Molecular genetic analysis of *Giardia intestinalis* isolates at the glutamate dehydrogenase locus

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SUMMARY

Samples of DNA from a panel of *Giardia* isolated from humans and animals in Europe and shown previously to consist of 2 major genotypes – 'Polish' and 'Belgian' – have been compared with human-derived Australian isolates chosen to represent distinct genotypes (genetic groups I–IV) defined previously by allozymic analysis. Homologous 0·52 kilobase (kb) segments of 2 trophozoite surface protein genes (*tsa417* and *tsp11*, both present in isolates belonging to genetic groups I and II) and a 1·2 kb segment of the glutamate dehydrogenase (*gdh*) gene were amplified by the polymerase chain reaction (PCR) and examined for restriction fragment length polymorphisms (RFLPs). Of 21 'Polish' isolates that were tested, all yielded *tsa417*-like and *tsp11*-like PCR products that are characteristic of genetic groups I or II (15 and 6 isolates respectively) in a distinct assemblage of *G. intestinalis* from Australia (Assemblage A). Conversely, most of the 19 'Belgian' isolates resembled a second assemblage of genotypes defined in Australia (Assemblage B) which contains genetic groups III and IV. RFLP analysis of *gdh* amplification products showed also that 'Polish' isolates were equivalent to Australian Assemblage A isolates (this analysis does not distinguish between genetic groups I and II) and that 'Belgian' isolates were equivalent to Australian Assemblage B isolates. Comparison of nucleotide sequences determined for a 690 base-pair portion of the *gdh* PCR products revealed ⩾ 99·0% identity between group I and group II (Assemblage A/'Polish') genotypes, 88·3–89·7% identity between Assemblage A and Assemblage B genotypes, and ⩾ 98·4% identity between various Assemblage B/'Belgian' genotypes. The results confirm that the *G. duodenalis* isolates examined in this study (inclusive of *G. intestinalis* from humans) can be divided into 2 major genetic clusters: Assemblage A (= 'Polish' genotype) containing allozymically defined groups I and II, and Assemblage B (= 'Belgian' genotype) containing allozymically defined groups III and IV and other related genotypes.

Key words: *Giardia*, protozoa, genetic analysis, polymerase chain reaction, systematics, nucleotide sequences, glutamate dehydrogenase.

INTRODUCTION

Giardia are intestinal parasitic protozoa found in a wide range of vertebrate hosts. The genus currently comprises 5 species – *G. agilis*, *G. ardeae*, *G. duodenalis*, *G. muris* and *G. psittaci* – distinguished on the basis of morphological and electrokaryotypic characteristics (Filice, 1952; van Keulen *et al.* 1993). Isolates classified as *G. duodenalis* have been recovered from several mammalian species but those from humans are usually assigned to a separate species, *G. intestinalis* (syn. *G. lamblia*). Considerable phenotypic and genotypic diversity exists within *G. intestinalis/G. duodenalis* as evidenced by antigenic, isoenzymic and karyotypic heterogeneity among axenic cultures (Nash & Keister, 1985; Korman *et al.* 1986, 1992; Kasprzak, Winiecka & Majewska, 1987; Meloni, Lymbery & Thompson, 1988; Upcroft, Boreham & Upcroft, 1989; Campbell *et al.* 1990; Nash, 1992; Safaris & Isaac-Renton, 1993) and by detection of polymorphisms at the DNA level (Nash *et al.* 1985; Meloni, Lymbery & Thompson, 1989; de Jonckheere, Majewska & Kasprzak, 1990; Nash & Mowatt, 1992; Weiss, van Keulen & Nash, 1992; Morgan *et al.* 1993; Carnaby *et al.* 1994).

Analysis of isoenzyme banding patterns has led to the description of a multitude of zymodemes – essentially 'fingerprints' of individual isolates (Meloni *et al.* 1988; Proctor *et al.* 1989) – which correlate with undefined restriction fragment length polymorphisms (RFLPs) and with random amplified polymorphic DNA differences (Meloni *et al.* 1989; Morgan *et al.* 1993; Thompson & Meloni, 1993). However, distinct genetic groups have been identified by other investigators. Using a combination of antigenic and genetic characteristics, Nash (Nash & Keister, 1985; Nash *et al.* 1985; Nash & Mowatt, 1992) has allocated isolates of axenically cultured *G. lamblia* into 3 genetic groups (1, 2 and 3) which are distinguishable by nucleotide substitutions

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identified at 2 sites within a 183 bp amplified segment of the 18S ribosomal RNA gene (Weiss *et al.* 1992). Andrews *et al.* (1989) identified 4 major genetic groups (I–IV) within axenized Australasian isolates of *G. intestinalis*, using an allozymic interpretation of data obtained from electrophoretic studies of enzymes encoded at 26 loci. By comparing the magnitude of fixed genetic differences that distinguished these groups with the levels (measured by the same technique) that are found between morphologically distinct species in other parasite genera, they proposed that *G. intestinalis* is a species complex comprising at least 2–4 cryptic species. The groups identified by Andrews *et al.* (1989) are supported by RFLPs identified in genomic DNA (Ey *et al.* 1992), by analysis of DNA amplified by the polymerase chain reaction (PCR) from genes encoding cysteine-rich surface proteins (Ey *et al.* 1993*a*,*b*), and by allozyme analysis at 27 loci of a diverse collection of Australian *G. intestinalis* established by growth in suckling mice (Mayrhofer *et al.* 1992, 1995). The latter study revealed the existence of 2 major genetic clusters, designated Assemblage A (containing genetic groups I and II) and Assemblage B (including genetic groups III and IV). Finally, a panel of predominantly European *Giardia* isolates from humans and animals has been classified by Homan and others into 2 major genetic groups ('Polish' and 'Belgian') on the basis of polymorphisms detected by isoenzyme, PCR and RFLP analyses (Homan *et al.* 1990; van Belkum *et al.* 1993). This grouping was consistent with earlier evidence of antigenic (Kasprzak *et al.* 1987) and genetic (de Jonckheere *et al.* 1990) heterogeneity among isolates from the same collection.

With increasing interest in the use of genetic techniques to classify *Giardia* and the obvious value of such information in exploring important clinical issues such as host specificity and pathogenicity, there is a clear need to correlate the genetic groups that have been defined in the various laboratories. This will aid production of a meaningful systematics for the genus and provide a sound genetic basis for comparison of ultrastructural, biochemical, immunological and clinical characteristics in *Giardia* of medical and veterinary significance. We describe herein comparative data from Australian and European isolates which match and define further the genetic groups described by Andrews *et al.* (1989), Homan *et al.* (1992) and Mayrhofer *et al.* (1995).

