Molecular Systematics of the Parasitic Protozoan *Giardia intestinalis*

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The long-standing controversy regarding whether *Giardia intestinalis* is a single species prevalent in both human and animal hosts or a species complex consisting of morphologically similar organisms that differ in host range and other biotypic characteristics is an issue with important medical, veterinary, and environmental management implications. In the past decade, highly distinct genotypes (some apparently confined to particular host groups) have been identified by genetic analysis of samples isolated from different host species. The aim of this study was to undertake a phylogenetic analysis of *G. intestinalis* that were representative of all known major genetic groups and compare them with other *Giardia* species, viz. *G. ardeae, G. muris,* and *G. microti.* Segments from four ''housekeeping'' genes (specifying glutamate dehydrogenase, triose phosphate isomerase, elongation factor 1α, and 18S ribosomal RNA) were examined by analysis of 0.48–0.69-kb nucleotide sequences determined from DNA amplified in polymerase chain reactions from each locus. In addition, isolates were compared by allozymic analysis of electrophoretic data obtained for 21 enzymes representing 23 gene loci. The results obtained from these independent techniques and different loci were essentially congruous. Analyses using *G. ardeae* and/or *G. muris* as outgroups supported the monophyly of *G. intestinalis* and also showed that this species includes genotypes that represent at least seven deeply rooted lineages, herein designated assemblages A–G. Inclusion of *G. microti* in the analysis of 18S rRNA sequence data demonstrated the monophyly of *Giardia* with the same median body morphology but did not support the monophyly of *G. intestinalis,* instead placing *G. microti* within *G. intestinalis.* The findings support the hypothesis that *G. intestinalis* is a species complex and suggest that *G. microti* is a member of this complex.

Introduction

The systematics of many parasite groups remain poorly resolved because limited morphological differences and complex host-dependent life cycles have hindered traditional taxonomic analysis (Monis 1999). Protozoans have presented additional difficulties because of the absence of fossil records and the frequent absence of sexuality, which limits the application of the biological species concept to delineate species (Corliss 1960). In the case of the genus *Giardia,* whose members are gastrointestinal parasites found in almost all vertebrate species, all of these factors have limited the development of an adequate species-level taxonomy (Kulda and Nohýnková 1996).

Giardia are of broad scientific interest because they represent one of the earliest known branches of the eukaryotic lineage (Hashimoto et al. 1994). The taxonomy of the genus is based on morphology—in particular, the shape of the trophozoite, the size of the ventral adhesive disc relative to the length of the cell, and the shape of the median bodies (Kulda and Nohýnková 1996). Using these criteria, Filice (1952) defined three species: *Giardia agilis, Giardia muris,* and *Giardia duodenalis* (syn. *Giardia intestinalis, Giardia lamblia*). Two species have subsequently been described on the basis of ultrastructural features identified by scanning electron microscopy of trophozoites (*Giardia ardeae,* Erlandsen et al. 1990; *Giardia psittaci,* Erlandsen and Bemrick 1987). A sixth species, *Giardia microti,* has been proposed on the basis of cyst morphology (Feely 1988) and small-subunit rRNA sequence analysis (van Keulen et al. 1998). The distinctiveness of *G. ardeae, G. intestinalis* (which has precedence over *G. duodenalis*; Kulda and Nohýnková 1996), and *G. muris* is also supported by small-subunit rRNA sequence data (van Keulen et al. 1991, 1993).

Giardia intestinalis includes organisms that have been recovered from many different mammalian species. The morphological uniformity of these isolates masks a considerable biotypic and genetic diversity, and their taxonomy is considered inadequate (Kirkpatrick and Green 1985; Andrews et al. 1989; Binz et al. 1992; Homan et al. 1992; Nash and Mowatt 1992; Erlandsen 1994; Meloni, Lymbery, and Thompson 1995; Kulda and Nohýnková 1996). Allozymic analysis of multilocus enzyme electrophoresis (MLEE) data has placed all isolates from humans within two major genetic assemblages (A and B) which encompass four genetic clusters, groups I–IV (Andrews et al. 1989; Mayrhofer et al. 1995). These studies revealed that the genetic differences between isolates belonging to assemblage A and those belonging to assemblage B were similar in magnitude to the differences between *G. intestinalis* and *G. muris* (Mayrhofer et al. 1995). Nucleotide sequence analysis of a 690-bp segment of the glutamate dehydrogenase (*gdh*) gene (Monis et al. 1996) has confirmed that assemblages A and B are highly divergent lineages. These assemblages correspond, respectively, to the ''Polish'' and ''Belgian'' genotypes of Homan et al. (1992) and groups (1+2) and group 3 of Nash (Nash and Keister 1985; Nash et al. 1985; Nash and Mowatt 1992). Analysis of *G. intestinalis* from animals has identified three additional lineages: assemblages C and D, defined by isolates recovered from dogs (Monis et al. 1998), and a ''Hoofed livestock'' lineage, defined by isolates from sheep, goats, cattle, and pigs (Ey et al. 1997). The restricted host range of these latter genotypes

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Abbreviation: MLEE, multilocus enzyme electrophoresis.

