Trends in fishery genetics

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Published in:
The Future of Fishery Science in North America. Fish & Fisheries Series,

DOI:
10.1007/978-1-4020-9210-7

Publication date:
2009

Document Version:
Final published version

Chapter 24
Trends in Fishery Genetics

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Abstract Fishery genetics has a history of half a century, applying molecular biological and genetic techniques to answer fisheries-related questions in taxonomy and ecology of fishes and invertebrates. This review aims to provide an overview of the developments in fishery genetics of the last decade, focussing on DNA-based species and stock identification. Microsatellites became the 'gold standard' in genetic stock identification, but accumulating sequence information for commercially important species opens the door for genome-wide SNP (Single Nucleotide Polymorphism) analysis, which will support or even displace microsatellites in the future. Recent advancements in DNA analytics, such as DNA microarrays and pyrosequencing, are highlighted and their possible applications in fishery genetics are discussed. Emphasis is also given to DNA barcoding, a recently advocated concept using a fragment of the mitochondrial cytochrome oxidase I (COI) gene as a standard marker for the identification of animals. DNA barcoding becomes more and more accepted in the scientific community and the international initiative Fish-BOL (Fish Barcoding of Life) aims to barcode all fish species. These novel technologies and concepts will enable a tremendous progress in fishery genetics.

24.1 Introduction and Historical Overview

Fishery genetics bridges the gap between fisheries research and molecular biology by applying molecular methods to fisheries-related questions in taxonomy and ecology of fishes and invertebrates. In a broader sense, fisheries genetics can also deal with the evolution of species relevant in fisheries, as well as breeding in aquaculture.

The application of molecular genetic approaches in fisheries research started in the 1950s, investigating blood group variants in tunas, salmonids and cod to analyse population structure (review by Ligny 1969). However, these methods have not been broadly adapted in fisheries research, because genetic variation could be detected

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R.J. Beamish and B.J. Rothschild (eds.), *The Future of Fisheries Science in North America*, Fish & Fisheries Series, © Springer Science + Business Media B.V. 2009

much easier by determining protein polymorphism using starch gel electrophoresis (Ward and Grewe 1994). Studies on the variation of protein polymorphism in fish started in the early 1960s by investigating hemoglobin variants in cod (*Gadus morhua*) and whiting (*Merlangius merlangus*) (Sick 1961). The focus was then changed to enzymatic proteins (so-called allozymes). Because they were easy to handle, results were reproducible, and the method was reasonably inexpensive (Ferguson 1994; Ward and Grewe 1994). Allozymes are differing in electrophoretic mobility due to allelic differences at a single gene. In the 1970s, allozyme analysis by protein electrophoresis was widely applied, providing new knowledge about populations, such as size, migration and isolation (Utter 1994). This is reflected in the number of fish- and fishery-related publications, which started to grow in the late 1970s (Fig. 24.1). The prime use of allozyme variation was the application as a molecular marker for stock identification, in the sense of identifying reproductively isolated populations for fisheries management, as well as species identification. However, such genetic markers have to be neutral, because polymorphism between populations caused by selection will not provide information if stocks will be reproductively isolated. There has been a long debate whether allozyme variation is subject to selection and it has been shown that at least some protein coding loci are not neutral (Ferguson 1994). Additionally, allozyme analysis is only an indirect measure for genetic variation in deoxyribonucleic acid (DNA), and therefore has a lower resolution (= variation) than direct assessment of DNA (Ward and Grewe 1994). Another disadvantage of protein electrophoresis is the handling of samples, which have to be

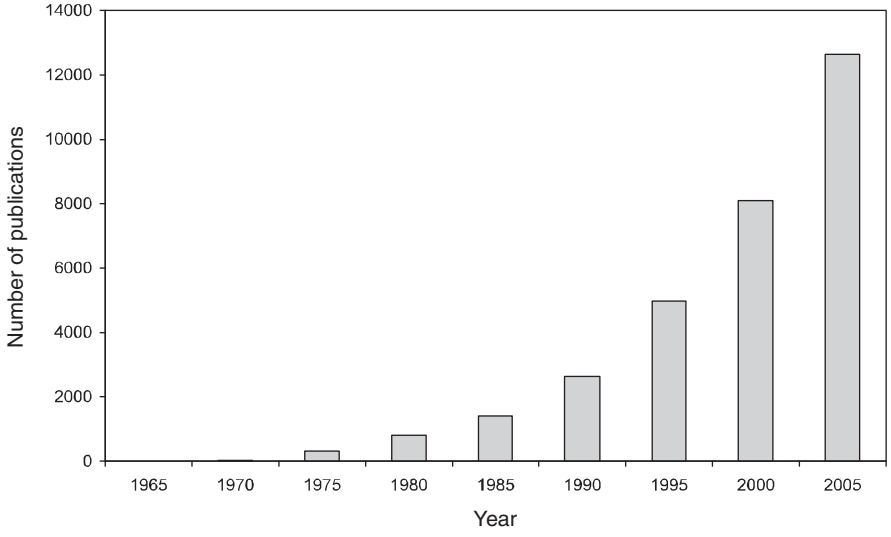

Fig. 24.1 Cumulative number of publications related to fishery genetics in the literature data base 'Aquatic sciences and fisheries abstracts (ASFA)' with the search string: (Title = fish* or Keywords = fish* or Abstract = fish*) and (Title = genetic* or Keywords = genetic* or Abstract = genetic*)

analysed fresh immediately after sampling or have to be kept frozen until analysis. This is not problematic on modern research or commercial fishing vessels, but is a logistic problem in land-based surveys in remote areas with no infrastructure, e.g., in many developing countries. Problematic is also the large amount of tissue needed, which can usually only be obtained by sacrificing the specimens. This is especially problematic when the species under study is endangered or protected (Park and Moran 1994). In contrast, only minute amounts of tissue are needed for DNA analysis and such tissue samples can be stored at ambient temperature in 96% ethanol, but for long-term preservation storage of such samples at 4°C is recommended (Zhang and Hewitt 1998). Therefore, direct analysis of DNA is advantageous.

Even though it was revealed already in the 1940s that DNA is the substance of inheritance (Avery et al. 1944) and the molecular structure was known since the 1950s (Watson and Crick 1953), first attempts to analyse DNA directly in population genetics have only been made in the 1970s by studying the Restriction Fragment Length Polymorphism (RFLP) of mitochondrial DNA (e.g., Brown and Vinograd 1974; Upholt and Dawid 1977). At the same time, other studies important for the application of mtDNA RFLPs in population genetics were the discovery of the predominantly maternal inheritance of mtDNA in higher animals (Dawid and Blackler 1972) and their high rate of evolution (Brown et al. 1979), as well as the development of a statistical method to calculate the divergence between mtDNA haplotypes from RFLP banding pattern (Upholt 1977). The basis for direct analysis of DNA sequences was the development of the 'dideoxy' method by Sanger et al. (1977), but routine analysis of DNA sequences was finally accelerated by the invention of the polymerase chain reaction (PCR) by Mullis and colleagues (Saiki et al. 1985, 1988). Since the 1990s, the application of PCR and DNA sequencing became a routine method in fisheries genetics and the number of publications in that area, which started with only four in 1965, was rapidly increasing to >12,000 in 2005 (Fig. 24.1). The development of these methods lead to a growing interest in DNA sequencing for the study of the evolution and population genetics of fishes, which is reflected in a growing number of DNA sequences derived from bony fishes. Starting with seven sequences from bony fishes in 1993, the number of sequences in international sequence databases reached more than 5.5 million by the end of 2007 (Fig. 24.2). Most of data entries (64%) are nuclear EST (expressed sequence tag) sequences, derived from gene expression studies with model fish species such as zebra fish (*Danio rerio*), but also from species important in fisheries and aquaculture; e.g., Atlantic cod (*Gadus morhua*), turbot (*Psetta maxima*), European eel (*Anguilla anguilla*), and Atlantic salmon (*Salmo salar*). GSS (genome survey sequences) entries represent 18% and are mainly obtained from nuclear genome sequencing projects on zebra fish (*Danio rerio*) and Japanese pufferfish (*Takifugu rubripes*). CoreNucleotide sequences also have a proportion of 18% and comprise mitochondrial as well as nuclear DNA sequences, many of them obtained for studies on phylogenetics, species identification, and population genetics. These sequences sum up to one million and are the ones most users are interested in.

This review aims to provide an overview of recent developments and trend in genetics and their utilisation and potential application in fisheries research. Even though

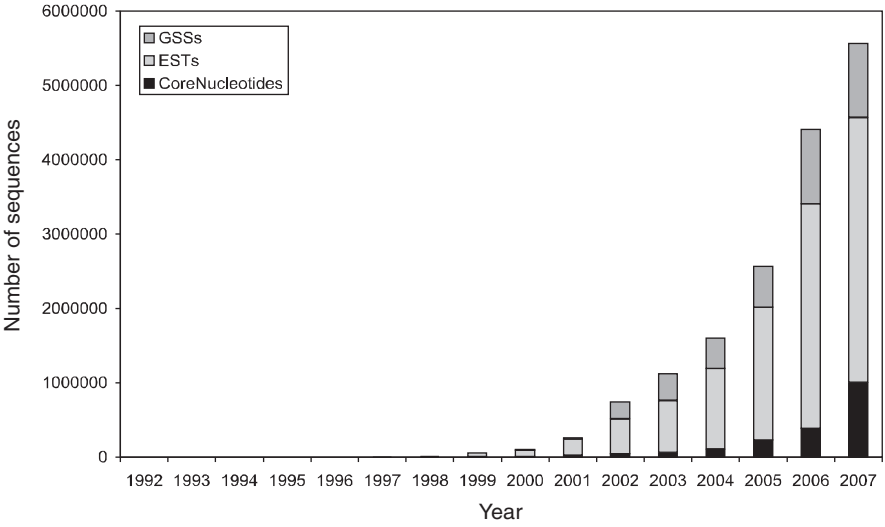

Fig. 24.2 Number of DNA sequences for bony fishes (Teleostei) in international DNA databases (derived from GenBank, December 2007). GSSs: Genome survey sequences; ESTs: Expressed sequence tags; CoreNucleotides: all nucleotide sequences that are not GSSs or ESTs

fishery genetics deal with a variety of freshwater as well as marine fishes and invertebrates, this review will be restricted to species and stock identification of marine fishes. Since the direct analysis of DNA sequences is the 'gold standard' in the era of genomics, protein analysis will not be considered in this review. Excellent overviews on the state of the art in fishery genetics, including protein analysis, at the end of the last century are provided in a special issue of *Reviews in Fish Biology and Fisheries* (e.g., Carvalho and Hauser 1994; Park and Moran 1994; Ward and Grewe 1994) and by Ward (2000). This review will cover established DNA-based methods and examples of their application, as well as recent advancements, namely DNA barcoding, DNA microarrays, and pyrosequencing, which have the potential to revolutionise species and stock identification in fisheries research.