MATERIALS AND METHODS

Source of Giardia *isolates and isolation of genomic DNA*

The panel of *G. duodenalis*/*G. intestinalis* examined included 40 axenic isolates that had been typed in the Bilthoven laboratory by Homan *et al.* (1992) as belonging to the 'Belgian' genotype (19 isolates) or 'Polish' genotype (21 isolates). These cultures were established from samples collected from human subjects in hospitals in the Netherlands (AMC and Nij isolates; Homan *et al.* (1992), He-1 isolate, W. Homan unpublished), Belgium (LD isolates; Gordts *et al.* 1984), Poland (HP isolates; Majewska & Kasprzak, 1990), Israel (KC-8; isolated by S. H. Korman and described by de Jonckheere & Gordts, 1987), England (VNB-4 and VNB-5; Bhatia & Warhurst, (1981)), the USA (Portland-1, ATCC 30888; Meyer (1976)) and Australia (BAH-8; Meloni *et al.* (1988)). CP-117, GGPRP-114, LSLP-116, SLP-111 and SP-115 originated from animals in a zoo in Poland (Majewska & Kasprzak, 1990; de Jonckheere *et al.* 1990), GP-1 from a guinea-pig in the USA (Fortess & Meyer, 1976). Genomic DNA extracted in the Bilthoven laboratory according to Homan *et al.* (1992) was subjected to analysis in Adelaide, alongside samples of DNA from representative human-derived Australian isolates. The latter panel comprised cloned axenic cultures of isolates Ad-1, Ad-2, Ad-3, Ad-6 and BRIS/83/HEPU/136 (the last obtained from Dr P. Boreham, Queensland Institute of Medical Research, Brisbane), uncloned axenic cultures of isolates Ad-28 and Ad-45; and isolates Ad-7, Ad-19, Ad-52, Ad-62 and Ad-121 which were propagated by growth in suckling mice (Andrews *et al.* 1989, 1992; Mayrhofer *et al.* 1992, 1995; Ey *et al.* 1992, 1993). Axenic isolates were cultured at 37 °C in modified TYI-S-33 medium as described (Andrews, Chilton & Mayrhofer, 1992; Homan *et al.* 1992). Isolates used as standards for the allozyme-defined genetic groups I, II, III and IV of Andrews *et al.* (1989) were clones of Ad-1, Ad-3 and Ad-6 (all group I), Ad-2 and Bris-136 (both group II) (Ey *et al.* 1992, 1993*b*), and uncloned isolates Ad-19 (group III-like), Ad-7, Ad-28 and Ad-52 (group IV-like), as described elsewhere (Mayrhofer *et al.* 1995) and indicated in Table 1.The geographic origin (actual or deduced) and host origin of each isolate is indicated in Table 1.

Polymerase chain reactions (PCR)

Amplification of 0·52 kb segments from tsa417 *and* tsp11*-like genes.* This assay, described by Ey *et al.* (1993*a*), uses as PCR primer sites sequences that are conserved within the homologous promoter-distal portions of the genes encoding trophozoite surface antigen 417 (*tsa417*) and trophozoite surface protein 11 (*tsp11*). Trophozoites belonging to allozyme-defined genetic groups I or II possess both genes (Ey & Mayrhofer, 1993; Ey *et al.* 1993*b*) and the 0·52 kb DNA amplified from isolates of either genotype using oligonucleotides 432 (5′ primer) and 433 (3′ primer) is a mixture of sequences corresponding to *tsa417* (*Hin*d III$^-$, *Pst* I$^+$, *Kpn* I$^+$) and *tsp11*

(*Hind* III$^+$, *Pst* I$^-$, *Kpn* I$^-$). Group I and group II genotypes can be differentiated by the use of *Pst* I, which detects a novel RFLP specific to group II isolates (Ey *et al.* 1993*b*). Human-derived isolates belonging to genetic groups III or IV yield only trace amounts of a smaller (0·37 kb) amplification product in this assay (Ey *et al.* 1993*a*).

Amplification of a 1·2 kb segment of the glutamate dehydrogenase gene. To facilitate comparisons across the entire genus, the gene for glutamate dehydrogenase (gdh) was chosen as a genetic marker. Consensus sequences of conserved segments, identified near the promoter proximal and promoter distal portions of the single published giardial *gdh* gene (from the Portland-1 isolate of *G. duodenalis*, Yee & Dennis (1992)) and homologous *gdh* sequences from *E. coli* and *Chlorella sorokiniana* obtained from the NCBI Genbank database, were used to design 2 PCR primers (oligonucleotides 578 and 579; Fig. 1) which were synthesized on an Applied Biosystems 3181A DNA synthesizer. Amplifications (95 °C for 4 min, then 30 cycles comprising 30 s at 94 °C, 30 s at 56 °C and 2 min at 72 °C, followed by a final extension at 72 °C for 6 min) were performed on an FTS-320 thermal cycler (Corbett Research, Sydney) in reaction volumes of 50 μl containing 1 × *Tth* reaction buffer (67 mM Tris–HCl, 16·6 mM $(NH_4)_2SO_4$, 0·45% Triton X-100, 0·2 mg/ml gelatin, pH 8·8; Biotech International Ltd, Perth, W.A.), 4 mM $MgCl_2$, 0·2 mM of each dNTP, 0·8 μM of each primer, 5% dimethylsulphoxide; 1 unit of *Tth* '*plus*' DNA polymerase (Biotech International) and *Giardia* DNA (50–200 ng). As expected (Benachenhou-Lahfa, Forterre & Labedan, 1993), no amplification product was obtained using template DNA from mammals (human, mouse, rat). A PCR product of the expected size (1·17 kb) was obtained using genomic DNA from Gram-negative bacteria (*E. coli*, *V. cholerae*) and Gram-positive bacteria (*C. glutimacum*, *B. subtilis*), but restriction site differences distinguished these products from those amplified from *Giardia*. Furthermore, nucleotide sequences determined for products amplified from different axenic isolates of *G. duodenalis* differed at $< 12\%$ of nucleotide positions, whereas all of these sequences differed from published bacterial *gdh* gene sequences by $> 37\%$. DNA extracted from gut washings of *Giardia*-free suckling mice failed to yield any detectable *gdh* PCR product, indicating that contamination with gut microflora during harvesting of trophozoites was unlikely to be a significant problem in assays using DNA extracted from *Giardia* grown in suckling mice (Mayrhofer *et al.* 1992). To minimize sequencing errors arising from polymerase infidelity during PCR, uncloned amplified DNA (purified using BresaClean, Bresatec Ltd, Adelaide) was used in the sequencing reactions, which utilized *Taq* DNA polymerase and fluorescent dideoxynucleotides (Prism Ready Reaction Dye Deoxy Terminator Cycle sequencing kit, Applied BioSystems Inc.) in conjunction with oligodeoxynucleotide primers 578 (mentioned above), 862 (5′-AGTACGCGACGCTGGGATACT-3′), 913 (5′-ATGACCGAGCT(T/C)CAGAGGC-3′) or no. 914 (5′-TGAACTCGTTCCTNAGGCG-3′). Sequences were determined by automated analysis (Applied BioSystems 373A DNA sequencer), collated using the editing software SeqEd, and aligned using CLUSTAL V (Higgins, Bleasby & Fuchs, 1992). Phylogenetic analyses were performed using version 1.02 of the Molecular Evolutionary Genetics Analysis software (*MEGA*) of Kumar, Tamura & Nei (1993).

