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Division of *Giardia* isolates from humans into two genetically distinct assemblages by electrophoretic analysis of enzymes encoded at 27 loci and comparison with *Giardia muris*

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SUMMARY

Giardia that infect humans are known to be heterogeneous but they are assigned currently to a single species, *Giardia intestinalis* (syn. *G. lamblia*). The genetic differences that exist within *G. intestinalis* have not yet been assessed quantitatively and neither have they been compared in magnitude with those that exist between *G. intestinalis* and species that are morphologically similar (*G. duodenalis*) or morphologically distinct (e.g. *G. muris*). In this study, 60 Australian isolates of *G. intestinalis* were analysed electrophoretically at 27 enzyme loci and compared with *G. muris* and a feline isolate of *G. duodenalis*. Isolates of *G. intestinalis* were distinct genetically from both *G. muris* (approximately 80% fixed allelic differences) and the feline *G. duodenalis* isolate (approximately 75% fixed allelic differences). The *G. intestinalis* isolates were extremely heterogeneous but they fell into 2 major genetic assemblages, separated by fixed allelic differences at approximately 60% of loci examined. The magnitude of the genetic differences between the *G. intestinalis* assemblages approached the level that distinguished the *G. duodenalis* isolate from the morphologically distinct *G. muris*. This raises important questions about the evolutionary relationships of the assemblages with *Homo sapiens*, the possibility of ancient or contemporary transmission from animal hosts to humans and the biogeographical origins of the two clusters.

Key words: *Giardia intestinalis*, *Giardia duodenalis*, *Giardia muris*, allozyme electrophoresis, systematics.

INTRODUCTION

Giardia is a genus composed of binucleate flagellate protozoa that are parasitic for a wide range of vertebrates, including humans. At present the genus has no genetically based systematics and species have been allocated on the basis of morphology and/or presumed host specificity (Thompson, Lymbery & Meloni, 1990). Those *Giardia* that infect humans belong to the morphological species *G. duodenalis* (Filice, 1952) but they have been assigned to a separate species, *G. intestinalis* (syn. *G. lamblia*), on the basis of presumed host specificity. Disease patterns in humans vary (Gillon, 1984), possibly reflecting infections with genetically different organisms (Nash *et al.* 1987) and genetic heterogeneity within *G. intestinalis* has been demonstrated by serological (Nash & Keister, 1985), biochemical (Bertram *et al.* 1983; Baveja *et al.* 1986; Meloni, Lymbery & Thompson, 1988) and molecular genetic (Nash *et al.* 1985) analyses. Analysis of allozyme data obtained from electrophoretic studies of enzymes encoded at 26 loci in axenized Australasian isolates of *G. intestinalis* allowed Andrews *et al.* (1989) to identify 4 genetic groups (I–IV) and to propose the existence of a species complex comprising 2–4 cryptic species. We have shown recently that axenic culture selects preferentially for genotypes belonging to genetic groups I and II, while isolation from human clinical specimens by growth in suckling mice yields predominantly genotypes related to genetic groups III and IV (Mayrhofer *et al.* 1992; Andrews, Chilton & Mayrhofer, 1992).

In this study, we have examined 60 Australian isolates of *G. intestinalis* established in suckling mice from faecal cysts and compared them with axenized isolates representing each of the genetic groups identified previously (Andrews *et al.* 1989). Furthermore, all *G. intestinalis* isolates have been compared directly at 27 enzyme loci with a feline isolate of *G. duodenalis* and with a well-studied isolate of *G. muris* (Roberts-Thomson *et al.* 1976). The results are discussed in relation to the systematics of *Giardia* and possible explanations are advanced for the major genetic division within *G. intestinalis*.