24.2 DNA Marker

In fisheries genetics, mitochondrial and nuclear markers are utilised. In order to understand advantages and disadvantages of the different marker systems, a brief overview is given.

Mitochondrial DNA (mtDNA) sequences are the most used genetic markers in phylogenetics, phylogeography, as well as population genetics, and have replaced allozymes. In the late 1990s more than 80% of phylogeographic studies used mtDNA as a genetic marker (Avise 1998). Mitochondrial markers are widely used in studies

on fishes and a large number of 'universal' primers are available to amplify different fragments of the mitochondrial genome of many fish species (Meyer 1993, 1994). However, no marker is completely universal, and therefore, multiplex-PCRs or 'cocktails' with several 'universal' primers are needed to ensure that a certain fragment can be amplified from most fish species (Ivanova et al. 2007; Sevilla et al. 2007).

Mitochondria are the power plants of the cell and have a central role in cell metabolism. A vertebrate mitochondrial genome is a double-stranded, circular molecule of about 16,000 bp length that contains 13 protein, 2 rRNA and 22 tRNA genes. Compared to the nuclear genome it is extremely compact, about 93% of mtDNA are genes, whereas non-coding DNA has only a proportion of about 3% in the nuclear genome (Fig. 24.3). In higher animals, mitochondrial DNA is maternally inherited, does not appear to undergo recombination, and evolves about ten times faster than the nuclear genome. It is also believed that mtDNA is a neutral marker, not undergoing selection. These features made mtDNA extremely important in molecular systematics and population genetics.

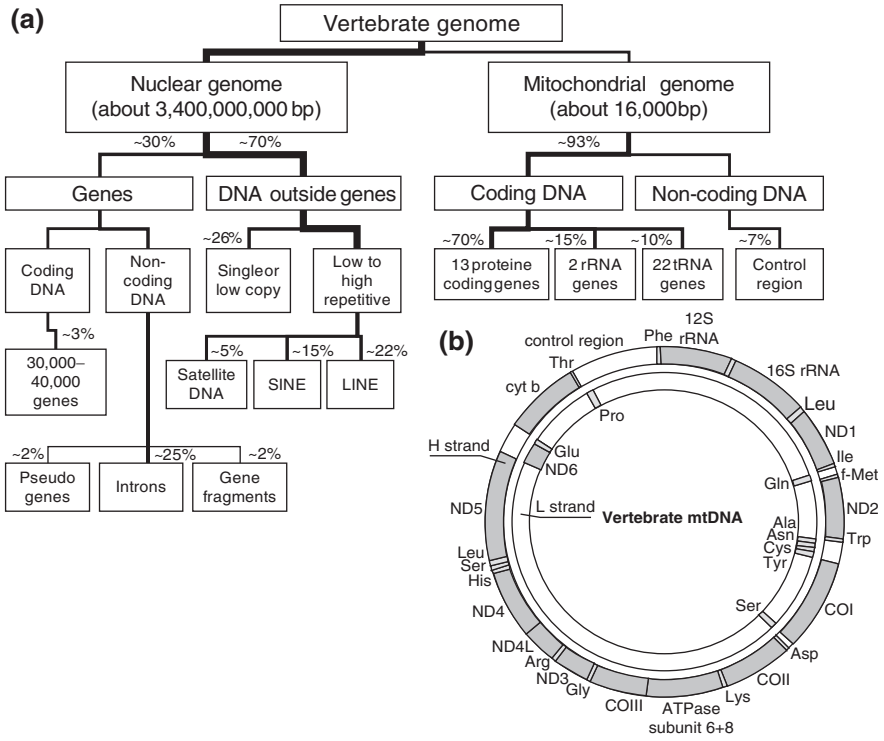

Fig. 24.3 (**a**) Vertebrate genome (Adapted from Haeseler and Liebers 2003); **(b)** Vertebrate mitochondrial genome. Protein and rRNA (dark grey) and tRNA (light grey) genes are located on the outer H (heavy) strand and the inner L (light strand) (Adapted from Page and Holmes 1998)

However, there are some pitfalls, because not all of the features mentioned above can be generalised. In some animals, recombination of mtDNA is known, e.g. in the mussel *Mytilus galloprovincialis* (Ladoukakis and Zouros 2001), and also biparental inheritance was observed (Ballard and Whitlock 2004).

Nuclear mitochondrial pseudogenes (Numts) can be another problem. Numts are copies of mitochondrial genes or fragments of them that have been transferred to the nuclear genome. These Numts evolve much differently than mtDNA and if accidentally utilised as mtDNA in data analysis, the results are flawed (Bensasson et al. 2001). The general notion is that fishes rarely have or do not have Numts at all. However, based on recent complete nuclear genomes of three fish species, the presence of Numts is under debate. An analysis of the complete nuclear genomes of *Fugu rubripes*, *Tetraodon nigroviridis*, and *Danio rerio* showed the presence of several Numts (Antunes and Ramos 2005). However, subsequent analysis of a new release of the complete nuclear genomes of *Fugu rubripes* did not confirm this result (Venkatesh et al. 2006). The authors concluded that methodological problems in shotgun sequencing and sequence assembly lead to the artificial incorporation of mitochondrial fragments in the consensus sequence. Other recent studies, however, indicate the presence of Numts in nuclear fish genomes (Teletchea et al. 2006; Knudsen et al. 2007).

Closely related species can hybridise and if these hybrids backcross, the maternally inherited mitochondrial genome can be passed from one species into the other by introgression (Ballard and Whitlock 2004). These species cannot be differentiated by mtDNA analysis, which is a potential problem in species identification.

The general assumption that mtDNA is a neutral marker is also questioned and it appears that mitochondria are often under selection (Ballard and Whitlock 2004; Bazin et al. 2006). A comparative study on genetic diversity in populations obtained from allozymes, nuclear and mitochondrial sequences did not find a correlation between mitochondrial genetic diversity and population size. In contrast, allozyme and nuclear sequence data showed such a correlation, which is congruent to the assumption in population genetics theory that genetic diversity is proportional to the effective population size. The authors concluded that mtDNA is not neutral and is subject to adaptive evolution (Bazin et al. 2006).

The most applied nuclear markers in population genetics are microsatellites, which are short tandem arrays composed of di-, tri- or tetranucleotide repeats with a length of tens to hundreds of base pairs (Tautz 1989). They are dispersed throughout the nuclear genome, are highly abundant and have a proportion of about 5% together with minisatellites (Fig. 24.3). Supposed to be non-coding and neutral, microsatellites are highly variable in length and are therefore an ideal tool to study intraspecific variation (Wright and Bentzen 1994; O'Connell and Wright 1997). When the flanking regions are known, locus-specific primers can be design and amplification of several microsatellite loci is possible in a multiplex-PCR (O'Connell and Wright 1997). However, microsatellites also have some drawbacks, such as problems in scoring, null alleles, and in some microsatellite loci selection. Problems in scoring can arise when so-called stutter bands are observed that are experimental artefacts caused by slipped-strand mispairing during PCR. It can be

difficult to differentiate the true band from the stutter band. Another problem is null alleles, which are not amplified during PCR due to mutations at the primer biding site. They may contribute significantly to the observed patterns in variation of microsatellites (O'Connell and Wright 1997). An excellent review on microsatellites in fish, including technical details, is provided by O'Connell and Wright (1997).

24.3 Species Identification

24.3.1 Identification of Eggs and Larvae

The identification of species is the prerequisite for any meaningful biological research. The identification of adults, especially of commercially important fish species, is usually not problematic, even though marine cryptic species are frequently revealed by genetic studies (Knowlton 2000). However, the morphological identification of eggs and larval stages of fishes and invertebrates can be extremely problematic and in many cases even impossible. Classical microscopy methods require a high degree of taxonomic expertise, which is currently falling short, and are very time consuming. Therefore, the identification of species is a major bottleneck, hampering the necessary monitoring of marine biodiversity. For instance, about one third of the specimens in 138 studies on invertebrate diversity in European seas were not identified to species level (Schander and Willassen 2005). Even more problematic are the species-rich tropical seas. Even though the adult fish fauna of Indo-Pacific coral reefs is very well investigated (e.g. Randall 1983; Smith and Heemstra 1991; Randall 1995; Randall et al. 1997; Allen 2000), the larval stages of these species are poorly known. The authors of the three standard works for the identification of Indo-Pacific coral reef fish larvae state that the aim of the books is the identification of families, not genera or species (Leis and Rennis 1983; Leis and Trnski 1989; Leis and Carson-Ewart 2000). Also eggs of many fish species are impossible to be distinguished by morphological character (Moser et al. 1984). However, the correct identification of fish eggs and larvae to species level is a prerequisite for proper fish stock assessment based on ichthyoplankton surveys.

A solution for this problem is the application of DNA-based identification methods. They are powerful tools with an unprecedented accuracy due to their inherently highest possible resolution, which can reach even the level of single base changes in a whole genome. Minute amounts of template from eggs or larvae can be amplified by PCR and sequenced or analysed by several other methods.

24.3.1.1 DNA Sequencing

One of the first studies using sequencing for the identification of fish larvae utilised a fragment of the mitochondrial cyt *b* gene (Hare et al. 1994). However, this study showed drastically possible pitfalls of this approach if PCR is not conducted

carefully. The universal primers used did not only bind to the DNA of the fish larvae, but also to human DNA, which lead to contaminations in this study. The PCR with templates from one adult specimen as well as all larvae investigated were contaminated and human cyt *b* sequences were obtained, leading to wrong conclusions about larvae identity as well as phylogenetic relationships (Hare et al. 1996). However, after processing the samples again, sequences from the species under study could be obtained, and larvae were identified by a phylogenetic analysis (Hare et al. 1998). Due to the large amount of sequence data available in international data bases, it is now possible to check if obtained sequences are the result of a contamination.