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Table 1
Isolates Examined in this Study

Giardia Isolate	Host Origin	Mode of Culture[a]	Defined Genotype[b]	GenBank Accession Numbers[c]			
				gdh	*tpi*	*ef1α*	18S rRNA
G. intestinalis							
Ad-1	Human	Axenic	Assemblage A, group I	L40509	AF069556	—	—
Ad-2	Human	Axenic	Assemblage A, group II	L40510	AF069557	AF069573	—
BAH12	Human	Axenic	Assemblage B, group III	AF069059	AF069561	AF069569	AF113897
Ad-28, Ad-19..	Human	S/m	Assemblage B, group IV	L40508	AF069560	AF069570	AF113898
Ad-23	Cat	S/m	Undefined	AF069057	AF069558	AF069572	AF113901
Ad-136	Dog	Clinical	Assemblage C	U60982	AF069563	AF069574	AF113899
Ad-137	Dog	S/m	Assemblage C	U60983	—	—	—
Ad-148	Dog	S/m	Assemblage D	U60986	—	AF069575	AF113900
P-15[d]	Pig	Axenic	''Hoofed livestock''	U47632	AF069559	AF069571	AF113902
Ad-157	Rat	S/m	Undefined	AF069058	AF069562	AF069568	AF113896
G. ardeae[e]	Blue heron	Axenic	NA	AF069060	AF069564	AF069567	—
G. muris	Mouse						
Ad-120[f]	Mouse	S/m	NA	—	AF069565	AF069566	AF113895

[a] Axenic = cultured *in vitro*; Clinical = unpropagated; S/m = propagated by infection of suckling mice; trophozoites obtained at autopsy.
[b] Determined by allozyme analysis (Andrews et al. 1989; Mayrhofer et al. 1995; Ey et al. 1997; Monis et al. 1998). NA = not applicable.
[c] Nucleotide sequences determined from this study have accession numbers starting with the letters AF.
[d] Koudela et al. (1991).
[e] Erlandsen et al. (1990).
[f] Mayrhofer et al. (1995).

suggests that they are biologically distinct from isolates belonging to assemblage A or B. These findings, together with evidence of growth rate differences that appear to be genetically based (Andrews, Chilton, and Mayrhofer 1992; Binz et al. 1992; Karanis and Ey 1998; Monis et al. 1998), provide support for proposals that *G. intestinalis* is a species complex (Andrews et al. 1989; Murtagh et al. 1992).

To examine more rigorously the phylogenetic relationships between these genotypes of *G. intestinalis*, we undertook a multilocus analysis of isolates of *G. intestinalis* that are representative of each major genetic group and compared these with two isolates of *G. intestinalis* (derived from a cat and a rat) which were known to fall outside assemblages A and B (Mayrhofer et al. 1995; unpublished data).

Materials and Methods

Sources of Isolates

The isolates used, along with their defined genotypes, are listed in table 1. Each was chosen as a representative of the main assemblages and genetic groups identified from our previous studies. Except for Ad-157, all have previously been described (Andrews et al. 1989; Mayrhofer et al. 1995; Ey et al. 1997; Monis et al. 1998). Isolate Ad-157 was established by infecting suckling mice with cysts purified from the feces of a laboratory rat according to the method of Mayrhofer et al. (1992). The culture of *G. ardeae* (Erlandsen et al. 1990) was kindly provided by Prof. R. C. A. Thompson (Murdoch University, Perth, Western Australia).

Enzyme Electrophoresis and Allozyme Analysis

Sonicates prepared from trophozoites grown in either axenic culture or experimentally infected suckling mice were examined for enzyme charge polymorphisms by electrophoresis on cellulose acetate (Andrews et al. 1989, 1993). A total of 21 enzyme activities were detected (table 2), representing products from a presumptive 23 gene loci. For each enzyme, bands were scored as allozymes (a, b, c, etc.) in order of increasing anodal migration. The proportion of loci showing ''fixed'' allelic differences (the absence of shared alleles) was used as a measure of genetic distance between pairs of isolates. This measure correlates with other genetic distance measures such as Nei's *D* (Richardson, Baverstock, and Adams 1986), and it has been used successfully to elucidate genetic relationships within protozoan and metazoan parasites (*Giardia*: Andrews et al. 1989; Mayrhofer et al. 1995; *Leishmania*: Andrews et al. 1988; helminths: Chilton, Beveridge, and Andrews 1992; arthropods: Andrews et al. 1992). Genetic distances were calculated from allelic profiles using software kindly provided by Mark Adams (South Australian Museum) and analyzed using PHYLIP (Felsenstein 1993).

Amplification of Gene Segments by the Polymerase Chain Reaction

*The Glutamate Dehydrogenase (*gdh*) Gene*

The polymerase chain reaction (PCR) conditions and oligonucleotide primers used to amplify and sequence a 1.17-kb segment of the *gdh* gene have previously been described (Monis et al. 1996). Primer GAGDH500 (5′-GAG ATG TGC AAG GAY AAC-3′) was used to sequence part of the *gdh* gene amplified from *G. ardeae* DNA.

*The Triose Phosphate Isomerase (*tpi*) Gene*

Two forward and three reverse primers were designed for the *tpi* gene, using published nucleotide sequences for the gene from *G. intestinalis* isolates WB and GS/M (Mowatt et al. 1994) and our own sequences as they became available during this study: TPIGENF