MATERIALS AND METHODS

Giardia isolates

G. intestinalis cysts were prepared from faecal specimens obtained from hospital diagnostic labora-

tories in metropolitan Adelaide, regional centres in South Australia and from Alice Springs in the Northern Territory. Cyst preparation, inoculation of suckling mice, propagation of trophozoites in suckling mice and designation of the resulting isolates have been described previously (Mayrhofer *et al.* 1992). Trophozoites from 60 of the isolates were purified from the intestines of suckling mice by adherence (Mayrhofer *et al.* 1992) and stored as pellets at −80 °C for electrophoretic analysis. Other isolates propagated in suckling mice were Ad-23 (feline isolate, Mayrhofer *et al.* 1992) and *G. muris* (Roberts-Thomson *et al.* 1976) obtained from Dr B. Underdown (McMaster University, Hamilton, Ontario). Axenic cultures of Ad-1, Ad-2, BAH 12 and Bris/87/HEPU/694 trophozoites were used as reference isolates representing genetic groups I–IV respectively (Andrews *et al.* 1989). A further genetic group IV-related isolate (Bris/87/HEPU/787) was kindly provided by Dr P. F. L. Boreham (Queensland Institute of Medical Research).

Allozyme electrophoresis

Each isolate was examined at 27 enzyme loci by electrophoresis on cellulose acetate gels as described elsewhere (Andrews *et al.* 1989). Each enzyme was compared directly in all isolates on a single gel. Also included on each gel were samples from axenic trophozoites representing *G. intestinalis* genetic groups I–IV and samples from *G. muris* and from the *G. duodenalis* isolate (Ad-23), both propagated in suckling mice. Loci examined were acid phosphatase (*Acp*), aldolase (*Ald*), diaphorase 1 and 2 (*Dia-1*, *Dia-2*), enolase (*Enol*), esterase (*Est*), fructose-1,6-diphosphatase (*Fdp*), glyceraldehyde phosphate dehydrogenase (*Gapd*), glutamate dehydrogenase (*Gdh*), aspartate aminotransferase (syn. glutamate-oxaloacetate transaminase, *Got-1*), glycerol-3-phosphate dehydrogenase (*Gpd*), glucose-6-phosphate dehydrogenase (*G6pd*), glucose phosphate isomerase (*Gpi*), alanine aminotransferase (syn. glutamate-pyruvate transaminase, *Gpt*), hexokinase (*Hk*), malate dehydrogenase (*Mdh*), malic enzyme (*Me*), nucleoside diphosphate kinase (*Ndpk*), purine nucleoside phosphorylase-2 (*Np-2*), peptidase (valine-leucine, *Pep-A*), peptidase (phenylalanine-proline, *Pep-D*), phosphoglycerate mutase (*Pgam*), 6-phosphogluconate dehydrogenase (*6-Pgd*), phosphoglycerate kinase (*Pgk*), phosphoglucomutase (*Pgm*) and triose-phosphate isomerase (*Tpi*). Enzyme Commission numbers and electrophoretic conditions are as detailed by Andrews *et al.* (1989) and Richardson, Baverstock & Adams (1986). A new locus for *Giardia*, referred to tentatively as *Got-x*, has been described elsewhere (Andrews *et al.* 1993).

Analysis of electrophoretic data

Phenograms illustrating the genetic distances between isolates of *G. intestinalis*, *G. muris* and *G. duodenalis* were constructed by the Unweighted Pair Group Method using Arithmetic Averages (UPGMA; Sneath & Sokal, 1973) using as a measure of genetic distance the percentage of loci showing 'fixed' allelic differences. This measure has been shown to correlate with other measures of genetic distance such as Nei' D (for detailed discussion, see Richardson *et al.* 1986). A fixed allelic difference exists when no member of a group of taxa shares any alleles with members of other groups at the particular enzyme locus in question. Reference samples from genetic groups I (Ad-1), II (Ad-2), III (BAH12) and IV (Bris/87/HEPU/694), described elsewhere (Andrews *et al.* 1989), were included in the electrophoretic analysis and in construction of the phenogram. Also included was the axenic genetic group IV-like isolate (Bris/87/HEPU/787), the feline *G. duodenalis* isolate (Ad-23) and the *G. muris* isolate. The latter provides a morphologically distinct *Giardia* for comparison with the isolates of *G. intestinalis* and *G. duodenalis*. For reasons discussed below, no attempt has been made to analyse the electrophoretic data by phylogenetic (i.e. cladistic) methods.