Other studies also used the approach of sequencing mtDNA for the identification of eel (16S rRNA; Aoyama et al. 1999), rockfish (cyt *b*; Rocha-Olivares et al. 2000) and coral reef fish larvae (control region; Pegg et al. 2006), as well as eggs of alfonsino (16S rRNA; Akimoto et al. 2002). A study on the identification of tuna and billfish larvae based on cyt *b* sequencing implemented an automated high-throughput system with a liquid-handling robot, reducing manual pipetting to a minimum. This system is able to sequence more than 800 specimens per week, reaching an identification success rate of 89% (Richardson et al. 2006).

24.3.1.2 Length Polymorphisms of PCR Products

Another approach is the amplification of a molecular marker with species-specific differences in length that can be detected by electrophoresis. Such species-specific differences in length occur, for example, in the 16S ribosomal RNA (16S rRNA) gene of the mitochondrial genome. This gene has a well-characterised secondary structure (Meyer 1993; Ortí et al. 1996) and especially the highly variable loop regions exhibit many insertions and deletions, so-called indels, which cause species-specific differences in length. Additionally, the mitochondrial 16S rRNA gene exhibits a very low intraspecific, but sufficient interspecific variation to discriminate different species. This was, for example, shown in a study on lionfishes (Kochzius et al. 2003), which revealed that individuals of the same species exhibit identical 16S rRNA haplotypes even though they were sampled at sites thousands of kilometres apart, but clear differences could be detected between closely related lionfish species. The detection of length differences with an automated sequencer was successfully applied for the identification of the very similar eggs of European hake (*Merluccius merluccius*), megrim (*Lepidorhombus whiffiagonis*), and four-spotted megrim (*Lepidorhombus boscii*) (Perez et al. 2005). This was even possible with formaldehyde-fixed eggs, which is usually very problematic due to degradation of DNA and cross-linking of DNA with denatured proteins.

24.3.1.3 Species-Specific Primers

The above-mentioned method utilises the natural variation in length of ribosomal genes by amplifying a certain fragment with a single primer pair. However, differences

in length of PCR products that are detectable in a gel electrophoresis can also be obtained by using species-specific primer pairs in a multiplex-PCR. Species-specific primers for a cyt *b* fragment allowed the identification of eggs and larvae of blue marlin (*Makaira nigricans*), dolphinfish (*Coryphaena equiselis*), shortbill spearfish (*Tetrapturus angustirostris*), swordfish (*Xiphias gladius*) and wahoo (*Acanthocybium solandri*). This methodology is rather simple and does not require sophisticated laboratory equipment and can be therefore set up on a research vessel for shipboard identification of samples within 3 h, allowing researchers to adopt sampling protocols for more efficient study of egg and larval distribution (Hyde et al. 2005). In other studies, multiplex-PCRs amplifying fragments of mtDNA allowed the differentiation of two garfish (control region; *Hyporhamphus* spp; Noell et al. 2001) and four rockfish species (cyt *b*; *Sebastomus* spp; Rocha-Olivares 1998).

24.3.1.4 PCR-SSCP

An even simpler method is the detection of single strand confirmation polymorphism (SSCP) by a polyacrylamide gel electrophoresis (PAGE), which does not even require prior knowledge of the sequence. The detection of SSCP is based on differences in the sequence of the PCR-amplified molecular marker which results in a different secondary structure and mobility in electrophoresis (Sunnucks et al. 2000). Species can be again discriminated by different banding pattern. The feasibility of PCR-SSCP for the identification of fish eggs based on a fragment of the 16S rRNA gene was shown for formaldehyde-fixed eggs of European hake (*Merluccius merluccius*), megrim (*Lepidorhombus whiffiagonis*), Atlantic mackerel (*Scomber scombrus*) and longspine snipefish (*Macrorhamphosus scolopax*) (García-Vásquez et al. 2006).

24.3.1.5 PCR-RFLP

Differences in banding pattern after electrophoresis are also utilised by the RFLP (*restriction fragment length polymorphism*), even though the principle behind it is different. Here, a certain genetic marker is amplified by PCR and the product is digested with restriction enzymes. Due to sequence variation, the restriction enzymes will cut the PCR product at different positions, resulting in species-specific fragments of different lengths that can be visualised by gel electrophoresis. This approach was for example used to identify larvae of five gobiid fishes (Lindstrom 1999) and eggs of three European horse mackerel (*Trachurus* spp) species (Karaiskou et al. 2003) based on restriction digests of PCR-amplified cyt *b* fragments. Using the nuclear locus BM32-2 in a PCR-RFLP, larval billfishes of four species could be identified. A single eye of a 3 mm billfish larvae yielded sufficient DNA for analysis (McDowell and Graves 2001; Luthy et al. 2005).

24.3.1.6 Molecular Probes

An even more difficult task is the identification of digested larval remains in the gut of predatory fish. Such studies can be important to reveal if the recovery of a fish stock is negatively influenced by predation on larval fish. Even though fishing pressure on north-western Atlantic cod (*Gadus morhua*) was reduced after the collapse of the fisheries, stocks did not recover, which could be due to predation on their larvae. However, identification of larval remains is extremely difficult and many times impossible. In addition, larvae of cod can not easily be distinguished from other gadid fish species based on morphological characters. Therefore, an assay based on PCR to amplify a fragment of the 16S rRNA gene and a dot-blot hybridisation procedure was developed to identify larval remains of cod. In a first step, DNA was extracted from homogenised stomach content. Then, a PCR with gadid-specific primers for a fragment of the 16S rRNA gene was conducted and the PCR product was fixed to a nylon membrane. Afterwards, a species-specific probe was hybridised to the immobilised PCR product. Hybridisation of the biotin-labelled probe to the sample was visualised by chemiluminescence. Identification of cod larvae from stomach content was possible with this methodology (Rosel and Kocher 2002). However, the described methodology only provides a qualitative answer and can not quantify the amount of detected larvae. Additionally, this method only unfolds its advantages completely if probes for the detection of several species are utilised. Much more powerful is the species-specific detection of PCR products by immobilised probes on a DNA microarray, which will be discussed later.

24.3.1.7 Real-Time PCR

The latest development in identifying fish eggs and larvae is the use of the TaqMan™ technology, which is a PCR monitored in real time by detecting the signal of a fluorophore (reporter dye) that is covalently bound at the 5′-end of a species-specific oligonucleotide probe. Signal emission of the reporter dye (e.g. FAM, 6-carboxyfluorescein) is suppressed by a so-called quencher dye (TAMRA, 6-carboxy-tetramethylrhodamine) at the 3′-end of the oligonucleotide probe. Since Taq polymerase has a 5′ nuclease activity, it cleaves the non-extendible hybridisation probe during the extension phase of PCR. The reporter dye is released from the quencher dye and its fluorescence signal can be detected, which is a direct measure of the amplification rate (Heid et al. 1996). Using a probe for Japanese eel (*Anguilla japonica*) in a TaqMan™ assay based on the 16S rRNA gene, identification of eggs and leptocephali larvae on board of a research vessel was possible within 3–4 h (Watanabe et al. 2004). Since fluorophores with different emission spectra are available, a multiplex reaction with different probes is possible. This approach was utilised in a survey on the abundance and distribution of cod eggs (*Gadus morhua*) that are difficult to distinguish from whiting (*Merlangius merlangus*) and haddock (*Melanogrammus aeglefinus*). These ‘cod-like’ eggs are usually believed to be cod eggs in ichthyoplankton surveys conducted to estimate the spawning stock biomass

by the annual egg production method (AEPM). Probes for these three species, carrying different reporter dyes, were used in a multiplex PCR. The study revealed that only 34% of 'cod-like' eggs were actually cod, and that 58% were whiting and 8% were haddock, inflating estimates of the cod stock biomass (Fox et al. 2005).

24.3.2 Species Identification in Food Control

Most seafood products are processed and diagnostic features are removed, making species identification based on morphology impossible. Therefore, DNA-based identification methods are the only possibility to identify species. Species identification in food control is important to prevent commercial fraud, because substitution of lower value species for high price species frequently occurs (Sweijd et al. 2000). For instance, the European Union (EU) has strict regulations for seafood labelling, which must include, e.g. the species name (EU Council Regulation No 104/2000; EU Commission Regulation No 2065/2001). However, about 420 species of fish are on the German market alone, making a reliable identification urgently necessary to protect the customer.

24.3.2.1 Isoelectric Focusing of Proteins

Even though this review focuses on DNA-based identification methods, isoelectric focusing (IEF) will be briefly mentioned, because it is a well-established technique for the identification of fish species and regularly used in food control (Rehbein 1990). IEF separates proteins in an electrophoresis along a pH-gradient. Depending on their electric charge, the proteins will move to the anode or cathode through the gel. Due to the pH-gradient the proteins will change their electrical charge until they reach the isoelectric point. At the isoelectric point the protein no longer has a net electrical charge and stops moving through the gel. Therefore, species-specific banding pattern of muscle proteins can be produced with this method. In several countries catalogues for commercial species with IEF banding patterns, as well as photographs and a description of the fish species are available, e.g. France (Durand et al. 1985), Belgium (Bossier and Cooreman 2000), Australia (Yearsley et al. 2001; Yearsley et al. 2003), the United States (Tenge et al. 1993) and Germany (Rehbein and Kündiger 2005). The German data base also contains PCR-RFLP and PCR-SSCP pattern, as well as DNA sequences. However, the banding pattern produced by IEF can be influenced by the freshness of the fillet or fish, type of muscle (light or dark), and conditions of frozen storage (Rehbein 1990), hampering the identification by comparing them with reference banding pattern. Heat-sterilised canned fish, for example can not be identified by IEF, because proteins are severely denatured. Another problem is that protein profiles are not able to differentiate closely related species, e.g. in tuna, sardine and salmon (Mackie et al. 1999). Additionally, IEF requires a certain amount of material, which might not be available in all cases.