Table 2
Allele Profiles of *Giardia* Isolates at 23 Enzyme Loci

	Enzyme Locus																						
Isolate	*Acp*	*Ald*	*Dia-1*	*Dia-2*	*Enol*	*Est*	*Gdh*	*Got-1*	*G6pd*	*Gpt*	*Hk*	*Mdh*	*Me*	*Ndpk*	*Np-1*	*Np-2*	*Pep-A*	*Pep-D*	*Pgam*	*6Pgd*	*Pgk*	*Pgm*	*Tpi*
Ad-1.....	e[1]	e	c	c	e	d	b	b	c	e	e	a	f	a	a	a	b	d	d	a	d	d	a
Ad-2.....	e	e	d	c	e	d	b	b	c	f	e	a	f	a	b	b	b	d	d	a	d	e	a
P-15.....	f	e	a	d	g	d	e	e	b	e	d	a	e	a	b	c	b	d	d	a	d	—	a
Ad-23.....	f	g	—	—	e	a	a	c	a	e	e	c	e	c	b	c	b	e	b	a	d	d	d
BAH-12....	b	df	f	c	d	e	bc	c	f	d	g	b	ac	a	d	d	a	e	bc	cd	b	bc	a
Ad-19.....	a	g	f	—	d	d	b	b	f	d	f	b	—	a	—	d	a	d	b	b	b	c	a
Ad-28.....	b	g	f	b	d	d	b	d	e	d	f	b	c	a	d	d	a	d	b	b	d	c	a
Ad-148	c	e	c	c	e	a	c	b	g	b	d	e	g	a	b	b	b	c	b	c	b	a	c
Ad-137	d	b	e	c	b	d	c	a	g	a	c	e	f	a	e	e	c	c	b	b	b	a	c
Ad-136	d	b	f	b	b	g	c	a	g	b	c	d	h	a	e	e	a	a	b	c	b	a	b
Ad-157	f	g	a	c	c	f	c	d	f	e	a	a	f	a	g	g	c	e	b	a	e	b	a
G. ardeae ..	b	h	f	d	d	g	c	f	c	g	c	e	j	—	g	g	c	d	b	—	—	b	b
Ad-120	f	i	g	d	f	i	d	e	c	h	b	c	g	c	h	h	c	c	a	c	d	b	d

Note.—Alleles are designated alphabetically in order of increasing anodal migration. Where present, multiple loci are designated numerically according to increasing electrophoretic mobility (—, not scoreable).

(5′-ATCGGYGGTAAYTTYAARTG-3′) and TPI16F (5′- CCCTTCATCGGYGGTAAC-3′); TPI533R (5′-CCCGTGCCRATRGACCACAC- 3′), TPI572R (5′-ACRTGGACYTCCTCYG-CYTGCTC-3′), and TPIGENR (5′-CACTGGCCAAGYTTYTCRCA-3′). Reactions (94°C for 2 min, then 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final 72°C extension for 7 min) were performed in 0.2-ml thin-walled tubes on a Perkin Elmer GeneAmp PCR System 2400 using 50 μl of 1 × *Taq* DNA polymerase 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, Western Australia) containing 4 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate, 0.8 μM of both the forward and the reverse primers, 1 U of *Taq* DNA polymerase, and *Giardia* DNA (50–200 ng). These conditions were adequate for all primer pairs, although the amplification of the *tpi* segment using *G. ardeae* DNA as a template required the inclusion of dimethyl sulfoxide (DMSO) at a final concentration of 5%.

*The Elongation Factor 1 Alpha (*ef1α*) Gene*

Nucleotide sequences for the *ef1α* gene from *G. intestinalis, Trypanosoma cruzi, Entamoeba histolytica, Plasmodium falciparum, Euglena gracilis, Saccharomyces cerevisiae, Drosophila melanogaster, Artemia* sp., *Mus musculus, Gallus gallus, Xenopus laevis,* and *Lycopersicon esculentum* were obtained from GenBank (accession numbers D14342, D29834, X86144, X60488, X16890, X01638, X06869, X00546, X13661, L00677, X55324, and X14449, respectively). The sequences were aligned using CLUSTAL W (Thompson, Higgins, and Gibson 1994), and adjustments to indels were made to maintain codon integrity. A degenerate primer was constructed from a highly conserved region and designated EF1AR (5′-AGCTCYTCGTGRTGCATYTC- 3′). More specific primers, designated GLONGF (5′-GCTCSTTCAAGTACGCGTGG-3′) and GLONGR (5′-GCATCTCGACGGATTCSACC- 3′), were designed using only the *G. intestinalis*/*T. cruzi* sequences. Reactions were performed as described above for *tpi*, using primer combinations GLONGF + GLONGR or GLONGF + EF1AR and including DMSO (5% final).

The 18S Ribosomal RNA Gene

The *G. muris, G. ardeae,* and *G. intestinalis* 18S rRNA sequences reported by van Keulen et al. (1991) were obtained from GenBank and aligned using CLUSTAL W (Thompson, Higgins, and Gibson 1994). Conserved regions were identified, and primers designated G18S2 (5′-TCCGGTYGATTCTGCC-3′) and G18S3 (5′-CTGGAATTACCGCGGCTGCT-3′) were constructed. Amplifications were performed as described above (for *tpi*) but with an annealing temperature of 60°C and with DMSO at a final concentration of 10%. Amplification of rDNA from *G. intestinalis, G. muris,* or (in the case of samples grown in vivo) contaminating host (murine) DNA varied with reaction conditions. Lowering the annealing temperature to 55°C and omitting DMSO allowed mouse rDNA (distinguishable from the *Giardia* rDNA sequences on the basis of size) to be amplified, while use of 5% DMSO facilitated amplification of both mouse and *G. muris* rDNA (not shown).

Nucleotide Sequence Determination and Phylogenetic Analyses

Uncloned amplified DNA (purified using BresaClean DNA purification kits; Bresatec Ltd., Adelaide, Australia) was used for cycle sequencing reactions (Prism Ready Reaction Dye Deoxy Terminator Cycle sequencing kit, Applied BioSystems Inc. [ABI], Foster City, Calif.). Sequences were determined by automated analysis (ABI 373A or 377 DNA sequencers) and collated and aligned using the ABI software SeqEd. Neighbor-joining (NJ) analyses were performed using Tamura-Nei distances as implemented by MEGA (version 1.02; Kumar, Tamura, and Nei 1994). Maximum-likelihood analyses were conducted using PUZZLE (version 4.1; Strimmer and von Haeseler 1996) with the default settings, except that parameter estimation was exact and the model of rate heterogeneity was gamma-distributed

