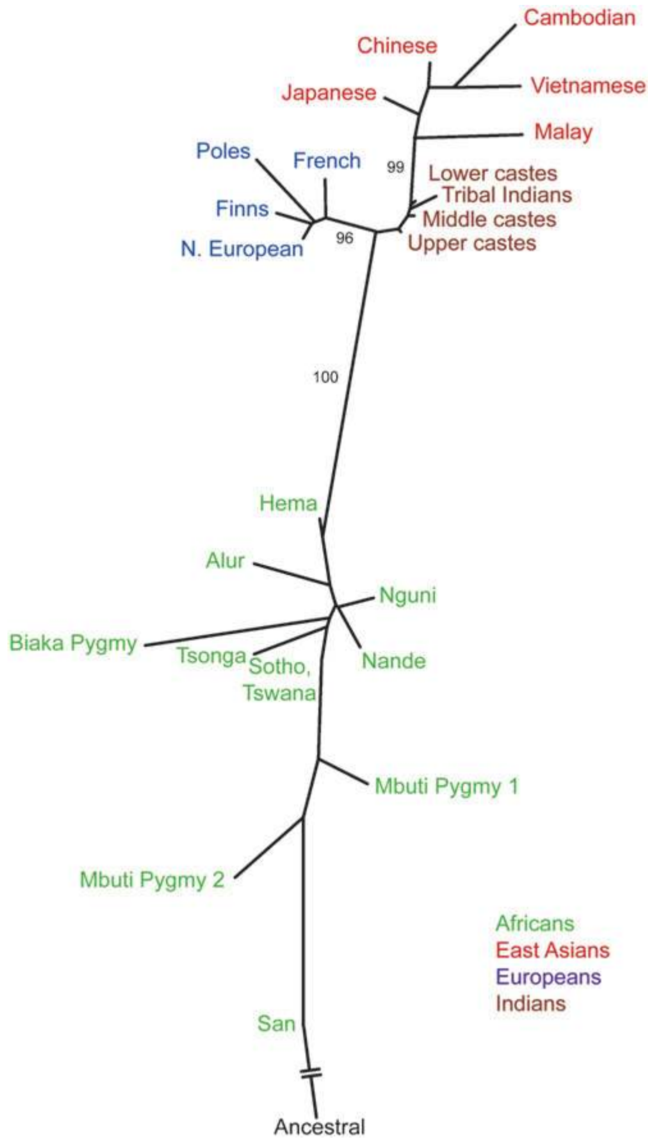


Plate 4.2 Phylogenetic trees based on modern molecular markers. The overall pattern is comparable to that of Figure 1, clearly distinguishing between classical ethnic groups, although the overall difference between groups is limited [this page: neighbour-joining network of population similarities based on the frequencies of 100 *Alu* insertion sequences (Jorde and Wooding, 2004); next page: least-squares tree for 37 populations based on 80 independent loci (41 haplotyped loci, 36 biallelic loci and 3 STRPs) with about 620 statistically independent alleles, after Tishkoff and Kidd (2004)].

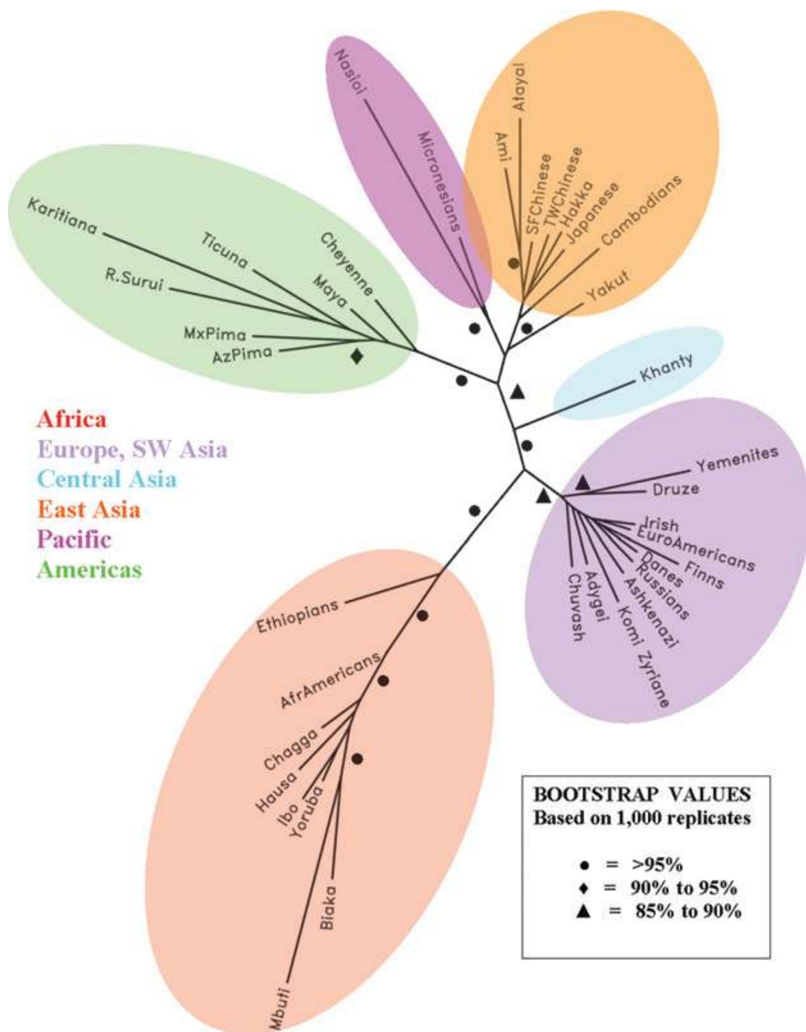


Plate 4.2 (continued)