Such problems are not encountered in DNA analysis, which requires only minute amounts of DNA that can be amplified by PCR. Therefore, nowadays DNA-based identification is the method of choice, providing a good alternative to protein electrophoresis (Mafra et al. 2007)

24.3.2.2 DNA Sequencing

A study on commercial fraud on the American fish market revealed by sequencing of a 953 bp cyt *b* fragment that three quarters of fish sold as red snapper (*Lutjanus campechanus*) were mislabelled and belonged to other species (Marko et al. 2004). In another study, flatfishes were differentiated successfully based on sequences of a 464 bp cyt *b* fragment (Sotelo et al. 2001). However, both studies showed also the general limitation of such an approach: exact identification is only possible if corresponding reference sequences are available. In order to differentiate four species of anchovies (*Engraulis* spp), a cyt *b* fragment of 540 bp length was sequenced. Analysis of sequences obtained from canned and frozen anchovies identified the species correctly in all commercial samples (Santaclara et al. 2006). A study aiming to identify canned tuna showed that even a short cyt *b* sequences of 126 bp is sufficient to differentiate six tuna species (Quinteiro et al. 1998).

24.3.2.3 Length Polymorphisms of PCR Products

In order to detect the substitution of Greenland halibut (*Reinhardtius hippoglossoides*) for sole (*Solea solea*) fillets, the length polymorphism of the nuclear 5S rRNA gene was utilised. The two species could be clearly distinguished by the size of the PCR fragments in a gel electrophoresis (Céspedes et al. 1999). Length polymorphism in the same gene was also used to identify the three horse mackerel species (*Trachurus* spp) occurring in European seas (Karaiskou et al. 2003).

24.3.2.4 Species-Specific Primers

An identification assay based on species specific primers for a mitochondrial control-region fragment was developed for four Mediterranean grey mullet species (Mugilidae) in order to identify the origin of bottarga (salted and semi-dried ovary product). High-quality bottarga is produced from *Mugil cephalus* in Sardinia, but might be substituted by lower quality products from other species and regions. The developed assay differentiated the four grey mullet species and was even able to confirm the Sardinian origin of bottarga from *Mugil cephalus* (Murgia et al. 2002). Another study on six grouper (*Epinephelus aeneus*, *E. caninus*, *E. costae*, *E. marginatus*, *Mycteroperca fusca* and *M. rubra*) and two substitute species (nile perch, *Lates niloticus* and wreck fish, *Polyprion americanus*) showed that group specific primers targeting the 16S rRNA gene in a multiplex PCR produced fragments of

different length. Grouper species were represented by a 300 bp fragment and its substitute species nile perch and wreck fish by 230 bp and 140 bp, respectively. These fragments could be detected in a gel electrophoresis. By this method, the groupers could be differentiated from the two substitute species, and no cross-reaction with was observed with DNA samples from 41 marketed fish species. However, identification of the grouper species was not possible (Trotta et al. 2005).

A special case in the application of specific primers is the detection of genetically modified coho salmon (*Oncorhynchus kisutch*), which contains an 'all-salmon' gene-construct. The genetic alteration was detected by the presence of a PCR product that was amplified with specific primers annealing within the gene-construct (Rehbein et al. 2002).

24.3.2.5 PCR-SSCP

High-priced tuna species are subject to commercial fraud, especially in canned products (Mackie et al. 1999). Due to the processing, DNA of canned fish is degraded and therefore, only short fragments can be amplified. PCR-SSCP analysis of a 123 bp fragment from the cyt *b* gene allowed the correct identification of eight tuna species in 90% of the cases, even in mixed samples (Rehbein et al. 1999).

PCR-SSCP was also successfully used to differentiate ten salmon species of the genera *Salmo*, *Oncorhynchus* and *Salvelinus* in raw and cold-smoked salmon, as well as salmon caviar. The identification was based on PCR amplified fragments of 300–460 bp length of the mitochondrial cyt *b* and nuclear parvalbumine and growth hormone genes (Rehbein 2005).

24.3.2.6 PCR-RFLP

The most widely applied PCR-based method for the identification of fish species in food control is PCR-RFLP. Several genetic markers are utilised, such as nuclear 5S rRNA (Aranishi 2005) or mitochondrial 16S rRNA (Chakraborty et al. 2007), but species identification is mainly based on fragments of the mitochondrial cyt *b* gene (Mafra et al. 2007). All following examples have utilised this gene.

A fragment of 464 bp length was amplified from a variety of smoked, pickled and heat-treated salmon products. RFLP pattern of the PCR products in a framework of ten species allowed the identification of all commercial samples (Hold et al. 2001). In order to differentiate the three European horse mackerel species, a fragment of about 370 bp length was digested with restriction enzymes, detecting mislabelling of blue jack mackerel (*Trachurus picturatus*) as Mediterranean horse mackerels (*T. mediterraneus*) on the Greek and Italian market (Karaiskou et al. 2003). Based on an amplified 126 bp fragment, six canned tuna species could be identified by RFLP pattern (Quinteiro et al. 1998). A study on flatfishes developed an assay for the identification of 24 species. PCR-RFLP was based on a fragment of 464 bp length, allowing the identification of 11 commercial samples of frozen

fish to species and of three to family level (Sotelo et al. 2001). Investigations on six different sardines and four allied species based on short fragments of 142 and 147 bp allowed the unambiguous differentiation of *Sardina pilchardus* from all other studied species in canned and raw products (Jérôme et al. 2003). Analysis of the closely related anchovies (*Engraulis* spp) by PCR-RFLP based on a fragment of 284 bp length allowed only the discrimination of two species (*E. anchoita* and *E. ringens*) and a species pair (*E. japonicus/Engraulis encrasicolus*), showing the shortcomings of the method. The latter two species could only be identified by DNA sequencing (Santaclara et al. 2006).

In all examples mentioned above, restriction fragments have been separated by conventional gel electrophoresis. In order to enhance the resolution and reproducibility, fragment size analysis was carried out by a lab-on-a-chip capillary electrophoresis. Using this system, the identification of ten fish species based on a 464 bp fragment was possible, also in mixtures of two species (Dooley et al. 2005a, b).

24.3.2.7 Real-Time PCR

As described already above in the paragraph on species specific primers, the aim of a study on six groupers was their discrimination from two substitute species. In order to avoid gel electrophoresis, a conventional real-time PCR assay based on a fragment of the mitochondrial 16S rRNA gene was developed. The different-sized fragments (groupers: 300 bp; nile perch 230 bp; wreckfish: 140 bp) could be detected at the end of the multiplex PCR according to different melting temperatures (Trotta et al. 2005). A dye (e.g. SYBR Green I) is added to the PCR reaction, which only emits a continuously monitored fluorescence signal when intercalated to double-stranded DNA. By gradually heating up the double-stranded PCR products, the two strands will dissociate at a specific melting temperature. The dye will be released from the DNA and the reduced fluorescence signal can be measured. Such a real-time PCR assay could be used for an automated high-throughput system.

24.3.3 Species Identification in Fisheries and Trade

Identification of species is also very important to enforce fishery regulations and international agreements (Sweijd et al. 2000). On the one hand, for many species fishery regulations are implemented that restrict fishing activity in order to reduce the fishing pressure on exploited stocks. On the other hand, for some species the level of exploitation is not even known due to problems in morphological identification, preventing the development of management strategies.

An example is the fishery on sharks, because the catch is usually processed on board of the fishing vessels and morphological features important for identification such as head, fins and tails are removed in order to save space for storage

(Pank et al. 2001; Greig 2005). On the contrary, another problem is the identification of shark fins that receive high prices on the East Asian markets. After removal of the fins, the animals are discarded; making an identification based on morphological characters impossible (Hoelzel 2001; Pank et al. 2001). In order to solve this identification problem for the monitoring of the US Atlantic shark fishery, a 1,400 bp fragment spanning the 3′-end of the 12S rRNA gene, the complete valine tRNA gene, and 5′-end of the 16S rRNA gene was sequenced for 35 shark species, based on archived voucher specimens. This mitochondrial marker showed sufficient variation to discriminate the species under study (Greig et al. 2005) and the data could be used to develop easy-to-handle identification assays. In another study, a multiplex PCR method using the nuclear ribosomal ITS2 region was developed to distinguish two Atlantic shark species (*Carcharhinus plumbeus* and *C. obscurus*) based on size differences of the PCR products (Pank et al. 2001). In order to identify the origin of processed shark products such as dried fins, fin soup or cartilage pills in international trade, the above mentioned approaches using DNA fragments of more than 1,000 bp are not applicable. DNA in these processed products is degraded and therefore such long fragments can not be amplified. In these cases, only shorter fragments of less than 200 bp can be amplified by PCR and used for identification. This approach was chosen in a study by Hoelzel (2001) who sequenced small fragments of 188 bp from the mitochondrial cyt *b* gene to identify the origin of fin soup (hammerhead shark, *Sphyrna lewini*) and cartilage pills (basking shark, *Cetorhinus maximus*).

Other examples for species identification in fisheries assessment and control are North Atlantic sandeels as well as Patagonian and Antarctic toothfish. Sandeels are targeted by an industrial fishery in the North Sea and catches mainly consist of lesser sandeel (*Ammodytes marinus*). However, the occurrence of the other two sandeel species *A. tobianus* and *Gymnammodytes semisquamatus* is not generally quantified, which would be important in assessing the impact of the fishery on these species. In order to address this problem, a PCR-RFLP assay based on a fragment of the mitochondrial 16S rRNA/ND1 gene was developed that could discriminate all North Atlantic sandeel species (Mitchell et al. 1998).

In the Southern Ocean a large fishery with growing catches of toothfish has developed in recent years. Most of the catches are believed to consist of the Patagonian toothfish (*Dissostichus eleginoides*), but since the fishery is extending further South to Antarctic waters, there is also an unregulated fishery on the Antarctic toothfish (*D. mawsoni*), probably exceeding the total allowable catch (TAC) set by the Commission for the Conservation of Antarctic Marine Living Resources. Additionally, toothfish are mislabelled as hake or bass on the market. Since head, gut and tails are removed already on board of the fishing vessels before freezing, and the fish is often processed to filets on land, identification by morphological characters is impossible. Therefore, three molecular identification methods have been developed in order to monitor the fishery on the Antarctic toohfish. On the one hand, two mtDNA-based methods have been utilised: PCR-RFLP of a 16S rRNA fragment and length polymorphism in the control region, both discriminating the two species. On the other hand, isoelectric focusing (IEF) of muscle proteins also enabled the identification of the two species (Smith et al. 2001).