Detection of restriction fragment length polymorphisms (RFLPs)

Aliquots of PCR reaction mixtures were incubated overnight at 37 °C with 2 units of restriction endonuclease (Boehringer-Mannheim) in 20 μl of the appropriate 1 × digestion buffer. Cleavage was assessed by subjecting samples to electrophoresis in 1% agarose gels using Tris-borate-EDTA (TBE) buffer and staining the DNA with ethidium bromide. Fragment sizes were calculated from electrophoretic mobilities by regression analysis using DNASIS, utilizing *Eco*R I cleavage fragments of *Bacillus subtilis* bacteriophage SPP-1 DNA as size standards.

RESULTS

Amplification and analysis of 0·52 kb segments from the tsa417 *and* tsp11 *genes*

Samples of DNA from the different *Giardia* isolates were screened initially for amplification of 0·52 kb segments of the trophozoite surface protein genes *tsa417* and *tsp11*, as these segments are known to be conserved among isolates of *G. intestinalis* belonging to allozymically defined groups I or II (Ey *et al.* 1993*a*,*b*). The size and yield of the DNA amplified using this assay is summarized in Table 1, together with biographical information on each isolate. Australian isolates representing group I (Ad-1, Ad-3, Ad-6) or group II (Ad-2, Bris-136) of Assemblage A (Mayrhofer *et al.* 1995) all yielded the expected 0·52 kb PCR products in high yield, whereas 5 isolates belonging to Assemblage B (Ad-19, which has close affinity with allozymically defined genetic group III, and isolates Ad-7, Ad-28, Ad-45 and Ad-52 which have affinity with allozymically defined genetic group IV) each yielded only a 0·37 kb product (described previously – Ey *et al.* 1993*a*) in trace amounts. A sixth Assemblage B isolate (Ad-121) yielded a 0·52 kb product – barely detectable in

Table 1. Classification of *Giardia* isolates by the size and RFLP pattern of DNA amplified by PCR from *tsa417* and *tsp11* surface protein genes

Isolate(s) and clone no.	Geographic origin/site of infection	Host origin	Previously established genotype*	PCR product (bp)†	Genotype predicted by RFLP‡
Ad-1 no. 1	Australia	Human	A-I	**520**	(Standard)
Ad-3 no. 2, Ad-6 no. 1	Australia	Human	A-I	**520**	(Standard)
Ad-2 no. 2	Australia	Human	A-II	**520**	(Standard)
Bris-136 no. 2	Australia	Human	A-II	**520**	(Standard)
Ad-19	Australia	Human	B-(III)	370 (f)	(Standard)
Ad-7, -28, -45	Australia	Human	B-(IV)	370 (f)	(Standard)
Ad-52	Australia	Human	B-(IV)	370 (f)	(Standard)
Ad-121	Australia	Human	B	520 (f)	(mix?)§
AMC-6	Nicaragua	Human	'Polish'	**520**	A-I
AMC-7	Indonesia	Human	'Polish'	**520**	A-I
GP-1	USA	Guinea-pig	'Polish'	**520**	A-I
HP-42, -88, -98	Poland	Human	'Polish'	**520**	A-I
HP-100, -101, -108	Poland	Human	'Polish'	**520**	A-I
LD-1	Africa	Human	'Polish'	**520**	A-I
Nij-2 no. 8	Netherlands	Human	'Polish'	**520**	A-I
Portland-1	USA	Human	'Polish'	**520**	A-I
SLP-111	Poland/S.E. Asia	Slow loris	'Polish'	**520**	A-I
VNB-4, VNB-5	England	Human	'Polish'	**520**	A-I
Nij-1	Netherlands	Human	'Polish'	**520**	A-II
AMC-1	Africa	Human	'Polish'	**520**	A-II
AMC-12	Chile	Human	'Polish'	**520**	A-II
AMC-13	Netherlands	Human	'Polish'	**520**	A-II
HP-10	Poland	Human	'Polish'	**520**	A-II
KC-8	Israel	Human	'Polish'	**520**	A-II
AMC-2, -3, -4, -5	Africa and Neth.	Human	'Belgian'	(–)	[not 'A']
AMC-9	India	Human	'Belgian'	(–)	[not 'A']
BAH-8	Western Aust.	Human	'Belgian'	370/520 (f)	B [+A mix?]§
CP-117,	Poland	Cuis (rat)	'Belgian'	370/520 (f)	B [+A mix?]§
GGPRP-114	Poland/Africa	Pouched rat	'Belgian'	370 (f)	B
He-1	Netherlands	Human	'Belgian'	370 (f)	B
LD-18, -19	Africa	Human	'Belgian'	(–)	[not 'A']
LD-20, -21, -22, -26	Africa	Human	'Belgian'	570 (f)	[not 'A']
LSLP-116	Poland	L. slow loris	'Belgian'	370 (f)	B
Nij-4 no. 2, Nij-5 no. 7	Netherlands	Human	'Belgian'	370 (f)	B
SP-115	Poland	Siamang	'Belgian'	370 (f)	B

* Assemblage A (containing genotypes I and II) and Assemblage B (containing genotypes III and IV) as defined by Mayrhofer *et al.* (1995); 'Polish' and 'Belgian' genotypes as defined by Homan *et al.* (1992).
† Bold type '520' indicates product obtained in high yield; (f) = faint band(s), product(s) detected in trace amount only; (–) = no product detected.
‡ Amplification of 520-bp products identifies isolates as belonging to genetic Assemblage A; RFLP analysis using *Hin*d III, *Kpn* I and *Pst* I determined whether Assemblage A isolates belonged to allozymically defined genetic groups I or II. Australian isolates used as genotypic standards are denoted as 'Standard'.
§ Possible mixtures (predominant 'B' genotype and a minor 'A' genotype).

comparison with the strong band observed for Assemblage A isolates – indicating either the presence of a second (Assemblage A) genotype or suboptimal amplification of a distantly related sequence. Of the 21 isolates typed as 'Polish' (Homan *et al.* 1992), all yielded a strongly stained 0·52 kb band, showing that they were similar to Assemblage A isolates from Australia (Table 1). Subsequent RFLP analysis of the amplified DNA revealed only 2 patterns – one with the restriction pattern characteristic of allozymically defined genetic group I (15 isolates), the other with the pattern characteristic of genetic group II (6 isolates).