RESULTS

Each enzyme was compared directly in all isolates (including samples from genetic groups I–IV, Bris/87/HEPU/787, *G. muris* and Ad-23) by electrophoresis on a single gel. Isolates could be scored at between 22 and 27 loci. The allozyme pattern of each isolate was examined and isolates were excluded if more than one allozyme (electrophoretic band) was detected for any of the enzymes analysed. From the original 60 isolates of *G. intestinalis*, this process yielded 28 in which single allozymes were detected for all enzymes. We have adopted this conservative approach to avoid the possible ambiguities that are inevitable in interpreting allozyme patterns in uncloned isolates, especially as the ploidy of *Giardia* remains uncertain and there is evidence in some isolates of heterozygosity (Andrews *et al.* 1989) and in others of mixed genotypes (Andrews *et al.* 1989; Mayrhofer *et al.* 1992; Andrews *et al.* 1992). Allelic profiles from representatives of the 28 new *G. intestinalis* isolates are shown in Table 1 and summarized by the phenogram in Fig. 1. A number of important features are evident in these data. Firstly, *G. intestinalis* contains hitherto unsuspected heterogeneity, while retaining the general structure detected by Andrews *et al.* (1989). It consists of 2 distinct assemblages (A and B), which are characterized by fixed genetic differences at over 60% of the loci examined. Assemblage A incorporates genetic groups I and II. However, it also contains other genotypes falling into at least 3 clusters (each represented by 1 or more isolates) distinguished by

Table 1. Allelic profiles of enzymes encoded at 27 loci in trophozoites produced from isolates of *Giardia muris* and *G. duodenalis* (cat isolate), from axenic human isolates representing genetic groups I–IV and from new human isolates representative of the main clusters shown in Fig. 1

	Enzyme locus*								
	Acp	*Ald*	*Dia-1*	*Dia-2*	*Enol*	*Est*	*Fdp*	*Gapd*	*Gdh*
G. muris	e	d	c	b	c	—	f	a	d
Group I	d	a	c	b	b	a	d	a	b
Group II	d	a	—	b	b	a	c	a	b
Ad-62	d	a	a	b	b	a	—	—	b
Ad-43	d	a	—	b	b	—	d	a	b
Ad-12	d	a	a	b	a	a	b	—	b
Group III	b	—	b	b	a	b	—	a	ac
Ad-19	a	b	b	b	a	a	b	a	b
Group IV	b	c	b	b	a	a	b	a	b
Ad-52	b	b	b	b	a	a	—	a	b
Ad-16	b	b	b	b	a	b	a	a	b
Ad-28	b	b	b	b	a	a	b	a	b
Ad-50	b	b	b	b	a	a	—	a	b
G. duodenalis	c	—	c	a	b	—	e	a	a
	Got-1	*Got-x*	*Gpd*	*G6pd*	*Gpi*	*Gpt*	*Hk*	*Mdh*	*Me*
G. muris	d	b	—	b	c	c	a	d	e
Group I	b	a	a	a	c	b	b	a	d
Group II	b	a	c	a	a	c	b	a	d
Ad-62	b	a	d	d	c	c	e	a	c
Ad-43	a	a	c	a	b	c	b	a	g
Ad-12	a	—	c	—	b	c	b	a	c
Group III	c	a	c	cg	—	a	e	b	—
Ad-19	b	a	c	g	d	a	d	b	a
Group IV	bd	a	e	g	c	a	e	b	c
Ad-52	d	a	e	g	b	a	e	b	c
Ad-16	a	a	—	e	e	a	e	b	b
Ad-28	d	a	d	f	d	a	d	b	b
Ad-50	d	a	d	e	c	a	c	b	b
G. duodenalis	c	a	b	a	c	b	b	c	c
	Ndpk	*Np-2*	*Pep-A*	*Pep-D*	*Pgam*	*6Pgd*	*Pgk*	*Pgm*	*Tpi*
G. muris	—	d	d	—	d	e	b	b	c
Group I	a	a	b	a	c	a	b	b	a
Group II	a	c	c	a	c	a	b	c	a
Ad-62	a	c	b	a	c	a	b	c	a
Ad-43	a	c	c	c	—	—	b	d	a
Ad-12	a	b	c	—	c	—	b	c	a
Group III	a	b	a	—	ab	cd	b	—	a
Ad-19	a	b	a	a	a	b	b	b	a
Group IV	—	b	a	b	a	b	b	b	a
Ad-52	a	b	—	c	—	b	a	b	a
Ad-16	a	b	a	a	a	b	a	a	a
Ad-28	a	b	a	a	a	b	b	b	a
Ad-50	a	b	a	a	a	b	b	b	a
G. duodenalis	a	a	b	—	b	—	b	a	b