24.4 Stock Identification

A basic concept in fisheries management is the 'sustainable yield', which is a harvestable surplus that can be exploited by fisheries without jeopardising the stock. However, the main problem with this concept is the identification of a stock and the question how to utilise information on stock structure in fisheries management (Carvalho and Hauser 1994). In this review the term 'stock' always refers to the 'biological' or more precisely 'genetic stock' concept, which is defined as a reproductively isolated unit that is genetically distinct.

Several methods for stock identification have been used, such as parasite distribution, morphometrics and meristics, allozymes and DNA analysis. This review will focus on state-of-the-art PCR-based DNA analytical methods such as sequencing and microsatellite analysis. A comprehensive overview on allozyme and RFLP analysis is, for example, provided by Carvalho and Hauser (1994) and Park and Moran (1994).

For many centuries cod (*Gadus morhua*) was an important protein resource for Europe's population, armies and, naval as well as merchant fleets (Kurlansky 1997), but due to steadily increasing fishing pressure the stocks in the North Atlantic declined since the 1970s (Marteinsdottir et al. 2005) and the north-western Atlantic stocks even collapsed in the late 1980s and, early 1990s (Pauly et al. 2002). Atlantic cod is one of the best studied marine fish of commercial importance and genetic studies on stock structure have been conducted for more than 40 years on both sides of the Atlantic Ocean (Marteinsdottir et al. 2005). Therefore, examples of genetic investigations on cod are chosen to highlight the development, power and pitfalls of genetic tools to study genetic stock structure. Emphasis is also given to novel tools for genetic data analysis that go beyond genetic stock identification.

First studies on cod genetic stock structure began in the 1960s by investigating haemoglobin variants (Sick 1961, 1965a, b), but the discovery of selection on this locus made it unreliable for such studies (Mork and Sundnes 1985). A large-scale study throughout the species range utilising allozymes showed a very low amount of genetic differentiation and the most divergent population was from the Baltic Sea (Mork et al. 1985). Allozyme studies in the north-western Atlantic also did not show a significant genetic structure (Pogson et al. 1995).

However, does a very low amount of genetic differentiation reflect a high level of gene flow or limited resolution of the genetic marker? This question could only be answered by studying genetic variation directly on DNA level. The most basic technique to study genetic variation directly on DNA level is RFLP-analysis of mtDNA. A study utilising this technique did not detect a higher level of genetic differentiation and supported former results on the differentiation of Arctic and coastal cod in the north-eastern Atlantic obtained by haemoglobin and allozyme analyses (Dahle 1991). Analysis of partial cyt *b* sequences could detect significant genetic structure between populations in the north-western (Newfoundland) and north-eastern Atlantic (Greenland, Iceland, Faroe Islands, Norway, Baltic and White Sea). Within the north-eastern Atlantic, only the population from the Baltic Sea was

significantly different (Árnason 2004), which was also detected by an earlier study using allozymes (Mork et al. 1985). Samples from the Baltic Sea as well as Faroe Islands showed no significant genetic structure within these regions using cyt *b* sequences (Árnason et al. 1998; Sigurgíslason and Árnason 2003). No significant genetic structure was also found by utilising cyt *b* sequences of cod from several sites in the north-western Atlantic (Carr and Crutcher 1998). Since mtDNA did not provide a sufficient resolution to reveal predicted population structure in cod, nuclear markers, such as nuclear RFLP loci and microsatellites, were applied.

The first utilised nuclear DNA markers were DNA fingerprints (Dahle 1994). They were studied by Southern blot analysis to screen for 17 nuclear restriction fragment length polymorphism (RFLP) loci scored by 11 anonymous cDNA clones (Pogson et al. 1995). In contrast to previous allozyme studies, significant genetic differences between all sites in the northern Atlantic (Nova Scotia, Newfoundland, Iceland, North Sea, Balsfjord and Barents Sea) were detected. A regional study in the north-eastern Atlantic (Nova Scotia and Newfoundland) using the same methodology also revealed significant but weak genetic structure (Pogson et al. 2001). The mean F_{ST}-value, which can reach values from 0 (no structure) to 1 (complete separation of populations), of 10 loci was only 0.014, but the mean value for significant differences was 0.068. Both studies revealed isolation-by-distance (IBD), indicating limited dispersal that contrasts previous results based on allozymes and mtDNA analysis. Another large scale analysis also based on nuclear DNA RFLP variation detected a significant genetic difference between the Barents Sea and all other sites in the north-eastern Atlantic (Celtic Sea, Loftstaahraun, North Sea, Trondheimsfjorden; F_{ST}-values ranging from 0.48 to 0.67) and north western Atlantic (Scotian Shelf; F_{ST}-values ranging from 0 to 0.60). There was also a separation between north-western and north-eastern Atlantic, but pairwise comparison of samples from the Scotian Shelf and Celtic Sea did not show a significant differentiation. No significant differentiation was revealed between samples from the Celtic Sea, Loftstaahraun, North Sea, and Trondheimsfjorden (Jónsdóttir et al. 2003). A small-scale study in southern Iceland indicated sub-structuring of cod populations, which supported earlier tagging experiments (Imsland et al. 2004).

One of these nuclear RFLP loci, pantophysin I (*Pan* I) (originally called GM798 or *Syp* I; Pogson et al. 1995), has two main alleles (*Pan* I^A and *Pan* I^B) that show different frequencies in cod from the Barents Sea and Norwegian coast. The *Pan* I^A allele is predominant among Norwegian coastal cod (allele frequency up to 0.91), whereas the *Pan* I^B dominates in cod from the Barents Sea (allele frequency up to 0.89). F-statistics between groups showed significant values between populations from the Barents Sea and Norwegian coast (0.36) and between populations within the two groups (0.04), indicating a strong genetic stock structure (Pogson and Fevolden 2003). In order to allow rapid and cost-effective genotyping of these two alleles, a PCR with allele specific, fluorescent labelled primers was developed to detect length differences of PCR products from the two alleles by an automated sequencer. This assay can be included in a multiplex PCR for microsatellite analysis in order to reduce costs (Stenvik et al. 2006a). Concordance of the *Pan* I locus with microsatellites supports that genetic structuring observed in the *Pan* I locus is

due to restricted gene flow, even though this locus might be to some extent under selection (Skarstein et al. 2007).

The most used nuclear markers in population genetics of fish are microsatellites. These markers were first developed for cod in the early 1990s (Brooker et al. 1994) and since then a large number of studies using microsatellites for genetic stock analysis of cod were published. Even though many loci are known and utilised, newly developed microsatellites are recently published (Stenvik et al. 2006b; Westgaard et al. 2007), based on data mining of published EST (expressed sequence tags) sequences in Genbank. This approach is very elegant, because time consuming and expensive laboratory work for the development of microsatellites is avoided.

Microsatellite studies on the genetic stock structure of cod on different spatial scales in the north-western Atlantic revealed genetic heterogeneity even on a small geographic scale. Analysis of a larval cod aggregation on the Western Bank of the Scotian Shelf revealed a high genetic heterogeneity, but single cohorts on the basis of age-at-length were genetically homogenous. These results indicate that the different cohorts of larvae originated from different spawning events and that oceanographic processes, such as eddies, retain eggs and larvae within well defined geographic areas on the Scotian Shelf. Comparison with adult cod showed that the genetic structure of the cod larvae was most similar to adults from Western Bank. This study gave interesting insights into reproduction and larval dispersal of cod, suggesting differential reproductive success among spawning groups and local retention of eggs and larvae by eddies (Taggart et al. 1998). A spatio-temporal study showed that a coastal population of cod was significantly different from an offshore population and that the genetic structure of the inshore population was temporally stable over the study period of 4 years (Taggart et al. 1998). A similar pattern was found by nuclear RFLP analysis in the Norwegian Barents Sea (Pogson and Fevolden 2003). A study investigating cod on Georges and Browns Bank as well as Bay of Fundy revealed a small but significant genetic differentiation, which could be attributed to existing gyres and separation of the banks by the deep Fundian Channel (Taggart et al. 1998). Summarising the above-mentioned results, Taggart et al. (1998) concluded that genetic stock structure can be revealed on scales of 60–100 nautical miles. However, a genetic differentiation between Georges and Browns Bank was not detected in another study using microsatellites (Lage et al. 2004). This difference can have two reasons: On the one hand, this difference might reflect a temporal variation; on the other hand, it might be due to methodological differences, because different microsatellite loci have been used in the two studies. Additionally, Lage et al. (2004) applied SNP analysis in the *Pan* I locus. Sampling was also extended further south, showing that the population from Nantucket Shoals was significantly different from Georges and Browns Bank (Lage et al. 2004). A detailed study off Newfoundland and Labrador tested the hypothesis of discrete 'bay stocks' of cod, which could not be confirmed. However, the significant genetic differentiation of coastal and off-shore populations, as well as between different banks was supported (Beacham et al. 2002).