When the *tsa417/tsp11* PCR was applied to the 19 isolates typed (Homan *et al.* 1992) as 'Belgian', only 2 isolates (BAH-8 and CP-117) yielded any detectable 0·52 kb product. However, in both cases this was present in trace amounts only, together with a faintly stained 0·37 kb product (Table 1). These cultures probably contain a mixture of genotypes. Six isolates yielded only a 0·37 kb amplified DNA (also in trace quantities), 4 yielded small amounts of a single 0·57 kb product, whilst the remaining 7 isolates gave no detectable product (Table 1). These results confirm that the 'Belgian' group is distinct from the 'Polish' group and indicate that it is

	Forward Region	Reverse Region
Portland-1:	GAGAGGATGCTTGAGCCGGAGCGCGTCATC	GGCGCGAACATCGCCGGGTTCCTGAAGGT
Chlorella:	A..CA...CG.....T.....G...	.....C.....G..C...ACC.....
***E. coli*:**	...C.TC..G....A.....G...	.....T.....T..TG.....
Consensus:	VMRMDKVTGBYTGWDCCDGARMGMRTSATM	GGYGCBAAYATYGCVRGBTTYVYSAAGGT
PRIMERS:	GAGAGGATCCTTGARCCNGAGCGCGTNATC	CCGCGNTTGTADCGNCCNAAGATCTTCCA
	5' ——————————> 3'	3' <—————————— 5'
	OLIGO #578	**OLIGO #579**

Fig. 1. Promoter proximal and promoter distal segments of glutamate dehydrogenase genes from *Giardia duodenalis* (Portland-1; Yee & Dennis (1992); GenBank accession number M84604), *Escherichia coli* (K02499) and *Chlorella sorokiniana* (X58831) aligned using CLUSTAL V (Higgins *et al.* 1992) and selected as PCR primer sites for the amplification of a 1·17 kb gene segment (inclusive of primers) from various *Giardia* isolates. The consensus sequence and derived oligodeoxynucleotide primer sequence is shown for both forward and reverse regions (the latter depicted as the complementary strand), corresponding to nucleotides 145–174 and 1288–1316 respectively of the Portland-1 *gdh* coding sequence. For the single letter code, B = C, G or T, D = A, G or T, K = G or T, M = A or C, N = A, C, G or T, R = A or G, S = C or G, V = A, C or G, and Y = C or T.

Table 2. Restriction characteristics of the 1·2 kb segment of the glutamate dehydrogenase gene amplified by PCR from isolates of *Giardia duodenalis*

Isolate	Known genotype*	Cleavage† observed by:						RFLP type‡
		Apa I	*Eco*R I	*Kpn* I	*Bsp*H I	*Sac* I	*Xho* I	
Ad-1 no. 1	A-I	−	+	+	+	+	+	A
Ad-3 no. 2	A-I	N.D.	+	+	+	+	+	A
Ad-6 no. 1	A-I	N.D.	+	+	+	+	+	A
Ad-2 no. 2	A-II	−	+	+	+	+	+	A
Ad-62	A-(IIa)§	N.D.	+	+	+	+	+	A
Bris-136 no. 2	A-II	−	+	+	+	+	+	A
Ad-7	B-(IV)	+	−	−	−	+	+	B
Ad-19	B-(III)	+	−	−	−	+	+	B
Ad-28	B-(IV)	+	−	−	−	+	+	B
Ad-52	B-(IV)	+	−	−	−	+	+	B
Ad-121	B	+	−	−	−	+	+	B
AMC-1	'Polish' (A-II)	N.D.	+	+	+	+	+	A
Nij-1	'Polish' (A-II)	−	+	+	+	+	+	A
Nij-2 no. 8	'Polish' (A-I)	−	+	+	+	+	+	A
Portland-1	'Polish' (A-I)	−	+	+	+	+	+	A
AMC-3	'Belgian'	+	−	−	−	+	+	B
LD-19	'Belgian'	N.D.	−	−	−	+	+	B
LD-20	'Belgian'	+	−	−	−	+	+	B
LD-21	'Belgian'	N.D.	−	−	−	+	+	B
LSLP-116	'Belgian' (B)	+	−	−	−	+	+	B
Nij-5 no. 7	'Belgian' (B)	N.D.	−	−	−	+	+	B

* Established from previous studies. Where possible, the allozymically defined genetic group classification of 'Polish' and 'Belgian' isolates (predicted from Table 1) is also shown.
† + = cleavage; − = not cleaved; N.D. = not determined.
‡ Genotype (Assemblage A or Assemblage B) of characterized Australian isolates or predicted from RFLP patterns obtained for 'Polish' and 'Belgian' isolates.
§ Allozymic analysis has shown the genotype of Ad-62 to be distinct from genetic groups I and II, but with greater affinity with group II.

heterogeneous – containing at least 3 subgroups, one of which shares the 0·37 kb amplified sequence with the majority of the group III and group IV genotypes detected in Australia. A second subgroup (containing the isolates which yield no *tsa417/tsp11* PCR product) may be similar to Australian isolates represented by Ad-121.

Comparison of isolates by RFLP analysis of DNA amplified from the gdh *gene*

The preceding findings, together with previous studies (Ey *et al.* 1993*a*, *b*) indicate that the products amplified using PCR primers complementary to sequences in the *tsa417* and *tsp11* genes are useful for