* Allozymes are designated alphabetically, in order of increasing electrophoretic mobility (Richardson *et al.* 1986). A dash indicates that enzyme activity was insufficient to score confidently.

fixed allelic differences at 20% or more of the loci examined. It is noteworthy that isolate Ad-1 (group I control) represents 17 other axenic isolates (identical at 26 loci) from a previous study, while Ad-2 (group II control) represents 5 other axenic isolates identical at a similar number of loci (Andrews *et al.* 1989).

Assemblage B is more heterogeneous than assemblage A. It includes genetic groups III and IV (each represented previously by single isolates, Andrews *et al.* 1989). The genetic group III control (BAH 12) forms a cluster with 3 other isolates (Ad-19, Ad-25 and Ad-42), from which it is distinguished by fixed allelic differences at approximately 25% of the loci examined. The genetic group IV control (Bris/87/HEPU/694) and a further 3 isolates (Ad-

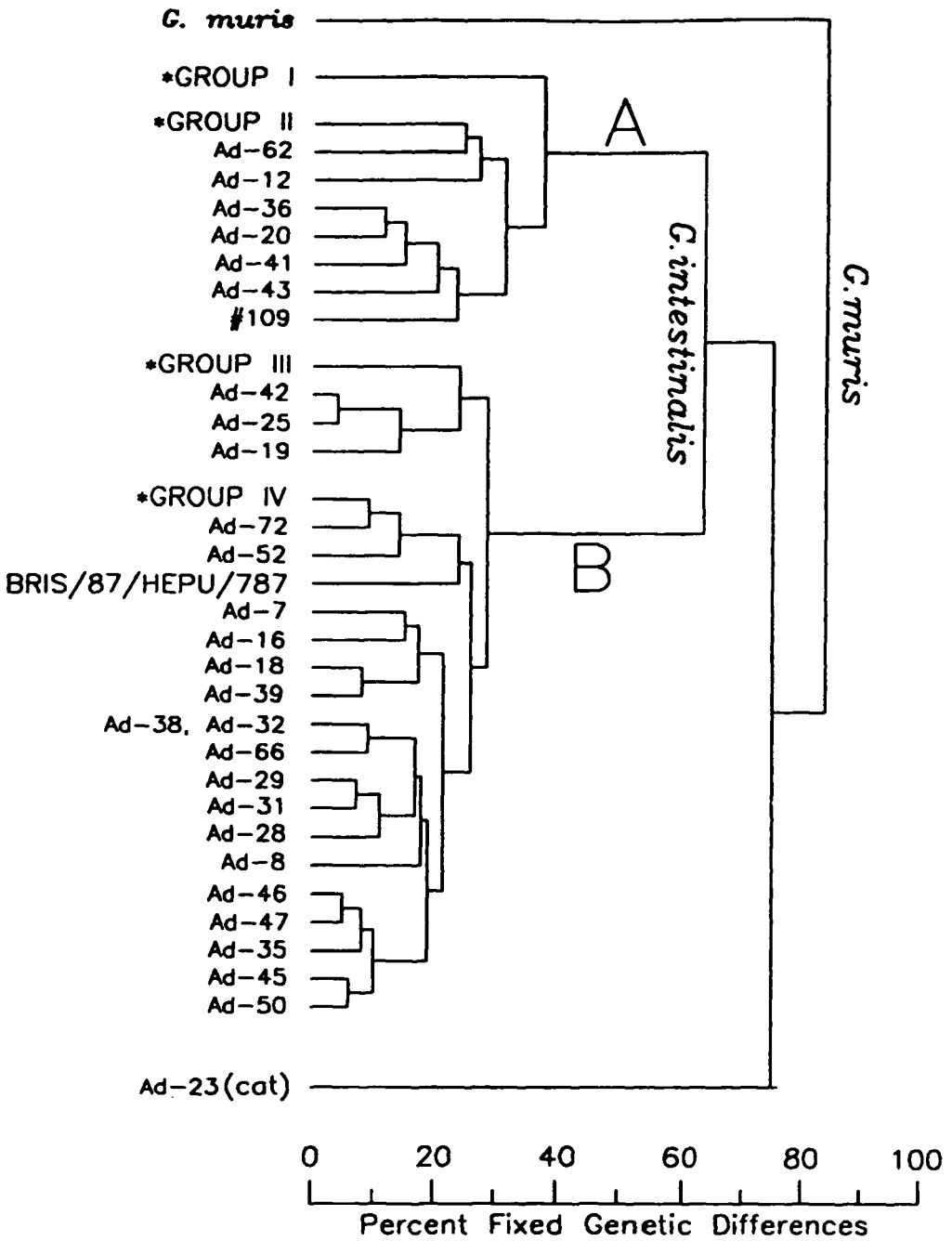

Fig. 1. Phenogram illustrating the genetic distances between isolates of *Giardia intestinalis*, *G. muris* and a feline isolate of *G. duodenalis*. The phenogram was constructed as described in the text. Included in the phenogram are results obtained from axenic cultures representing genetic groups I–IV (I, Ad-1; II, Ad-2; III, BAH12; IV, Bris/87/HEPU/694 and Bris/87/HEPU/787; Andrews *et al.* 1989), from new isolates of *G. intestinalis* isolated by growth in suckling mice (Mayrhofer *et al.* 1992), from *G. muris* (Roberts-Thomson *et al.* 1976) and from an isolate of *G. duodenalis* (Ad-23, domestic cat origin; Mayrhofer *et al.* 1992). Indicated are the two major assemblages of *G. intestinalis* (A and B).