In contrast to tagging experiments, previous genetic studies on cod suggested high dispersal and limited structuring in European Seas. Therefore, genetic stock

structure was re-analysed using microsatellites and revealed formerly undetected genetic differentiation. All European populations were significantly different from populations on the New Scotian shelf and Barents Sea, and within the North Sea, for example, four genetically distinct stocks could be detected (Hutchinson et al. 2001). A large-scale analysis, including samples from the species' whole distribution range revealed a significant difference (F_{ST} = 0.03) between samples from the Scotian Shelf and Baltic Sea to all other sample sites (West and East Greenland, West and East Iceland, Faeroes Ridges, Barents Sea, and Celtic Sea), which indicates three major population groupings: western Atlantic, mid and east North Atlantic, and Baltic Sea (O'Leary et al. 2007). Analysis of samples collected in an area stretching from Spitzbergen to the North Sea using microsatellites and the *Pan* I locus was concordant for both markers in detecting genetic differentiation of north-east Arctic cod (NEAC), Norwegian costal cod (NCC) and North Sea cod (NSC). Both markers also revealed sub-structuring in NCC, but only microsatellites detected genetic differentiation in NEAC and NSC (Skarstein et al. 2007). A significant genetic structure was also revealed between populations from southern Iceland compared to populations from eastern Iceland and the Faroe Islands by using microsatellites and SNP analysis in the *Pan* I locus. The lack of genetic differentiation between cod from eastern Iceland and the Faroe Islands indicated larval exchange between these two regions (Pampoulie et al. 2008). Analysis of stock structure on a scale of 300 km along the Norwegian coast in the Skagerrak revealed a low (F_{ST} = 0.0023) but significant genetic structure (Knutsen et al. 2003). Another study in the same area even detected significant structure on a much smaller scale of only 30 km (Jorde et al. 2007). However, the question remains if these genetically distinct populations, e.g. coastal and offshore populations, are temporally stable. Microsatellite studies on juvenile cod collected in coastal waters of the Skagerrak showed that they are predominantly of North Sea origin in a year with high inflow of North Sea water, whereas in another year they were of local origin. These results indicate that current-mediated larval transport from offshore populations influences the composition of coastal cod populations (Knutsen et al. 2004).

The power of microsatellites in genetic stock structure analysis was demonstrated by assigning cod specimens from the Baltic Sea, North Sea and north-eastern Arctic Ocean to their origin with an accuracy of 97–100% (Nielsen et al. 2001). This application of microsatellite analysis can be a valuable tool in fisheries control to reveal poaching and can also aid control of correct labelling of seafood products.

Another innovative approach in the application of microsatellites and SNP analysis for fisheries genetics is the study of temporal genetic stock structure over several decades by the analysis of DNA extracted from archived otoliths using ancient-DNA techniques. A study covering three decades (1964–1994) revealed a temporally stable genetic stock structure of cod off Newfoundland and Labrador (Ruzzante et al. 2001) using microsatellites. Analysis of the genetic diversity of a population based on microsatellites in the North Sea between 1954 and 1998 showed reduced values between 1954 and 1970, followed by a recovery until 1998 (Hutchinson et al. 2003). Another study applying microsatellites compared the genetic composition of a cod population in the North Sea in 1965 and 2002, as

well as a population in the Baltic Sea in 1928 and 1997. No significant change in allele frequencies was found for the North Sea population, but a small but significant change in the population from the Baltic Sea. However, there was no evidence for a genetic bottleneck in both populations (Poulsen et al. 2006). This study was expanded with samples from the Faroe Bank (1978 and 1992) and Faroe Plateau (1969 and 2002) and additionally to microsatellites, the *Pan* I locus was used. Both markers showed temporal stability in allele frequencies for all studied populations (Nielsen et al. 2007).

Estimation of population size is also an important task in fisheries research and can be supported by fishery genetics. Based on genetic data and genetic models the effective population (N_e) size can be estimated, which is the number of individuals contributing to the gene pool by reproduction. This number can be relatively small compared to the total population, which seems to be the case in cod. A study on two populations in the North Sea and Baltic Sea estimated an effective population size of more than 500, but most probably ranging in the thousands, which is concordant to the reproduction biology of the species. The authors conclude that this effective population size is sufficient to maintain the evolutionary potential of the species and that this number is not likely to be of general concern (Poulsen et al. 2006). In contrast, N_e for another population in the North Sea was estimated to be as low as 69 individuals from 1954–1960 and 121 individuals from 1960–1970 (Hutchinson et al. 2003).

However, there are also some pitfalls in microsatellite analysis. The general notion that microsatellites are neutral markers was questioned by several recent studies, showing that the loci Gmo132, Gmo37, Gmo34 and Gmo8 in cod are not neutral (Nielsen et al. 2006; Skarstein et al. 2007; Westgaard and Fevolden 2007). These findings strongly question some of the results summarised above, especially cases where genetic differentiation was only or mainly found in these loci. This is, for example, the case in the above-mentioned 300 km-scale and 30 km-scale studies at the Norwegian coast, where most of the significant genetic differentiation was attributed to Gmo132 and Gmo37 (Knutsen et al. 2003) or Gmo132 and Gmo34 (Jorde et al. 2007). These examples show that a large number of microsatellite loci should be utilised to detect loci under selection and to ensure that they do not flaw the analysis. Caution is also recommended in cases where genetic differentiation is only detected by one or two loci (Nielsen et al. 2006).

However, the advances in molecular genetics described above completely changed the view on dispersal capability and gene flow in cod from large-scale panmixing to strong genetic stock structure on even very small scales. A comprehensive overview on utilised markers and genetic stock structure of cod is provided in O'Leary et al. (2007). Analysis of more SNPs (Wirgin et al. 2007) and automation (Stenvik et al. 2006a) will enhance genetic studies on cod and enables regular large scale screening of genetic stock structure. Findings and experiences based on cod genetics could be also transferred to other fish species.

As shown above, analysis of genetic stock structure provides information on the level of differentiation between populations. Based on these data, the amount and direction of migration between populations can be estimated by applying genetic

models and statistical methods. The computer programme MIGRATE (Beerli 2008) is a maximum likelihood estimator based on the coalescent theory. It uses a Markov chain Monte Carlo approach to investigate possible genealogies with migration events (Beerli and Felsenstein 2001). Migration and effective population size can be estimated from sequence, SNP, microsatellite and electrophoresis data. In order to study migration patterns of marine ornamental fish from the Red Sea, this method was applied to mitochondrial control region sequence data of lionfish (*Pterois miles*) and fourline wrasse (*Larabicus quadrilineatus*) in the Red Sea. The study on gene flow between populations of the coral reef dwelling lionfish *P. miles* indicated panmixia between the Gulf of Aqaba and northern Red Sea, but analysis of migration patterns showed an almost unidirectional migration originating from the Red Sea proper (Kochzius and Blohm 2005). The genetic population structure of the fourline wrasse *L. quadrilineatus* indicted limited larval dispersal distance of only about 5 km in the Red Sea. Analysis of molecular variance (AMOVA) detected the highest significant genetic variation between northern and central/southern populations ($\Phi_{ct} = 0.012$; $p < 0.001$), and migration analysis revealed several folds higher northward than southward migration, which could be linked to oceanography and spawning season. In order to enable a sustainable ornamental fishery on the fourline wrasse, the results of this study suggest managing populations in the northern and southern Red Sea separately as two different stocks. The rather low larval dispersal distance needs to be considered in the design of marine protected areas to enable connectivity and self seeding (Froukh and Kochzius 2007).

24.5 Emerging Approaches and Technologies

24.5.1 DNA Barcoding

Molecular genetic methods have been widely applied for species identification and phylogenetics of animals, but due to the application of different molecular markers it is impossible to implement a unifying identification system. As also shown above, another problem is the huge variety of utilised methods, ranging from banding pattern in gel electrophoresis to DNA sequences. The different zoological disciplines developed their own traditions, utilising different genetic markers. Ichthyologists focused mainly on the mitochondrial cyt *b* gene, but also used 16S and 12S rRNA genes frequently (Meyer 1993, 1994), whereas research on invertebrates was mainly based on COI (Folmer et al. 1994). This was due to the pioneering works of Kocher et al. (1989) and Folmer et al. (1994), being the first to publish universal primers for a plethora of animal taxa of vertebrates and invertebrates, respectively.

The first ‘standard gene’ for identification and phylogenetics was implemented more than 10 years ago in microbiology, utilising the sequences of the small subunit rRNA gene and setting up the data base ARB (Ludwig et al. 2004). The idea of using a ‘standard gene’ for a global bioidentification system of animals was later proposed

by Hebert et al. (2003a, b), showing that the 'Folmer fragment' (Folmer et al. 1994) of the mitochondrial COI gene can serve as a 'DNA barcode' to identify closely related species and higher taxa in all animal phyla, except cnidarians. The approach of DNA taxonomy, which gives DNA sequences a central role in defining species (Blaxter 2003; Tautz et al. 2002, 2003) provoked a controversial debate and was especially criticised by classical taxonomists (Lipscomb et al. 2003; Mallet and Willmot 2003; Seberg et al. 2003). Currently, taxonomy is in a crisis, facing the lack of prestige and resources (Godfrey 2002) and therefore classical taxonomists feared that funding will only be provided for DNA barcoding (Ebach and Holdrege 2005). However, DNA barcoding rather offers a unique opportunity of bringing together classical taxonomy, genetics and ecology in joint projects – and funding (Gregory 2005). DNA barcoding of metazoans is not reasonable without the expert knowledge in classical taxonomy based on morphology, because a DNA sequence that is not related to a precisely described voucher specimen is not of much value (Schindel and Miller 2005).

Exceptions are very diverse taxa that are not studied well yet taxonomically. In such cases 'molecular operational taxonomic units' (MOTU), also called 'phylospecies' or 'genospecies', can help to retrieve an overview on the genetic diversity, which can be translated in certain limits to taxon diversity (Blaxter 2004; Blaxter et al. 2004, 2005; Floyd et al. 2002). In the marine realm, this approach was for example successfully applied for estimating the diversity of nematods (Blaxter 2004) and stomatopods (Barber and Boyce 2006). However, in order to describe our planet's biodiversity properly in an integrative taxonomy approach, morphological, ecological and genetic data have to be combined (Dayrat 2005). Striking examples of this approach are the discoveries of two new whale species (Dalebout et al. 2002; Wada et al. 2003), a new dolphin species (Beasley et al. 2005) and a new giant clam species (Richter et al. 2008).

DNA barcoding utilises a short DNA sequence from an agreed-upon standard position in the genome for the identification of species. Such DNA barcode sequences usually have a length of 500–700 bp and can be obtained quickly and cheaply. Using a high-throughput sequencing system, the costs for a DNA barcode sequence are less than 1 Euro. Following the suggestion of Hebert et al. (2003a), the 5′-end of the mitochondrial COI gene, which is usually 648 bp long, is the standard barcode region for higher animals. Studies on various groups, such as birds (Hebert et al. 2004a; Kerr et al. 2007), moths and butterflies (Hebert et al. 2003a, 2004b; Hajibabaei et al. 2006a), bats (Clare et al. 2007) and fishes (Ward et al. 2005; Spies et al. 2006) have shown the feasibility of this approach. However, the suitability can not be generalised, because the 5′-end of the mitochondrial COI gene seems to be not well suited for amphibians (Vences et al. 2005), cnidarians (Hebert et al. 2003b) and sponges (Erpenbeck et al. 2005).