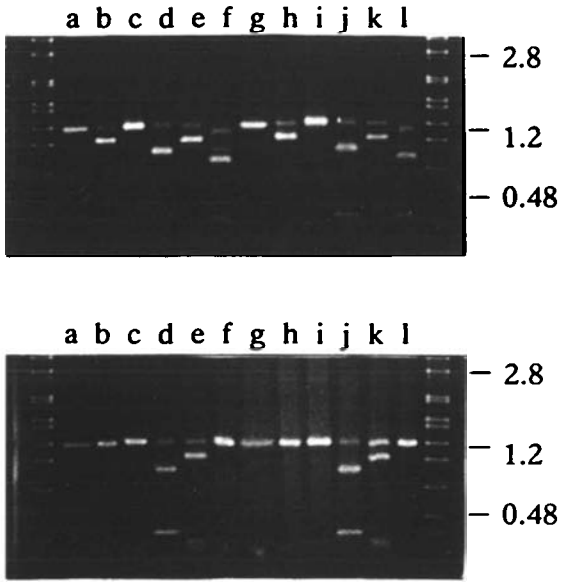

Fig. 2. Detection of assemblage-specific RFLPs in the glutamate dehydrogenase gene. Samples of 1·2 kb *gdh* DNA amplified by PCR from *Giardia intestinalis* isolates belonging to Assemblage A (top panel: Ad-2, Lanes a–f; AMC-1, Lanes g–l) or to Assemblage B (lower panel: Ad-52, Lanes a–f; LD-19, Lanes g–l) were assessed electrophoretically for cleavage after being incubated in buffer only (Lanes a, g) or with *Eco*R I (b, h), *Bam*H I (c, i), *Sac* I (d, j), *Xho* I (e, k) or *Bsp*H I (f, l). Marker DNA (bacteriophage SPP-1, cleaved by *Eco*R I) was applied to both side lanes, with 2·81 kb, 1·16 kb and 0·48 kb fragments indicated at right.

distinguishing genetic groups within Assemblage A and also for distinguishing Assemblage A isolates lying beyond this cluster, i.e. *Giardia* belonging to other genetic clusters, including Assemblage B. However, the assay is of uncertain value in analysing genetic differences within Assemblage B, because the products amplified from various isolates have not been characterized, while other isolates fail to yield an amplification product. Further genetic characterization of isolates that do not belong to Assemblage A requires, therefore, comparisons based on more highly conserved genetic loci. For this purpose, we synthesized oligonucleotide primers suitable for amplifying a 1·2 kb segment of the glutamate dehydrogenase gene in *Giardia* (Fig. 1). As described in the Materials and Methods section, a single 1·2 kb amplification product was obtained from every isolate tested, but only when the reaction was carried out in the presence of dimethylsulphoxide. DNA amplified from each of a panel of isolates selected from Table 1 was incubated with several different restriction endonucleases (chosen on the basis of cleavage sites identified within the published Portland-1 *gdh* sequence) to determine whether genotype-specific RFLPs could be detected.

As shown in Table 2, RFLP patterns identical to those predicted from the Portland-1 sequence (single cleavage sites for *Bsp*H I, *Eco*R I, *Kpn* I, *Sac* I and *Xho* I) were obtained for all tested isolates that belong to Assemblage A (Ad-1/clone 1, Ad-3 clone 2 and Ad-6/clone 1, group I; Ad-2/clone 2 and Bris-136/clone 2, genetic group II; Ad-62, genetic group II-like, Mayrhofer *et al.* (1995)) and the 'Polish' group (Nij-2/clone 8 and Portland-1, predicted genetic group I; AMC-1 and Nij-1, predicted genetic group II). In contrast, all of the Australian isolates belonging to Assemblage B (Ad-7, Ad-19, Ad-28, Ad-52 and Ad-121) and all of the 'Belgian' group of isolates (AMC-3, LD-19, LD-20, LD-21, LSLP-116 and Nij-5/clone 7) showed a distinct and invariant RFLP pattern, with cleavage by both *Sac* I and *Xho* I but not by *Eco*R I, *Kpn* I or *Bsp*H I. Representative RFLP patterns obtained for the DNA amplified from the *gdh* genes of isolates Ad-2, AMC-1, Ad-52 and LD-19 are shown in Fig. 2. In tests with other endonucleases (*Apa* I, *Bam*H I, *Hind* III, *Pst* I, *Sca* I) whose recognition sequences do not occur within the Portland-1 sequence, only *Apa* I cleaved amplification products from Assemblage B and 'Belgian' isolates (Table 2) – at a single common site, based on fragment sizes.

Nucleotide sequence alignments

The nucleotide sequences, determined for a portion of the 1·2 kb segment of the *gdh* gene amplified from 8 isolates, are shown aligned in Fig. 3. Inspection of the data reveals the following points. (1) Isolates Portland-1 and Ad-1 (both genetic group I) are identical over the full length of the alignment (690 nucleotides), which encompasses 4 of the 6 restriction sites listed in Table 2. (2) The sequence obtained for the Ad-2 isolate (genetic group II) differs from the Portland-1/Ad-1 sequence by single nucleotide substitutions at 7 sites (1·0%), all comprising transitions at codon third-base positions. (3) Sequences obtained for 5 Assemblage B isolates (Ad-7, Ad-121, AMC-3, LD-20 and LSLP-116 – representing all 3 subtypes detected by *tsa417/tsp11* PCR) differ at $\leqslant$ 1·6% of nucleotide sites. Therefore, only minor polymorphic differences exist between these isolates at this locus. (4) The latter sequences differ from those of Assemblage A isolates at 10·6–10·9% of the 690 nucleotide sites shown in Fig. 3 (or at 10·3–11·7% of the 498 sites determined for AMC-3 and LSLP-116). Of the 79 nucleotide substitutions (relative to the Portland-1 sequence) evident in Fig. 3, 66 (83·5%) are fixed mutations that distinguish Assemblage A from Assemblage B and 58 (87·9%) of these occur at codon third-base positions (56 as synonymous substitutions). This excludes DNA polymerase infidelity as a possible cause of these substitutions, as replication errors *per se* should show no codon site bias.