52, Ad-72 and Bris/87/HEPU/787) form a second cluster, the members of which are distinguished from each other by fixed allelic differences at between 10 and 24 % of loci examined. The cluster containing genetic group IV is itself part of a larger cluster that is separated from genetic group III by fixed allelic differences at approximately 30 % of the loci examined. Genetic group IV-like isolates have fixed allelic differences ranging from 0 to 25 % of loci examined and contain one cluster that includes Bris/87/HEPU/694 (group IV), Ad-72, Ad-52 and Bris/87/HEPU/787 and another larger cluster containing the remainder of the isolates. These remaining isolates form 4 smaller clusters (Ad-7, Ad-16, Ad-18 and Ad-39; Ad-28, Ad-29, Ad-31, Ad-32, Ad-38 and Ad-66; Ad-8; Ad-35, Ad-45, Ad-46, Ad-47 and Ad-50) that have fixed allelic differences ranging from 16 to 21 % of loci examined.

A second major finding, illustrated in Fig. 1, is the genetic relationship of *G. intestinalis* isolates to the feline isolate of *G. duodenalis* (Ad-23) and to the morphologically distinct *G. muris*. The feline isolate is distinguished from all *G. intestinalis* by fixed allelic differences at approximately 75% of loci examined. *G. muris* exhibits fixed allelic differences from all other isolates, human and feline, at approximately 84% of the loci examined.