DNA barcode sequences are stored in the Barcode of Life Data System (BOLD, www.barcodinglife.org, Hajibabaei et al. 2005; Ratnasingham and Hebert 2007), but also in Genbank (www.ncbi.nlm.nih.gov) where such sequences receive the keyword 'barcode'. Formal barcode sequences have to be related to the following data: (1) species name (although this can be interim), (2) voucher data (catalogue number and institution storing), (3) collection record (collector, collection date and location with GPS coordinates), (4) identifier of the specimen, (5) COI sequence

of at least 500 bp, (6) PCR primers used to generate the amplicon, and (7) trace files (Ratnasingham and Hebert 2007). In January 2008, BOLD contained 335,000 barcode sequences of 47,000 animal, fungi, protist and plant species. However, of these only about 128,000 barcodes of 12,000 species are validated. In Genbank, 11,000 sequences with the keyword 'barcode' were recorded.

Specimens can be identified with the BOLD Identification System (IDS) by submitting a sequence of the 5′end from the COI gene online. This query sequence is aligned to the global alignment of all reference sequences in BOLD. A positive identification is given, if the query sequence matches a reference sequence with less that 1% difference. If such a match could not be found, the query sequence will be assigned to a genus if the difference to a reference sequence is less than 3%. In cases where a query sequence can also not be related to a certain genus, a list with the 100 closest reference sequences is provided, giving information to which higher taxon level the query sequence belongs. Until BOLD is filled with barcodes of all taxa, such cases will appear frequently. However, with increasing taxon coverage, the number of precise identifications will increase. Additionally, a phylogenetic neighbour joining (NJ) tree with the 100 most similar sequences is provided (Ratnasingham and Hebert 2007).

In processed products and forensic samples DNA degradation can prevent the amplification of DNA barcodes that usually have a length of 650 bp. However, 'minimalist barcodes' of about 100 bp length can be obtained from samples with degraded DNA and these short sequences still contain enough information for species assignment (Hajibabaei et al. 2006b).

One of the large barcoding projects is Fish-BOL (Fish barcode of life initiative, www.fishbol.org), aiming to barcode all of the 30,000 known fish species. This initiative was launched in June 2005 on a workshop at the University of Guelph (Canada) with the aim to barcode all marine fish species until 2010. The programme and all presentations can be downloaded at www.fishbol.org/meeting_june05.php. In January 2008, 23,000 barcodes of 4,300 fish species have been generated and about 3,000 are public at BOLD. In Genbank, 1,400 COI sequences of bony fishes with the keyword 'barcode' are available.

However, other projects are also collecting sequence information of fishes and setting up sequence data bases, such as MitoFish (http://mitofish.ori.u-tokyo.ac.jp), FishTrace (www.fishtrace.org), and Fish & Chips (www.fish-and-chips.uni-bremen.de). The aim of MitoFish is the compilation of mitochondrial DNA sequences for evolutionary research (e.g. Saitoh et al. 2006; Lavoué et al. 2007). In January 2008, 82,000 mtDNA sequences from 9,800 fish species, including complete mitochondrial genomes of 395 species, were available in the MitoFish data base. The FishTrace data base provides 2,700 complete mitochondrial cyt *b* and nuclear rhodopsin sequences of 220 commercial fish species from Europe, all of them linked to voucher specimens. In the framework of 'Fish & Chips' (Kochzius et al. 2007a, b), more than 1,400 fragments of the mitochondrial 16S rRNA, cyt *b*, and COI genes from about 80 species have been sequenced as the basis for the development of DNA microarrays for the identification of commercial fishes from European seas (Kochzius et al. 2008). Additionally, the Fish & Chips data base compiled 4,750 16S rRNA, cyt *b*, and COI sequences of European marine fishes

obtained from Genbank. Fish & Chips, FishTrace and MitoFish allow the comparison of query sequences to the reference sequences by using the algorithm BLAST (Altschul et al. 1990), which guides species identification. Currently, the Fish & Chips data base is not public, but it will be freely available as soon as the development of the microarrays is finished.

24.5.2 DNA Microarrays

A DNA microarray is a systematic arrangement of oligonucleotide probes that are immobilised on a solid surface. These probes are complementary to DNA target sequences to be detected (Pirrung 2002). There are many technologies available, differing in fabrication and signal detection. The surface material can be glass, silicon, plastic or metal (Pirrung 2002; Dufva 2005), different surface chemistries for the immobilisation of the probes are available (Benters et al. 2002; Pirrung 2002), and the application of the probes can be done by spotting or in situ synthesis (Pirrung 2002; Dufva 2005). A microarray can contain thousands and even upto many hundred thousands of spots with different oligonucleotide probes, enabling a high redundancy (Dufva 2005). Also different detection systems are available, using electrochemistry (Metfies et al. 2005) or fluorescent dyes (Relógio et al. 2002).

However, currently glass microscope slides with a chemically modified surface on which oligonucleotide probes are spotted are very common. Usually, the DNA target is labelled during PCR amplification with a fluorophore and its hybridisation to the probe on the microarray can be detected with a fluorescence scanner (Fig. 24.4).

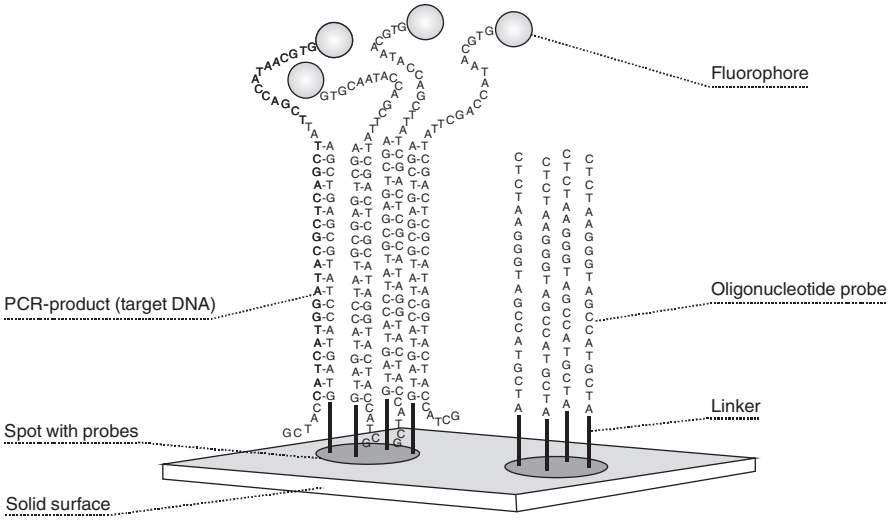

Fig. 24.4 Schematic illustration of a DNA microarray

The application of DNA microarrays for gene expression analysis has already reached the routine level of high-throughput systems (Blohm and Guiseppi-Elie 2001; Hoheisel 2006), but they have been only recently used for the identification of organisms, such as microbes (Wang et al. 2002; Call et al. 2003; Korimbocus et al. 2005; Loy and Bodrossy 2005), plants (Rønning et al. 2005), and animals (Pfunder et al. 2004). In the marine realm, DNA microarrays are used for the identification of bacteria (Peplies et al. 2003; Peplies et al. 2004), phytoplankton (Metfies and Medlin 2004; Metfies et al. 2005; Godhe et al. 2007), invertebrates (Chitipothu et al. 2007), and fishes (Kochzius et al. 2008). Microarrays are also used for gene expression analysis (Williams et al. 2003; Lidie et al. 2005; Wang et al. 2006; Cohen et al. 2007; Jenny et al. 2007) and genotyping of marine organisms in population genetics (Moriya et al. 2004, 2007).

In a fisheries research context, DNA microarrays can be applied for the identification of ichthyoplankton, processed fish in fishery and food control, as well as genotyping for stock identification. There is also a great potential for the application in gene expression analysis for breeding in aquaculture (e.g. Panserat et al. 2008), but this is not in the scope of this review. As shown above in many examples, DNA-based identification is a very important task for fishery genetics. In many cases, especially when analysing environmental samples, a very high number of target species needs to be detected and discriminated against a even much higher number of other species. DNA microarrays are believed to have the potential of identifying hundreds of species in parallel, making them a promising tool. However, the different microarray platforms are still error prone and quantification is difficult (Shi et al. 2006). Even receiving a clear qualitative result, i.e. presence or absence of a certain species, can be sometimes difficult. One methodical limitation is the design of species-specific probes that do not always exhibit the hybridisation properties they were selected for in silico. Therefore, the probes must be empirically tested in hybridisation experiments to ensure that they do not give false-negative or false-positive signals.

An important point in probe design is the choice of the molecular marker. On the one hand, intraspecific variation should be as low as possible to ensure that the designed probes match all individuals of a species and not only a certain populations. Therefore, it is important to obtain sequence information from a wide geographic range. On the other hand, interspecific variation has to be large enough to differentiate closely related species. Since all probes on a microarray will be exposed to the same experimental conditions, their features, such as length (usually 20–30 bp), melting temperature (T_m), and GC content (usually about 50%), have to be more or less identical. Additionally, secondary structures and dimer formations have to be avoided. A comparison of probes for the identification of fishes based on sequences of the mitochondrial 16S rRNA, cyt *b*, and COI genes have shown a very different performance at the same experimental conditions (Kochzius et al. 2007a). Inter- as well as intra-marker signal intensities are very variable, making quantification currently impossible (Kochzius et al. 2007a, 2008). However, a quantification of different species in a mixed sample would be desirable, because this would enable for example the identification and quantification

of eggs in plankton samples. Experiments with known mixtures of amplified target DNA and multiplex-PCRs amplifying the three markers from a mixture of species showed that the parallel identification of fish species is potentially feasible (Hauschild 2008).