Phylogenetic analysis of the aligned sequences shown in Fig. 3 yielded inter-assemblage distance

```
P-1,Ad-1   ATCTTCCGCGTGCCCTGGATGGATGACGCTGGACGCATCAACGTCAACCGCGGCTTCCGTGTCCAGTACAACTCTGCTCTCGGCCCCTACAAGGGTGGCC  100
Ad-2       ..................................................................................................
Ad-7       ..G.....T..C..........C.....C.....................................A.............C........G.............T.
Ad-121     ..G.....T..C..........C.....C.....................................A.............C........G.............T.
LD-20      ..G.....T..C..........C.....C.....................................A.............C........G.............T.

P-1,Ad-1   TCCGCTTCCACCCCTCTGTCAATCTTTCGATTCTCAAGTTCCTCGGTTTCGAGCAGATCCTGAAGAACTCCCTCACCACGCTCCCGATGGGCGGCGGCAA  200
Ad-2       ..................................................................................................
Ad-7       ......................C..C.....C..T..........C..T.....................T........T...........T..T..
Ad-121     ......................C..C.....C..T..........C..T.....................T........T...........T..T..
LD-20      ......................C..C.....C..T..........C..T.....................T....................T..T..
LSLP-116   ........................C.....C..T..........C..T.....................T....................T..T..
AMC-3      ........................C.....C..............C..T.....................T....................T..T..

P-1,Ad-1   GGGCGGCTCCGACTTTGACCCAAAGGGCAAGTCCGACAACGAGGTCATGCGCTTCTGCCAGTCCTTCATGACCGAGCTCCAGAGGCACGTCGGCGCCGAC  300
Ad-2,      ..................................................................................................
Ad-7       ..............C..T..T..........G.....................................T.....................G..T...
Ad-121     ..............C..T..T..........G.....................................T.....................G..T...
LD-20      ..............C..T..T..........G.....................................T.....................G..T...
LSLP-116   ..............C..T..T..........G.....................................T.....................G..T...
AMC-3      ..............C..T..T..........G.....................................T.....Y...............G..T...

P-1,Ad-1   ACTGACGTTCCTGCCGGCGACATCGGCGTCGGCGCCCGCGAGATCGGGTACCTGTACGGACAGTACAAGCGCCTGAGGAACGAGTTCACAGGCGTCCTCA  400
Ad-2       ..................................................................................................
Ad-7       ..C..........T.....T..T.........GT..........T..T....TT........T........C..........T..G.........
Ad-121     ..C..........T.....T..T.........GT..........T..T....TT........T........C..........T..G.........
LD-20      ..C..........T.....T..T.........GT.....Y..T..T....TT........Y........C..............G.........
LSLP-116   ..C..........T.....T..T.........GT..........T..T....TT........T........C..............G.........
AMC-3      ..C..........T.....T..T.........GT..........T..T....TT........T........C..............G.........

P-1,Ad-1   CAGGCAAGAACGTCAAGTGGGGCGGGTCTTTCATCAGGCCGGAGGCCACGGGCTATGGCGCTGTCTACTTCCTGGAGGAGATGTGCAAGGACAACAACAC  500
Ad-2       ..........................C.................T.....................................................
Ad-7       .G.........A.................C.......A..A........A..G.....A.....................................T........
Ad-121     .G.........A.................C.......A..A........A..G.....A.....................R.....T.....
LD-20      .R.........A..........T......C.......A..A........A..G.....A.....................................T........
LSLP-116   .G.........A.................C.......A..A........A..G.....A.....................................T........
AMC-3      .G.........A.................C.......A..A........A..G.....A.....................................T........

P-1,Ad-1   TGTGATCAGGGGTAAGAACGTCCTTCTTTCTGGCTCCGGCAACGTTGCCCAGTTTGCTTGCGAGAAGCTCATTCAGCTCGGCGCAAAGGTCCTCACCTTC  600
Ad-2       ........................C.....................................................T.....................
Ad-7       C..A........C...........C..C.........T..........T....AC..G...........C.C........T..G..............
Ad-121     C..A........C...........C..C.........T..........T....AC..G...........C.C........T..G..............
LD-20      C..A........C...........C..C.........T..........T....AC..G...........C.C........T..G..............
LSLP-116   C..A........C...........C..C.........T..........T....AC..G...........C.C........T..G..............
AMC-3      C..A........C...............C.........T..................AC..G...........C.C........T..G..............

P-1,Ad-1   TCAGACTCCAACGGGACCATTGTCGACAAGGACGGGTTCAACGAGGAGAAGCTGGCCCACCTCATGTACCTCAAGAACGAGAAGCGTGGG            690
Ad-2       ..............................T.....................T.............................C...
Ad-7       ..G......C.......T..C.....T.....T..C.....................................AC..........T........C...
Ad-121     ..G..............T..C.....T.....T..C.....................................AC..........T........C...
LD-20      ..G..............T..C.....T.....T..C.....................................AC..........T........C...
LSLP-116   ..G..............T..C
AMC-3      ..G..............T..C
```

Fig. 3. Alignment of glutamate dehydrogenase gene sequences, amplified from *Giardia duodenalis* isolates from Australia (Ad-1, Ad-2, Ad-121) and Europe (AMC-3, LD-20, LSLP-116), with the published *gdh* sequence of the Portland-1 isolate of *G. duodenalis* (Yee & Dennis (1992); GenBank accession number M84604) using CLUSTAL V (Higgins *et al.* 1992). The first 3 nucleotides of the alignment correspond to the 59th codon of the Portland-1 gene. For each position in the alignment, nucleotide identity between the Portland-1 sequence (P-1, top row) and each of the amplified sequences is depicted by a dot. The Ad-1 sequence was identical to P-1 over the entire 690-bp segment. The MAC-3 and LSLP-116 sequences were determined over a shorter segment (598 nucleotides) and the missing portions have been left blank. Positions at which nucleotide identity remains uncertain are shown (R = A or G, Y = C or T). The sequences have been deposited in the GenBank database under accession numbers L40509 (Ad-1), L40510 (Ad-2) and L40508 (Ad-7).

estimates (mean number of substitutions per nucleotide site) of 0·119–0·123 (approximately 12 times the distances calculated between genetic groups I and II within Assemblage A or between isolates belonging to Assemblage B – 0·0104 and $\leqslant$ 0·0102 respectively). As indicated in Fig. 4, the true magnitude of the dichotomy within *G. duodenalis*/*G. intestinalis* is underestimated by allozyme analysis. Similar comparisons (not shown) between the *Giardia* sequences and the same 0·69 kb segment in the published sequences of *gdh* genes (encoding, like the Portland-1 *Giardia* gene, NADP-dependent enzymes) from *Chlorella* (GenBank accession no. X58831), *Aspergillus* (X16121) and *Neurospora* (K01409) showed that the distance between Assemblage A isolates and Assemblage B isolates is approximately half (47%) that which separates *Aspergillus* from *Neurospora* (0·258 substitutions per nucleotide site) and approximately 31% of the distance that separates *G. duodenalis* from *Chlorella* (0·395 substitutions per nucleotide site).