DISCUSSION

The results obtained in this study allow analysis of 27 conserved genetic loci, in all likelihood scattered throughout the *Giardia* genome. They represent, therefore, a more significant and representative sampling of genetic diversity within *G. intestinalis* than other studies based on substantially fewer electrophoretic characters and offer some advantages over comparison of nucleotide sequence differences within a single gene. The *in vivo* technique used to obtain and propagate the isolates has precluded the use of cloned trophozoite populations. However, the analysis has been limited to only those isolates which are monomorphic at all loci examined, thereby effectively eliminating those samples which might contain mixtures of trophozoites with dissimilar genotypes. Although this conservative approach has reduced the sample size by half, the inclusion of the other isolates (not shown) does not alter substantially either the clustering of *G. intestinalis* into two assemblages or the proportion of fixed genetic differences that delineate *G. intestinalis*, *G. duodenalis* and *G. muris* in Fig. 1.

The allozymic interpretation of the data and analysis by UPGMA yields a quantitative estimate of genetic distances between isolates, providing a phenetic foundation for a systematics of the genus *Giardia*. On the other hand, a phylogenetic (i.e. cladistic) analysis of the electrophoretic data is premature for two reasons. Firstly, allozyme electrophoresis may not be the most suitable technique for analysis of the higher order systematics of the genus. The genetic differences between *G. intestinalis* and the morphologically similar *G. duodenalis* (approximately 75% fixed allelic differences) and between the two assemblages of *G. intestinalis* (approximately 62% fixed allelic differences) are so great that any similarities that exist have a significant probability of having arisen by chance rather than by retention of ancestral alleles (Richardson *et al.* 1986). Secondly, the choice of outgroup is critical and this depends on definition of what levels of genetic diversity are consistent with population polymorphism within the relevant taxa on the one hand and biologically relevant species level differences (e.g. morphology, host preference, geographical range) on the other. It will be necessary to analyse more conserved characters (e.g. nucleotide sequences of conserved genes encoding rRNA, structural proteins or housekeeping enzymes) in order to construct a phylogeny. For this reason we have commenced analysis of the *gdh* genes from a range of *G. duodenalis* and *G. intestinalis* isolates and the preliminary data support the wisdom of this conservative approach.

The organisms embraced by the species name *G. intestinalis* (Fig. 1) are so named because they have all been isolated from humans. The results confirm the existence of the 4 genetic groups proposed earlier by Andrews *et al.* (1989). However, the isolates examined here have not been exposed to the selective effects of axenic culture (Mayrhofer *et al.* 1992; Andrews *et al.* 1992) and they reveal a greater level of genetic heterogeneity than has been observed in axenic isolates from humans. Furthermore, the phenogram in Fig. 1 indicates that *G. intestinalis* consists of two main assemblages which are separated by fixed genetic differences at approximately 62% of the loci examined. The magnitude of the genetic differences between the isolates comprising assemblages A and B approaches those which divide other protozoan genera such as *Leishmania* (Andrews *et al.* 1988) and *Naeglaria* (Adams *et al.* 1989) into species that differ in morphology, ecology and pathogenicity. The major genetic discontinuity between the two assemblages provides additional support for the suggestion that *G. intestinalis* is a species complex (Andrews *et al.* 1989).

The allozymic evidence for a fundamental division within *G. intestinalis* is supported by studies at the DNA level (Nash, 1992; Homan *et al.* 1992; Ey *et al.* 1992). Our recent analyses have involved use of the polymerase chain reaction to amplify DNA from conserved segments of trophozoite surface protein genes (Ey, Andrews & Mayrhofer, 1993*a*; Ey *et al.* 1993*b*). All isolates belonging to assemblage A yield amplification products indistinguishable from the predicted size (0·52 kilobase) and restriction analyses of these products have identified differences that are diagnostic for organisms belonging to the allozymically defined genetic groups I and II. In contrast, use of the same primers with DNA from some isolates belonging to assemblage B yielded only trace amounts of a smaller (0·37 kilobase) amplification product, while other isolates yielded no detectable product (Ey *et al.* 1993*a*). There is, therefore, close agreement between the results obtained by two different techniques, each of which analyses different parts of the *G. intestinalis* genome.