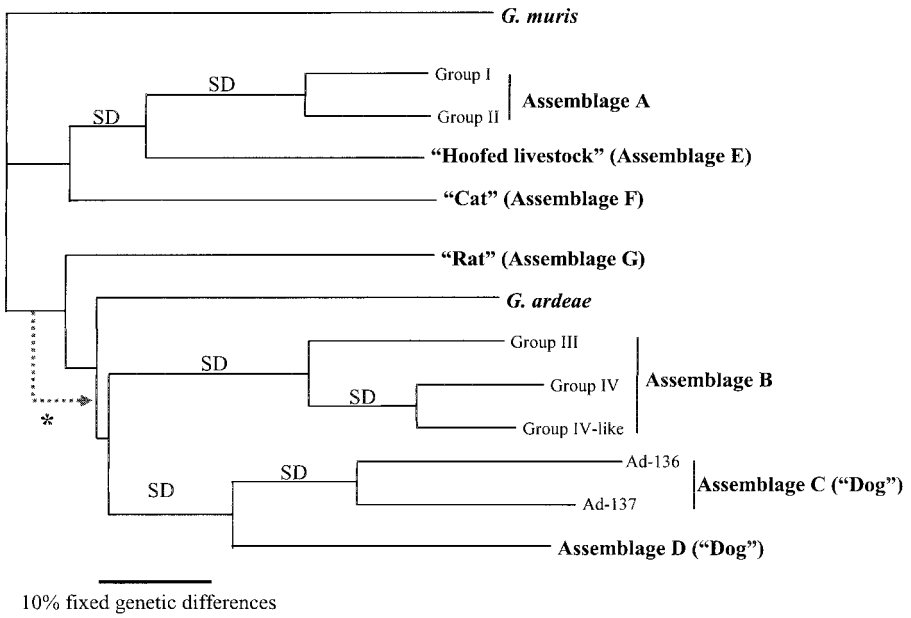

FIG. 1.—Genetic relationships of representative isolates of *G. intestinalis* and single isolates of *G. muris* and *G. ardeae*, inferred from allozyme data by NJ analysis. SD = nodes supported by Split Decomposition analysis; * = alternative placement of this branch by UPGMA.

with eight rate categories. Parsimony analyses were conducted using PAUP (Swofford 1993). Dendrograms were drawn using TREEVIEW (Page 1996). Split decomposition and spectral analyses were performed using SplitsTree (Dress, Huson, and Moulton 1996; Huson 1998) and Spectrum (version 2.0; Charleston 1998). All three codon base positions were utilized for distance estimations.

Results

Analysis of Allozyme Data

A genetic interpretation of the MLEE data yielded the allelic profiles listed in table 2. The proportion of loci exhibiting fixed allelic differences was calculated for each pairwise comparison, and phylogenetic relationships were subsequently inferred by NJ and unweighted pair grouping method with arithmetic means (UPGMA) analysis. The topology of the NJ tree is illustrated in figure 1. The branch lengths were similar for all of the lineages, suggesting that they evolved at similar rates. Two main clusters of isolates were evident within *G. intestinalis.* One contained the cat-derived isolate Ad-23 (''Cat''), as well as representative isolates of assemblage A and isolates from domestic livestock (''Hoofed livestock''). The other cluster included the rat-derived isolate Ad-157 (''Rat''), the *G. ardeae* isolate, and the representatives of assemblages B, C, and D. The topology of the UPGMA tree (not shown) was similar to that of the NJ tree, with the exception of the placement of the branch joining *G. ardeae* and the Rat isolate with the representatives of assemblages B, C, and D (fig. 1). Analysis of the distance data by split decomposition showed clear support for the previously identified assemblages (e.g., assemblages A–C, as indicated in fig. 1). Support was also found for the clustering of assemblage A with the Hoofed livestock group and the clustering of the dog-specific assemblages C and D. Spectral analysis (threshold setting = 0.01) found support for all of the clusters identified by split decomposition, with the exception of the clustering of assemblages C and D (not shown). A Manhattan tree produced from the spectral analysis (not shown, but its expected spectrum was closest to that observed for the data) possessed the same topology as the NJ tree.

Analysis of *gdh* Sequences

We were able to amplify a 1.17-kb segment of the *gdh* locus from all isolates except *G. muris.* Approximately half (690 bp from the 5′ end) of each amplified segment was sequenced. The nucleotide sequences (accession numbers listed in table 1) were aligned and subjected to phylogenetic analyses using distance-based, parsimony, and maximum-likelihood (ML) methods. The resulting trees, represented by the ML tree (fig. 2*A*), were largely compatible with the allozyme-based tree. In all cases, the same two main groups of *G. intestinalis* isolates were identified. The topology of the ML tree (fig. 2*A*) differed from the allozyme-based NJ tree in placing the Rat isolate external to the other *G. intestinalis* isolates. In contrast, the NJ tree (not shown) inferred by analysis of the *gdh* nucleotide sequence data placed the Rat isolate within the cluster containing assemblage A (but with <50% bootstrap support). The topology of this tree was not affected by the distance measure employed (corrected or uncorrected). Parsimony analysis (exhaustive search) identified two trees, each 256 steps in length (not shown). Both had topologies similar to that of the ML tree (fig. 2*A*), except for the placement in one tree of the assemblage B clade as a sister group to the assemblage A, C, and D, Cat, and Hoofed livestock clade. Strong bootstrap support (>90%) was found for the nodes linking isolates from the same as-