DNA-microarrays can also be used for screening genotypes in fish stock identification. In a study on chum salmon (*Oncorhynchus keta*), a microarray was developed to detect 30 known mtDNA control region haplotypes (Moriya et al. 2004). This microarray was applied to screen 2,200 chum salmon to investigate the stock composition in the Bering Sea and North Pacific Ocean (Moriya et al. 2007). The study showed that a rapid and accurate identification of haplotypes with DNA microarrays is possible on board of a fisheries research vessel. Based on this data, the proportion of Japanese, Russian and North American stocks and their geographic distribution was estimated (Moriya et al. 2007). Another application for such a technology is the localisation of the geographic origin of the catch in fisheries control. If a fishery on a species is closed in a certain region, but not in another, fisheries authorities could use such a tool to control the origin of the catch. For instance, the European Union (EU) has strict regulations for seafood labelling, which must include for example geographic origin (EU Council Regulation No 104/2000; EU Commission Regulation No 2065/2001). In cases of a known strong genetic population structure, where certain genotypes can be assigned to certain geographic areas, such an approach is realistic. The recently launched EU-funded research project 'FishPopTrace' (https://fishpoptrace.jrc.ec.europa.eu) aims to obtain such data and to develop methods to trace the geographic origin of commercially important species.

24.5.3 New Sequencing Technologies

Modern sequencing methods were introduced about 30 years ago, with the development of the dideoxy method of Sanger et al. (1977) and the chemical method of Maxam and Gilbert (1977). Since then several other sequencing methods have been introduced, but the refined Sanger sequencing method (Smith et al. 1986; Prober et al. 1987) still remains as the 'gold standard' used in genome sequencing projects and other applications (Marziali and Akeson 2001; Shendure et al. 2004; Metzker 2005; Hudson 2007; Hutchison 2007). However, this 'gold standard' is now challenged by new sequencing technologies, such as 454 sequencing (pyrosequencing; www.454.com), Solexa/Illumina 1G SBS technology (sequencing by synthesis; www.illumina.com), and Agencourt/ABI SOLiD technology (sequencing by oligonucleotide ligation and detection; www.appliedbiosystems.com) (Hudson 2007).

A common principle of these three technologies is the random fragmentation of genomic DNA that is immobilised on a solid support. This could be either microscopic beads (454 sequencing and SOLiD) or a flow cell (SBS technology). Afterwards the immobilised fragments are amplified by PCR in an emulsion

phase (emPCR) that prevents cross-contamination in order to receive clonal PCR products. The single beads are afterwards either placed in microscopic wells (454 sequencing) or are immobilised on a planar surface (SOLiD) and the sequencing chemistry is applied, which differs between platforms. The 454 platform is based on pyrosequencing and detects chemiluminescence signals, Solexa/Illumina applies reversible-terminator sequencing by synthesis, measuring different fluorescence signals for each base, and SOLiD ligates sequence specific labelled oligonucleotides, also using four different fluorophores (Hudson 2007). The main disadvantage of these technologies is the short read length of about 100 bp for the 454 Life Sciences Genome Sequencer GS20, 35 bp for the Solexa SBS technology and only 25 bp for SOLiD. This makes an assembly of hundreds of thousands of short fragments necessary, which is a difficult task that needs new bioinformatic tools to be developed (Jeck et al. 2007; Warren et al. 2007). The new 454 Genome Sequencer FLX has an enhanced read length of 200 bp and if this can be extended to >500 bp, it might succeed Sanger sequencing (Hudson 2007).

Since 454 Life Sciences' Genome Sequencer GS20 was launched in 2005, 100 scientific papers using the technology were published until November 2007 (www.454.com), ranging from de novo genome sequencing of viruses (Thomas et al. 2007) and bacteria (Goldberg et al. 2006) to metagenomics in marine environmental research (Huber et al. 2007) and ancient Neandertal DNA (Green et al. 2006). In comparison, only a few studies using either the Solexa/Illumina 1G SBS or Agencourt/AB SOLiD technology are published and therefore this review will focus on 454 pyrosequencing. Comprehensive general overviews on DNA sequencing technologies are provided by Marziali and Akeson (2001), Shendure et al. (2004), Metzker (2005), Hudson (2007), and Hutchison (2007).

Pyrosequencing is a sequencing-by-synthesis method that detects the incorporation of nucleotides by the enzymatic luminometric inorganic phyrophosphate detection assay (ELIDA) that emits light (Hymann 1988; Nyrén et al. 1993). Since the nucleotides are added subsequently, the light signal can be related proportional to each type of nucleotide (A, T, G or C) and the sequence can be assembled. This method was enhanced in recent years (Ronaghi et al. 1996, 1998) and finally developed into a highly parallel 454 Genome Sequencer, capable of sequencing about 25 million bases in only 4 h, which is about 100 times faster than the conventional Sanger sequencing using capillary-based electrophoresis systems (Margulies et al. 2005). Reagents for one run of the 454 Genome Sequencer cost about US$ 5,000, reducing the cost per base ten times compared to Sanger sequencing (Hudson 2007). Whole genome shotgun sequencing is possible without cloning of DNA fragments into bacterial cells, because fragmented genomic DNA is ligated to linker sequences and single fragments are captured on the surface of a 28-µm bead. Single beads are isolated in an emulsion droplet and amplification is performed. It is also possible to capture PCR-products on those beads. The beads are arrayed in the 1.6 million 75-pl wells of a fibre-optic slide and sequencing is carried out with DNA polymerase by primed synthesis. As described above, incorporation of nucleotides produces a light signal, which is detected by a CCD imager that is coupled with the fibre-optic array. As mentioned earlier, read length reaches about

100 bp, but recent advancements enabled read length of up to 200 bp. In comparison to Sanger sequencing, which reaches a read length of about 700 bp, this is rather short, but due to the extreme high number of parallel sequencing reactions, this disadvantage can be compensated to some degree, because complete sequences can be assembled from an extremely high number of redundant and overlapping fragments (Margulies et al. 2005), demanding considerable computational power. Other sources of error in pyrosequencing are homopolymeric stretches of the DNA, e.g. seven 'C's. The incorporation of seven nucleotides will give a single light signal that will only differ in its intensity compared to a single nucleotide. Up to three identical nucleotides can be detected, but measurements of longer homopolymeric stretches become increasingly inaccurate (Hudson 2007).

Even though 454 sequencing is capable to sequence whole prokaryotic genomes de novo, the technology can still not sequence whole eukaryotic genomes de novo, which are by far more complex. Nevertheless, the 454 technology is well-suited for re-sequencing, because if a 'master sequence' is already known, the small re-sequenced fragments can be aligned to that. Re-sequencing can be utilised for model species, of which whole genomes are available, or their close relatives. Such approaches can give valuable insights into genome-wide intraspecific variation. However, the cost of several hundreds of thousands of dollars still limits the application of whole genome re-sequencing. A much cheaper alternative is the sequencing of expressed sequence tags (ESTs), which focuses on messenger RNA that encodes proteins. Re-sequencing of these specific loci opens an avenue to genome wide SNP screening and genotyping in non-model species (Hudson 2007), which can be used as high resolution markers for population genetics.

The high capacity of parallel sequencing of the 454 technology could also be utilised for simultaneous sequencing of multiple homologous PCR products in stock identification (population genetics) and species identification (DNA barcoding). However, these PCR products can not exceed the current maximum read length of 200 bp, which usually do not contain enough variability for studies in population genetics. An exception is the highly variable mitochondrial control region, showing high haplotype diversity in a fragment of less than 200 bp in lionfish (Kochzius and Blohm 2005). As mentioned above, 'minimalist barcodes' of about 100 bp length are sufficient to identify species (Hajibabaei et al. 2006b) and 454 sequencing is currently tested for barcoding and identification of mixed samples (Hajibabaei 2007). Such an approach could be applied to complete plankton samples in order to identify ichthyoplankton by extracting the complete DNA and performing a multiplex-PCR with universal primer cocktails for fishes (Ivanova et al. 2007; Sevilla et al. 2007). Species identification is then conducted by comparing the sequences to corresponding sequence data bases, such as BOLD or FishTrace. The number of retrieved sequences per species could be used as an indication of its relative abundance or biomass. Such an approach is frequently used in environmental metagenomic studies on microbial communities (Huber et al. 2007). In order to exploit the highly parallel pyrosequencing even further, multiple environmental samples or pooled PCR products can be analysed in a single run. Assignment of the sequences to the different environmental samples or single PCR products from

the pool can be done by specific tags that are part of the PCR primers. These tags are combinations of 2–4 nucleotides that will be sequenced together with the primer and PCR product. The feasibility of this approach was shown for short 16S rDNA sequences that can identify 13 mammal species (Binladen et al. 2007) and multiple environmental samples (Huber et al. 2007).

Even though 454 pyrosequencing is currently too expensive for routine application in fisheries research, it has the potential to revolutionise fisheries genetics in the future, if the technology becomes more cost-effective and cheaper.

24.6 Conclusions

DNA analytics have made tremendous progress in the last decade, facilitating sequencing of the human and other organisms' genome (Hutchison 2007). DNA microarray technology developed to a flourishing field (Blohm and Guiseppi-Elie 2001; Hoheisel 2006) and application of this technology was extended from model organisms to non-model species in gene expression analysis and species identification. PCR technology was advanced to real time PCR (Heid et al. 1996), including the development of the TaqMan™ assay. Just recently, novel DNA sequencing technologies such as 454 pyrosequencing emerged, producing huge amounts of sequence data in a single run by highly parallel sequencing (Margulies et al. 2005; Hudson 2007). Even though 'traditional' PCR-based techniques (e.g. PCR-RFLP, PCR-SSCP) are still used in species identification, the above mentioned novel technologies are more and more utilised (Fox et al. 2005; Kochzius et al. 2008). The concept of DNA barcoding is getting established in the scientific community and the tremendous effort of barcoding all fishes is currently undertaken by an international consortium in the framework of the Fish-BOL initiative. In the field of genetic stock identification, microsatellites have been established as the 'gold standard', but due to enhancements of sequencing technologies and a steadily increasing amount of sequence data, discovery and analysis of SNPs will become more and more important. Today, numerous novel tools and technologies are available to be used in fishery genetics and their application will enable a tremendous progress in this field.

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