The existence of these two major assemblages within *G. intestinalis* raises interesting questions about their origins and biological significance. It is possible that humans acquire *Giardia* from animal sources with some regularity, reflecting contemporary zoonotic transmission. This cannot be excluded on present evidence (reviewed by Thompson *et al.* 1990) and it may be that one or other of the assemblages (or some members thereof) represents

zoonotic transmission from animals, giving rise to either transient or longer term infections in humans. However, it is also noteworthy that the magnitude of genetic difference between assemblages A and B is comparable to that defined by allozyme electrophoresis between rodent and marsupial genera (Baverstock *et al.* 1981, 1982). Although rates of genetic divergence in protozoa have not been related to the fossil record, it is nevertheless relevant to consider whether, on the one hand the assemblages of *G. intestinalis* diverged in the ancestors of *Homo sapiens* and have since co-evolved with humans, or alternatively, whether they entered humans as genetically distinct founder populations acquired zoonotically from separate animal host species. If the latter is true, it is interesting to consider the biogeography of the Australian continent and the possibility that some *G. intestinalis* in Australia might have a Gondwanan origin.

A concluding point of significance is the utility of the genetic groups proposed by Andrews *et al.* (1989) in the light of the present findings. Andrews *et al.* (1989) detected 18 isolates with the group I genotype among axenic cultures established from various geographic localities in Australasia and a further 6 isolates with the group II genotype. More recently, we have detected the group I genotype by allozyme electrophoresis in both animal and human isolates obtained from North America (Andrews *et al.*, manuscript in preparation) and by analysis of surface protein genes (Ey *et al.* 1993*a*) in animal and human isolates from Switzerland (Ey, Bruderer, Wehrli & Köhler, personal communication). Furthermore, the extensively studied isolates PO-1 and WB also belong to genetic group I (Ey *et al.* 1992; Andrews, unpublished data.) These genotypes, which appear to be selected by *in vitro* culture (Mayrhofer *et al.* 1992; Andrews *et al.* 1992), have a global distribution and their genetic homogeneity may indicate that dispersal has occurred in relatively recent times, perhaps accompanying modern human migration. Because of its genetic homogeneity, the group I genotype has obvious taxonomic value within assemblage A. It seems convenient at this point to use the genotype of Group II as representative of the second major cluster in assemblage A. However, this cluster is more heterogenous, already containing evidence of two genetic subclusters and it may justify further refinement in the future when larger numbers of isolates are analysed.

In contrast, assemblage B consists of a large number of different genotypes, of which the genetic groups III and IV suggested by Andrews *et al.* (1989) are represented only by single isolates. It is in fact characteristic of this assemblage that in the entire collection, only two of the new isolates had the same allelic profiles at 27 enzyme loci. Therefore, it follows that the true magnitude of genetic diversity within assemblage B remains unknown. Genetic groups III and IV may be convenient representatives with which to compare isolates within assemblage B, but it is apparent that other isolates are equally informative.

In proposing that *G. intestinalis* is a species complex, Andrews *et al.* (1989) stressed that it is important to show that the genetic differences between proposed cryptic species are distinct from those which occur within such groupings. Only the former would constitute taxonomically informative characters. The allozymic data presented herein on a large sample of isolates, together with our earlier findings (Andrews *et al.* 1989) and more recent data from studies at the DNA level (Ey *et al.* 1993*a*, *b*), indicate that the genetic differences between assemblages A and B far exceed the levels of genetic heterogeneity observed within either of the assemblages. Furthermore, the allozymic homogeneity of all isolates of the group I genotype (see above) distinguishes them clearly from the other members of assemblage A represented by genetic group II. On genetic grounds, assemblages A and B and the major dichotomy within assemblage A have clear taxonomic significance and support the view that *G. intestinalis* is a species complex. The implications of further subclustering within the isolates related to genetic group II, and also within assemblage B, await more extensive sampling of genotypes from within Australia and from other parts of the world, using isolation techniques that do not rely exclusively on *in vitro* culture of trophozoites. It is now important to compare the above genetic groupings with those identified by other workers using a variety of different biochemical and antigenic characters (Nash, 1992; Homan *et al.* 1992) and to evaluate the biological and medical utility of these findings.

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