DISCUSSION

In this study, molecular genetic analysis of the *gdh* locus in *G. intestinalis* isolated from several continents has been shown to support our earlier independent divisions of Australian and European *G. intestinalis* into 2 major genetic assemblages based on results of allozyme electrophoresis (Mayrhofer *et al.* 1995) and analysis with DNA probes (Homan *et al.* 1992). Six isolates of *G. duodenalis* from animals are also accommodated within these assemblages. Allozymic

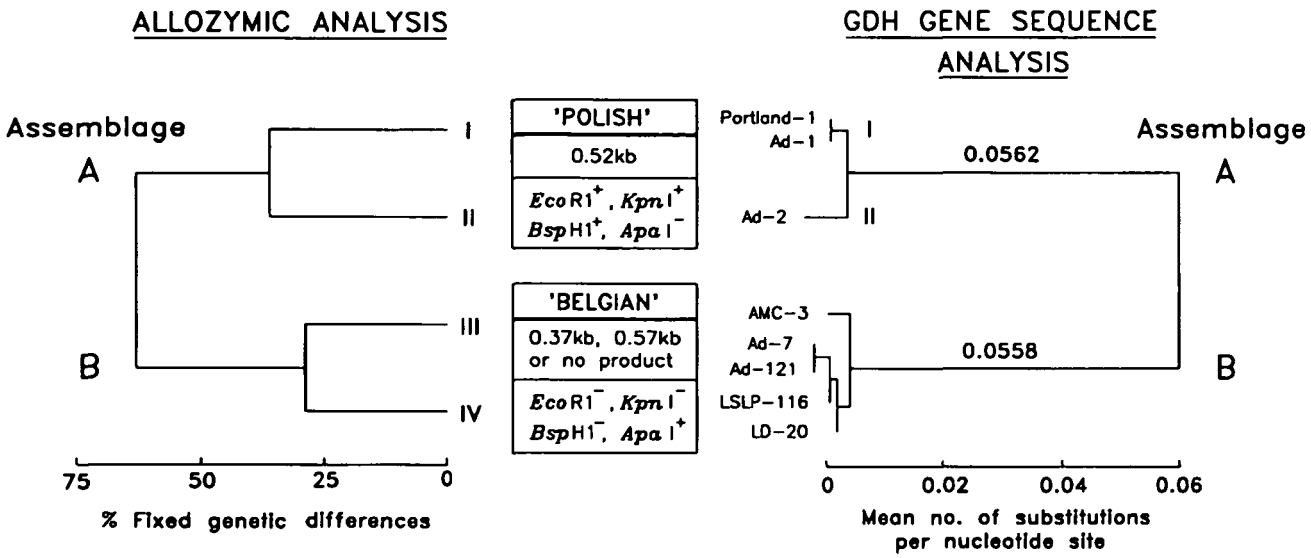

Fig. 4. Summary of data showing concordant relationships between the 'Polish' and 'Belgian' genotypes and allozymically defined genetic assemblages and groups identified in Australian isolates of *Giardia duodenalis*. Genetic differences separating the genetic groups (I and II, within Assemblage A; III and IV, within Assemblage B) are drawn to scale based (at *LEFT*) on the percentage of fixed genetic differences determined by allozymic analysis at 26 enzyme loci (Andrews *et al.* 1989), or (at *RIGHT*) on phylogenetic (Neighbour-Joining) analysis of Tamura-Nei distances calculated from the *gdh* nucleotide sequence data of Fig. 3 (all sites). Also depicted (boxed, at *CENTRE*) are the sizes of the *tsa417/tsp11* PCR products (0·52 kb for Assemblage A/'Polish' isolates; 0·37 kb, 0·57 kb, or no product, for Assemblage B/'Belgian' isolates), and diagnostic RFLP sites detected in the 1·2 kb *gdh* PCR products (Table 2).

analysis appears to produce an accentuated estimate of the genetic distances between the genetic groups identified within each assemblage, since on the basis of nucleotide substitutions in the conserved *gdh* locus these inter-group distances amount to only one-twelfth of the distance separating Assemblage A from Assemblage B. However, a clearer indication of the magnitude of the genetic distance between the 2 assemblages comes from comparison with the *gdh* sequences from *Chlorella* and two Ascomycete fungi (*Neurospora* and *Asperigillus*). *Giardia* (Archaezoa) and *Chlorella* (Plantae) belong to different kingdoms (Cavalier-Smith, 1993), while *Neurospora* (Sordariales) and *Aspergillus* (Eurotiales) belong to different Orders within the Subphylum Ascomycotina of the Kingdom Fungi (Kendrick, 1985; Berbee & Taylor, 1992). Accepting that there may be differences in the rates of evolution of the *gdh* genes in these diverse taxa, the results indicate that the genetic distance measured at this locus between Assemblages A and B in *G. intestinalis/G. duodenalis* is far greater than that between species in either the sexual Ascomycetes or the asexual unicellular algae, consistent with an ancient origin of these assemblages.

The 2 genetic groups of *G. duodenalis* defined in Europe by Homan *et al.* (1992) correspond precisely with the 2 major genetic assemblages defined by allozymic analysis of human-derived isolates from Australia (Andrews *et al.* 1989; Mayrhofer *et al.* 1995). Without exception, isolates typed in the Bilthoven laboratory as 'Polish' showed identity in both assays with Australian isolates belonging to genetic Assemblage A (Mayrhofer *et al.* 1995). Conversely, isolates typed as 'Belgian' showed identity in the *gdh* assay with Australian isolates belonging to genetic Assemblage B (Mayrhofer *et al.* 1995). The concordance of the allozymically defined groups (Andrews *et al.* 1989), the groups defined by the 2 PCR assays and the groups defined by Homan *et al.* (1992) are summarized in Fig. 4. Comparison of DNA amplified from the *gdh* locus in isolates belonging to the 2 assemblages show that there are fixed differences at 4 out of 6 restriction sites (within the 1·2 kb segment) and at approximately 11 % of nucleotide sites (within a 0·69 kb segment). These findings are consistent with the remarkably high level of fixed allelic differences found between Assemblages A and B (at approximately 63 % of loci examined) by allozymic analysis (Andrews *et al.* 1989; Mayrhofer *et al.* 1995).

However, *within* each assemblage there is a high level of nucleotide sequence identity ($\geqslant$ 98·5 %) in a portion of the amplified segment of the *gdh* gene and those nucleotide differences observed did not generate RFLPs. Evidence for the existence of distinct genetic subgroups within Assemblages A and B was obtained from analysis of the products amplified from the less conserved *tsa417* and *tsp11* gene loci. On the one hand, RFLP analysis of the 0·52 kb products amplified from 'Polish' isolates showed that, like isolates belonging to Australian Assemblage A, these organisms could be allocated to either genetic group I (15 isolates, 71 %) or genetic group

II (6 isolates, 29%). That is, the 'Polish' group of Homan *et al.* (1992) appears to consist entirely of organisms that belong either to genetic group I or II of Assemblage A. On the other hand, 'Belgian' and Australian isolates from Assemblage B could both be divided into those that yielded either no *tsa417/tsp11* PCR product or those from which 0·37 kb or 0·57 kb DNA could be amplified in trace amounts. Previous work from both laboratories has indicated genetic diversity among the latter subsets (Homan *et al.* 1992; Mayrhofer *et al.* 1995). Overall, the different genetic analyses used by the two laboratories yield complementary genetic groups within *G. duodenalis* isolates from humans and animals. Some genotypes within both assemblages appear to have a world-wide distribution and the homogeneity observed within genetic group I (in particular) by several analytical techniques suggests that it may be a successful clone that has become dispersed in relatively recent times by human migration.