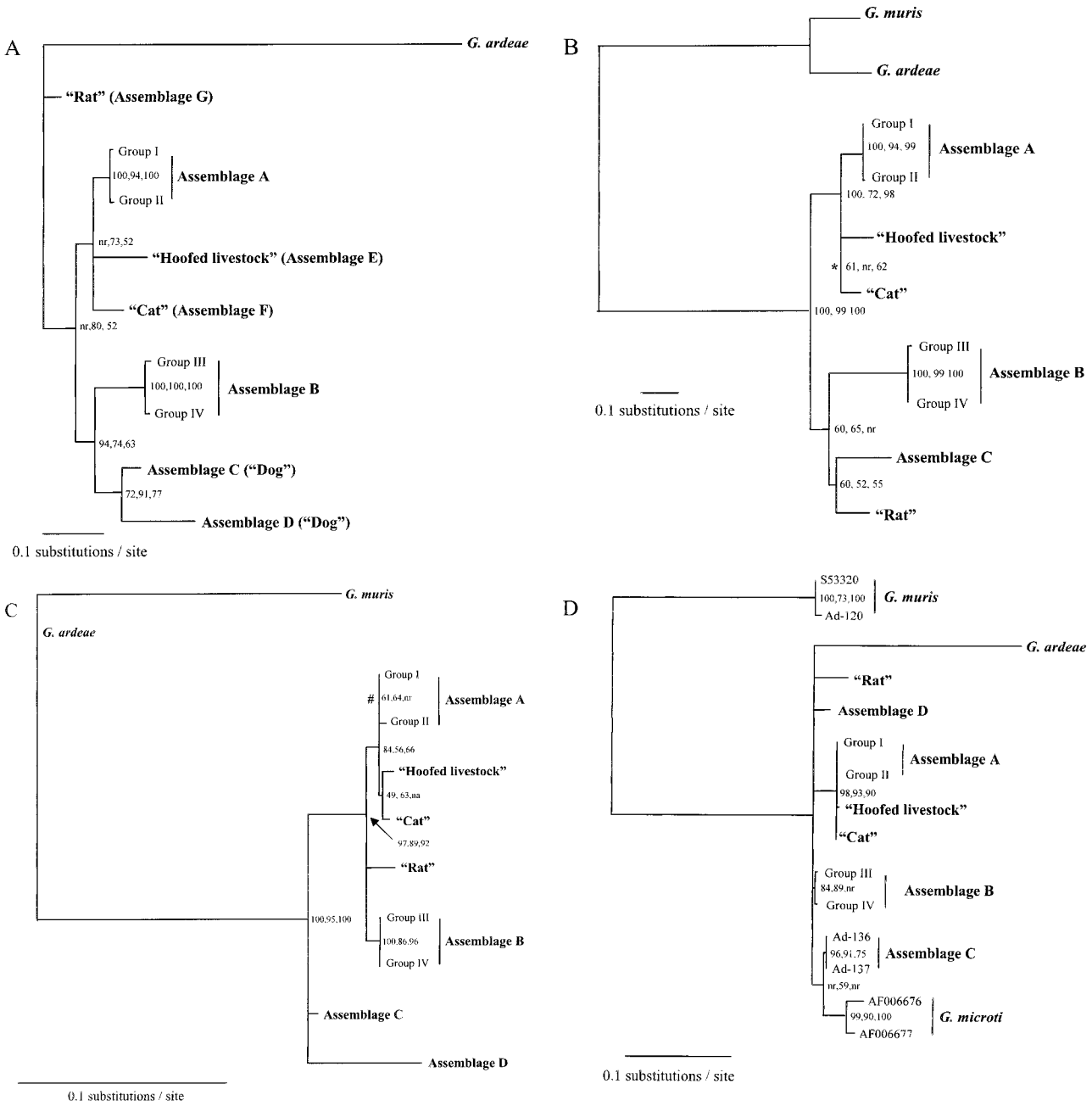

FIG. 2.—Phylogeny of *Giardia* isolates inferred by ML analysis of aligned nucleotide sequences derived from the genes encoding (*A*) glutamate dehydrogenase (GDH), (*B*) triose phosphate isomerase (TPI), (*C*) elongation factor 1α (EF1α), and (*D*) 18S rRNA. Numbers on the right of each node indicate the percentages of bootstrap support determined by NJ analysis (500 replicates), quartet puzzling (1,000 replicates), and parsimony analysis (heuristic search, 500 replicates), respectively. * = nodes not supported by ML analysis; # = nodes with branches that are too short to be visualized at the illustrated scale; nr = node not recovered by method employed.

semblage (e.g., group I and group II genotypes) (fig. 2*A*). Support (52%–94%, depending on the method of analysis) was found for all nodes of the tree except for the branching order of the assemblage A, Cat, and Hoofed livestock isolates. The evolutionary distances between isolates estimated from the ML analysis are summarized in table 3.

Additional analysis of the data by split decomposition supported the topology of the ML tree. Conflict was found for the placement of the Rat and Cat isolates, although this was not apparent if parsimony splits were used. Spectral analysis using Tamura-Nei distances (default threshold) supported two groups, one containing assemblages B, C, and D and the other containing assemblage A and the Hoofed livestock group, with very little conflict. The grouping of assemblage C with assemblage D and that of the Cat isolate with assemblage A and the Hoofed livestock group were also supported, although in these cases the level of conflict was larger (approaching 2/3 of the distances supporting the groups). Use of different distance measures in the analysis caused no noticeable change in the support (or con-

Table 3
Evolutionary Distances Estimated for Different Loci in *Giardia intestinalis*

Locus	Intraassemblage	Interassemblage	*G. intestinalis* vs. *Giardia ardeae*	*G. intestinalis* vs. *Giardia muris*
Allozymes[a]	0.22–0.43	0.50–0.96	0.90–0.94	0.76–1.00
Gdh[b]	0.01–0.02	0.07–0.3	0.69–0.91	—
Tpi[b]	0.004–0.01	0.11–0.41	1.42–1.92	1.44–1.57
Ef1α[b]	0–0.003	0.004–0.08	0.12–0.17	0.23–0.32
18S rRNA[b]	0–0.004	0–0.04	0.18–0.22	0.39–0.47

[a] Fraction of examined loci at which fixed allelic differences were detected by allozyme electrophoresis.

[b] Number of substitutions per nucleotide site, estimated using the HKY model of substitution (Hasegawa, Kishino, and Yano 1985) with gamma correction (eight categories) as implemented by PUZZLE (version 4.01).

flict) for any particular group. The Manhattan tree (not shown) was consistent with the ML tree (fig. 2*A*).

Analysis of *tpi* Sequences

The expected 520-bp segment of the *tpi* gene was amplified by PCR from all isolates except the assemblage D representative. These products were sequenced, and 480 bp (see table 1 for GenBank accession numbers) were used for phylogenetic analyses. The nucleotide sequence alignment required the insertion of a 3-bp gap at position 478 in the *G. muris* and *G. ardeae* sequences (relative to the published *G. intestinalis* sequence; Mowatt et al. 1994, GenBank accession number L02120). The results of the distance-based, ML, and parsimony analyses are summarized in figure 2*B* (ML tree illustrated). Parsimony analysis (exhaustive search) recovered a single tree of 382 steps in length (not shown). Both NJ and ML analyses recovered the same two main groups of *G. intestinalis* that were identified by allozyme-based analysis. However, the *tpi* data differed in placing *G. ardeae* as a sister taxon to *G. muris.* Bootstrap analysis produced strong support for the monophyly of *G. intestinalis* (>99%) and the grouping of the assemblage A representatives with the Cat and Hoofed livestock isolates (72%–100%). The grouping of the Rat isolate with the assemblage B and C representatives was supported weakly by NJ and ML analysis (60%–65%) and not recovered by parsimony analysis. Estimates for the evolutionary distances separating these isolates are summarized in table 3.

Split decomposition of the nucleotide sequence data using parsimony splits produced a tree with little structure except for the grouping of the assemblage A, Cat, and Hoofed livestock isolates, reflecting the levels of bootstrap support given to these nodes in the parsimony analysis. Split decomposition of hamming distances (essentially scaled Poisson distances) produced a tree compatible with the *tpi* ML tree (fig. 2*B*). Spectral analysis using hamming distances supported two groups: one containing the assemblage A, Cat, and Hoofed livestock isolates, and the other containing the assemblage C and Rat isolates. The number of conflicts for the first group increased when LogDet or Tamura-Nei distances were used, but not enough to outweigh the support.

Analysis of *ef1α* Sequences

The nucleotide sequence of a 650-bp segment of the *ef1α* locus was determined for every *Giardia* isolate used in this study (table 1). Analysis of the sequences by distance-based, ML, and parsimony methods produced trees with similar topologies (fig. 2*C,* ML tree illustrated). NJ analysis placed assemblage D as the earliest-branching *G. intestinalis* lineage (68% bootstrap support). Parsimony analysis (branch and bound) found three trees, each of 90 steps in length (not shown). Only one of the two main groups identified by the allozyme, *gdh,* and *tpi* analyses was apparent from the *ef1α* analyses (that containing the assemblage A, Cat, and Hoofed livestock isolates). The longest branches of the tree were the lineages giving rise to *G. muris* and *G. intestinalis.* The monophyly of *G. intestinalis* was strongly supported (95%–100% of bootstrap replicates). The level of support for the grouping of the assemblage A, Cat, and Hoofed livestock isolates varied depending on the method of analysis (56%–84%). The grouping of these isolates with the assemblage B and Rat isolates was strongly supported (89%–97%). Surprisingly, the monophyly of the two assemblage A representatives was poorly supported by this locus (<64% or not recovered)—probably because of the high level of conservation of *ef1α* and the apparent more recent emergence of the assemblage A, Cat, and Hoofed livestock lineages. However, the monophyly of the assemblage B representative isolates was strongly supported (>86%). The ML estimates of the evolutionary distances between isolates determined at this locus are summarized in table 3.

Split decomposition of hamming distances placed assemblages C and D external to the other isolates of *G. intestinalis* (consistent with the phylogenetic analyses), but otherwise, it was difficult to discern a clear branching order. Analysis using parsimony splits yielded the same divisions and indicated conflicting splits for many of the nodes of the tree. Spectral analysis of hamming, Tamura-Nei, and LogDet distances (default threshold) supported the results of the distance analysis. Minimal conflict was observed for the bipartition separating *G. intestinalis* from *G. muris* and *G. ardeae,* and no conflict was found for any of the well-supported nodes of the ML tree (fig. 2*C*). In addition, the placement of assemblage D as the earliest-diverging branch of the *G. intestinalis* lineage was supported.

Analysis of 18S Ribosomal RNA Gene Sequences

DNA corresponding in size to the expected segment of the *Giardia* 18S rRNA gene was amplified from the DNA of all of the isolates examined. Approximately

480 bp of the DNA amplified from the isolates listed in table 1 was sequenced. Sequences from a group I representative (Portland-1, accession number M19500), a group II representative (BRIS/83/HEPU/106, accession number X52949), *G. ardeae* (accession number S53312), *G. muris* (accession number S53320), and *G. microti* (accession numbers AF006676 and AF006677) were obtained from GenBank. Analysis of Tamura-Nei distances by NJ analysis produced a tree (not shown) with a topology similar to that of the tree inferred from analysis of the *ef1α* nucleotide sequence data (fig. 2*C*). The two differed only in the relative branching order of assemblage B and the Rat lineage. *Giardia microti* (not represented in the earlier analyses) was placed as a sister taxon to the *G. intestinalis* cluster (not shown). The monophyly of *G. muris* and *G. microti* (but not *G. intestinalis*) was supported by bootstrap analysis, as was the clustering of *G. microti* with *G. intestinalis.*

ML and parsimony analyses produced poorly resolved trees (fig. 2*D*, ML tree illustrated). In contrast to the NJ analysis, ML analysis placed *G. microti* as a sister taxon to assemblage C (59% bootstrap support), and *G. ardeae* was placed within the same clade as the *G. intestinalis* and *G. microti* isolates (73% bootstrap support). Parsimony analysis (branch and bound) found 72 trees of 232 steps in length. Only five clades were common to all trees and supported by bootstrap analysis (>75% support). Split decomposition produced a network with a topology similar to that of the NJ tree (not shown), but spectral analysis only found support for the bipartitions that were supported by the bootstrap analyses.