Of the 3 genetic groups identified by Nash and colleagues (Nash *et al.* 1985; Nash & Mowatt, 1992), only group 1 can be placed unambiguously within Assemblages A and B. Two widely used axenic isolates, Portland-1 and WB (both group 1, Nash) each belong to genetic group I of assemblage A, as shown (for Portland-1) in the present study and by Ey *et al.* (1992, 1993*b*) and (for WB) by allozymic analysis (R. H. Andrews, unpublished data). However, we are uncertain whether group 1 of Nash also includes genetic group II of Andrews *et al.* (1989) and thus corresponds with the Assemblage A/'Polish' genotype. In particular, it is not possible at present to correlate the remaining genetic groups depicted in Fig. 4 (group II of Assemblage A; Assemblage B and its perceived subgroups) with the genetic groups 2 and 3 defined by Nash (Nash & Mowatt, 1992). The group-specific differences detected in amplified rDNA by Weiss *et al.* (1992) – single nucleotide substitutions distinguishing group 1 from groups 2 and 3, two substitutions distinguishing group 2 from group 3 – provide insufficient characters for a reliable estimation of relative distances between these groups. However, isolates WB (Nash group 1) and GS/M-H7 (group 3, Nash & Mowatt 1992) or group 2?, Weiss *et al.* (1992) differ at 13·2% and 18·7% of nucleotide sites respectively in the genes encoding an ADP-ribosylation factor (ARF, Murtagh *et al.* (1992)) and triosephosphate isomerase (Mowatt *et al.* (1993, 1994)), a level of difference similar to that observed between Assemblage A and Assemblage B isolates at the *gdh* locus. The differences at the ARF locus led Murtagh *et al.* (1992) to postulate that isolates WB (Assemblage A) and GS/M-H7 (Assemblage B?) may have diverged from each other over 'a long period of time, possibly longer than the evolutionary period thought to separate human and bovine species'.

Thompson & Meloni (1993) have, in contrast, concentrated on the diversity (e.g. 47 zymodemes from 97 isolates) revealed by zymodemic interpretation of their electrophoretic data (Meloni *et al.* 1988; Thompson & Meloni, 1993) and 'rapdemes' identified from randomly amplified polymorphic DNA (Morgan *et al.* 1993). However, examination of their data shows evidence of 2 major zymodeme/rapdeme clusters and they appear to overlook the possibility that deeply rooted lineages can develop even in organisms that reproduce as asexual clones (Tibayrenc, Kjellberg & Ayala, 1990), when populations are founded from selected genotypes by events such as biogeographic separation or change of hosts by a parasite. It is significant that the BAH 12 isolate – defined allozymically as genetic group III of Assemblage B (Andrews *et al.* 1989) – falls within one major zymodemic cluster whilst both the Portland-1 and BAC 1 isolates (which also differ from BAH 12 in their rates of growth and ethanol production *in vitro*; Hall *et al.* 1992) fall within the other major cluster (Thompson & Meloni, 1993). These 2 major zymodemic clusters may, therefore, correspond to genetic Assemblages A and B.

We are less certain that karyotypic differences identified between *G. duodenalis* isolates by pulsed-field gel electrophoretic techniques (Upcroft *et al.* 1989; Campbell *et al.* 1990; Korman *et al.* 1992; Safaris & Isaac-Renton, 1993; Carnaby *et al.* 1994) are also consistent genetic markers defining major genotypes such as those mentioned above. Based on findings (in clones) of chromosomal rearrangements involving ribosomal DNA and other multiple repeat sequences near telomeres (reviewed by Le Blancq, 1994), marked variation of presumed hypervariable polymorphic minisatellite sequences (Upcroft, Mitchell & Boreham, 1990; Upcroft & Upcroft, 1994; Carnaby *et al.* 1994) and variable patterns of random amplified polymorphic DNA (RAPD; van Belkum *et al.* 1993; Morgan *et al.* 1993; Upcroft & Upcroft, 1994), it has been suggested that the karyotype of *Giardia* may be plastic. This would make karyotypes unsuitable as taxonomic characters. On the other hand, there is no evidence to indicate that individual genes encoding essential macromolecules (housekeeping enzymes, structural proteins, rRNA, etc.) are any less stable in *Giardia* than in other organisms. We prefer, therefore, to rely for systematic or phylogenetic analysis on data derived from structural genes rather than on data derived from regions of chromosomes (e.g. telomeric regions) that contain multiple repeats that may be prone to rearrangement. In this context, it is relevant that a limited study on European isolates using RFLP analysis with probes for the structural genes β-tubulin and α-giardin also differentiated 'Belgian' (Assemblage B) from 'Polish' (Assemblage A) genotypes (Homan *et al.* 1992). On the other hand, it should be noted that Southern hybridizations utili-

zing probes such as pGH311 (cf. Fig. 4 of Homan *et al.* 1992) allow the group I or group II status of some *but not all* 'Polish' isolates to be distinguished (W.L. Homan, unpublished data). This demonstrates, not unexpectedly, that the nature of an individual probe determines whether specific polymorphisms and fixed (group-specific) differences are detected within groups. The concordance between allozyme analyses (Andrews *et al.* 1989) and PCR assays based on the *tsa417/tsp11* genes (Ey *et al.* 1993*a*,*b*) suggest that the division between genetic groups I and II of Assemblage A is robust and useful taxonomically.

At present, there are no definite biological differences which are associated specifically with *G. duodenalis* isolates belonging to Assemblage A or Assemblage B. However, a search of the published literature on the double-stranded RNA *Giardia* virus (Miller, Wang & Wang, 1988; de Jonckheere *et al.* 1990; Sepp, Wang & Wang, 1994) indicates that axenic isolates bearing the virus, or those susceptible to infection by it, belong to Assemblage A and in particular to genetic group I. The differences mentioned above in the metabolism and growth rates between certain isolates (Hall *et al.* 1992) also appear to correlate with membership of Assemblage A (Portland-1 and BAC 1) or Assemblage B (BAH 12). The magnitude of the genetic dichotomy between the 2 assemblages suggests that other biological differences will also be found. The advantage of the genetic frameworks described herein and elsewhere (Nash & Mowatt, 1992; Homan *et al.* 1992; Mayrhofer *et al.* 1995) is that there now exists a rational basis for cataloguing such differences, some of which (e.g. host range, pathogenicity, endemicity in certain geographical areas) could have medical and veterinary importance.

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