Analysis of Amino Acid Sequences Predicted from Structural Genes

Distance-based and ML analyses were conducted on aligned amino acid sequences inferred from the *gdh*, *tpi*, and *ef1α* nucleotide sequences. Poisson-corrected distances were used for NJ analyses, and the JTT model of substitution (Jones, Taylor, and Thornton 1992) was used for the ML analysis. The topologies of the GDH- and TPI-based NJ trees (not shown) were identical. The EF1α-based NJ tree was similar, but it contained regions that could not be resolved because of the more conserved nature of this protein. ML analysis of the GDH and EF1α data (not shown) produced trees compatible with those of the NJ analyses except that the branch order of assemblages B and C could not be resolved. In all cases, the assemblage A, Cat, Hoofed livestock, and Rat representatives were grouped together. Bootstrap analysis supported these groups, but it gave weaker support for the rest of the tree. A combined analysis of the predicted GDH, TPI, and EF1α amino acid sequences (excluding those derived from the assemblage D and *G. muris* isolates) produced a result (fig. 3, ML tree illustrated) compatible with the analyses of the individual loci, but with higher bootstrap values. Within this composite polypeptide-based tree, significant bootstrap support was obtained for the placement of the Cat and Hoofed livestock lineages as sister taxa (81%–89%) and the placement of assemblage A as a sister taxon to this group (97%–99%). Split decomposition and spectral analysis of the composite amino acid distance data supported the topology of this tree.

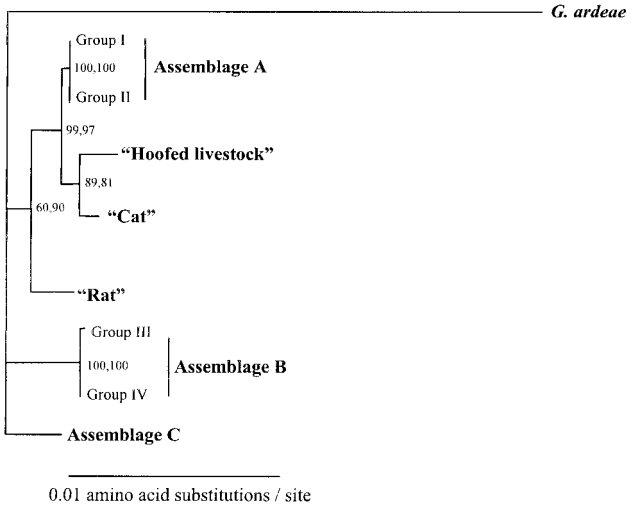

FIG. 3.—Phylogeny of *Giardia* isolates inferred by ML analysis of GDH, TPI, and EF1α amino acid sequences. Evolutionary distances, calculated using the JTT model of substitution (Jones, Taylor, and Thornton 1992) with gamma correction (eight rate categories), were determined for the combined (contiguous GDH + TPI + EF1α) sequences predicted from characterized segments of the respective genes. Numbers on the right of each node indicate the percentages of bootstrap support determined by NJ analysis of Poisson corrected distances (500 replicates) and quartet puzzling (1,000 replicates).

Discussion

In this study, we analyzed a panel of *G. intestinalis* isolates representative of all of the known genetic assemblages that constitute this species. Sequence data from genes encoding essential enzymes or rRNA, together with allozyme data derived by MLEE from ''housekeeping'' enzymes, were used to infer the evolutionary relationships of these genotypes and to compare estimates of genetic divergence between isolates of *G. intestinalis* with those of genetic divergence between morphologically defined species of *Giardia.* Several important conclusions may be deduced from the results. First, *Giardia* possessing the ''claw-hammer'' median body morphology are monophyletic. Second, *G. intestinalis* does not form a monophyletic group to the exclusion of *G. microti.* Third, in view of the fact that *G. microti* is considered a species distinct from *G. intestinalis* (Feely 1988; Kulda and Nohýnková 1996; van Keulen et al. 1998), genetic data showing that the major lineages of *G. intestinalis* are as divergent from each other as they are from *G. microti* provide strong grounds for considering these lineages distinct species also.

Comparisons of *G. intestinalis, G. ardeae,* and *G. muris* by MLEE (Mayrhofer et al. 1995) and rRNA sequence (van Keulen et al. 1993) analysis have supported the existing morphology-based taxonomy of the genus, with each data set indicating a large degree of genetic divergence between these species. Although allozyme analysis is not suitable for exploring higher-level systematic relationships due to the possibility of convergent

evolution or the reversion of electrophoretic variants (Richardson, Baverstock, and Adams 1986), this has not limited its utility for the study of population genetic phenomena or the comparison of sibling species. The technique has proven especially useful for defining the broad genetic relationships within *G. intestinalis* and provided some of the earliest indications that this ''species'' might be a species complex (Andrews et al. 1989; Mayrhofer et al. 1995; Monis et al. 1998). In contrast, the highly conserved nature of rRNA genes can limit their usefulness in many taxa to defining systematic relationships at or above genus level. We have previously used data from rapidly evolving genes to complement MLEE studies of *G. intestinalis* belonging to assemblage A (Ey, Andrews, and Mayrhofer 1993; Ey et al. 1996; Monis et al. 1996) and of the related complex isolated from domestic livestock (Ey et al. 1997). The present analysis of nucleotide sequences from genes that encode 18S rRNA and three housekeeping enzymes which appear to evolve at different rates (*tpi* > *gdh* ≫ *ef1α*), in combination with MLEE, has allowed us to investigate evolutionary relationships at three levels: within the genetic assemblages that compose *G. intestinalis,* between these assemblages, and between *G. intestinalis* and *G. ardeae* and *G. muris.*

The interspecific distances observed at the *ef1α* locus in comparisons between *G. intestinalis* and *G. muris* were only about half those observed for the same comparisons using 18S rRNA (van Keulen et al. 1993) and more than fivefold less than those observed using *gdh* or *tpi* (table 3). The *tpi* locus (the least conserved of the three structural genes examined) exhibited interspecific distances approximately twice those observed for *gdh.* Interestingly, the distances for some of the interspecific and intraspecific comparisons do not appear to be consistent when compared across the loci examined. For example, the interspecific distances determined from the *tpi* locus are twice those observed for the *gdh* locus, but the intraspecific distances are nevertheless similar.

The distinctiveness of assemblages A and B was strongly supported in all of the phylogenetic trees constructed from nucleotide sequence data and the predicted amino acid sequences. There was general broad agreement between all of the trees, especially regarding the clustering of the Cat and Hoofed livestock isolates with those belonging to assemblage A. Although the precise branching order of the Cat and Hoofed livestock lineages varied with the data source, it is clear that they form a clade in association with assemblage A isolates from humans, sheep, and dogs (Ey et al. 1997; Monis et al. 1998). The placements of the Rat and assemblage B, C, and D isolates are less certain. The Rat isolate was placed as an internal branch of *G. intestinalis,* except in the ML and parsimony analyses of the *gdh* nucleotide sequence data, where it was identified as the earliest-branching lineage of *G. intestinalis.* The *ef1α* sequence data supported placement of assemblage C and D isolates on the earliest branching lineages of *G. intestinalis.* NJ analysis of the 18S rRNA data also recovered this pattern but found no bootstrap support for it. In contrast, analysis of the *gdh* nucleotide sequence and allozyme data placed assemblages B–D in the same cluster. Analysis of additional loci will be required to elucidate the relationship of the Rat isolate and assemblages B–D to the other lineages within *G. intestinalis.*

On the basis of this study, *G. intestinalis* can be seen to consist of at least seven well-resolved lineages, only two of which (assemblages A and B) contain isolates from humans. Two other lineages (assemblages C and D) consist only of isolates obtained from dogs in Australia (Monis et al. 1998). The intraspecific distances that separate the remaining lineages are similar to those that separate assemblages A–D. The separation of these various lineages is similar to (or greater than) those between species in other genera of microorganisms (e.g., *Naegleria*: Adams et al. 1989; *Escherichia* and *Salmonella*: Nelson, Whittam, and Selander 1991). Available biological data suggest that a strong correlation exists between some biotypic characteristics, e.g., host range and growth rate in vitro or in vivo, and the genetic lineage to which an isolate belongs (Andrews, Chilton, and Mayrhofer 1992; Binz et al. 1992; Karanis and Ey 1998; Monis et al. 1998).

A difficulty with the systematics of asexual organisms (as outlined in the introduction) is that the biological-species concept cannot be applied for species delineation. The phylogenetic-species concept could be used as an alternative, but its use is fraught with difficulties because there is no method available for determining the level of evolutionary divergence that constitutes discrete species. We propose that correlations between the phylogeny and biological characteristics of a group of organisms can be used to determine this level. Such an approach, which would not preclude the use of morphological characters, would produce a taxonomy that reflects both the biology and the phylogeny of the taxa under investigation. The genetic and biological data appear to be sufficiently clear-cut to support the elevation of the lineages of *G. intestinalis* to species status, especially on the basis of the precedent set by the description of *G. microti* (van Keulen et al. 1998). However, it should be noted that biological data are available from only a limited number of isolates. Thus, until additional data are available to confirm the biotypic and genetic correlations, we will maintain the existing nomenclature by designating the Hoofed livestock lineage assemblage E and designating the lineages represented by the Cat (Ad-23) and Rat (Ad-157) isolates assemblages F and G, respectively.

Further clarification of the evolutionary relationships of the lineages comprising *G. intestinalis* may prove difficult. The data suggest that the internodal distances separating the lineages are relatively short, while the branch lengths are relatively long. If the genus *Giardia* has a clonal structure, as proposed by Tibayrenc, Kjellberg, and Ayala (1990), the observed pattern could indicate either that many other lineages remain to be discovered within *G. intestinalis* or that many have been lost by extinction of hosts. However, it is also possible that the extant lineages arose by an ancient, rapid radiation. If the latter is the case, then homoplasy and saturation effects could obscure the relationships inferred

from most gene sequences. It might be possible to overcome this problem by using amino acid sequences for phylogenetic analysis. This approach has been successfully used to examine early divergences in eukaryotes (Hashimoto et al. 1994). Combined analysis of predicted amino acid sequences from the *gdh*, *tpi*, and *ef1α* loci does appear to resolve the relationships within the group containing isolates from assemblage A and cats. Additional sequence data from other conserved protein-encoding genes will be required to further test the proposed relationships. Moreover, collection of *Giardia* from a larger range of mammalian hosts, especially from pristine environments, will allow the identification of any additional lineages and the completion of the systematics of *G. intestinalis.*

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