Retortamonad Flagellates are Closely Related to Diplomonads—Implications for the History of Mitochondrial Function in Eukaryote Evolution

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We present the first molecular phylogenetic examination of the evolutionary position of retortamonads, a group of mitochondrion-lacking flagellates usually found as commensals of the intestinal tracts of vertebrates. Our phylogenies include small subunit ribosomal gene sequences from six retortamonad isolates—four from mammals and two from amphibians. All six sequences were highly similar (95%–99%), with those from mammals being almost identical to each other. All phylogenetic methods utilized unequivocally placed retortamonads with another amitochondriate group, the diplomonads. Surprisingly, all methods weakly supported a position for retortamonads cladistically within diplomonads, as the sister group to *Giardia*. This position would conflict with a single origin and uniform retention of the doubled-cell organization displayed by most diplomonads, but not by retortamonads. Diplomonad monophyly was not rejected by Shimodaira-Hasegawa, Kishino-Hasegawa, and expected likelihood weights methods but was marginally rejected by parametric bootstrapping. Analyses with additional phylogenetic markers are needed to test this controversial branching order within the retortamonad + diplomonad clade. Nevertheless, the robust phylogenetic association between diplomonads and retortamonads suggests that they share an amitochondriate ancestor. Because strong evidence indicates that diplomonads have secondarily lost their mitochondria (rather than being ancestrally amitochondriate), our results imply that retortamonads are also secondarily amitochondriate. Of the various groups of eukaryotes originally suggested to be primitively amitochondriate under the archezoa hypothesis, all have now been found to have physical or genetic mitochondrial relics (or both) or form a robust clade with an organism with such a relic.

Introduction

Over the last two decades, the quest to understand the origin of eukaryotic cells has become established as an important field of research. During this time, the most important and influential idea has been the archezoa hypothesis (see Roger 1999). This hypothesis held that certain living unicellular eukaryotes that lack mitochondria had diverged before the acquisition of the mitochondrial symbiont, a seminal milestone in the history of eukaryotes. The hypothesis first crystallized when Cavalier-Smith grouped various mitochondrion-lacking (amitochondriate) eukaryotes in a new taxon, Archezoa, which he proposed to be the stem group for living eukaryotes (Cavalier-Smith 1983). The organisms then included were diplomonads, retortamonads, oxymonads, parabasalids, microsporidia, *Entamoeba*, and pelobionts (Cavalier-Smith 1983, 1987). The hypothesis achieved widespread popularity when small subunit ribosomal RNA (ssu rRNA, ssu rDNA) genes from diplomonads, parabasalids, and microsporidia were included in universal phylogenies and emerged as the basal branches among eukaryotes (Vossbrinck et al. 1987; Sogin 1989; Sogin et al. 1989). Broadly similar results were recovered with additional taxa and methods analyzing ssu rRNA (e.g., Leipe et al. 1993) and in phylogenies of proteins such as elongation factors (e.g., Hashimoto et al. 1994, 1995). By the early 1990s the archezoa hypothesis was almost orthodoxy.

In recent years, the archezoa hypothesis has fallen into disfavor primarily because of the discovery of genes of apparent mitochondrial origin within the nuclei of many putative Archezoa. The presence and phylogenetic affinities of these genes strongly suggest that the organisms bearing them had once had mitochondria but had subsequently lost or modified them. For example, mitochondrial isoform–related chaperonin 60 (cpn60) or heat shock protein 70 (hsp70) genes (or both) have now been found in diplomonads, parabasalids, microsporidia, and *Entamoeba* (see Roger 1999). Additionally, parabasalids and *Entamoeba* still harbor apparently mitochondrion-derived organelles, hydrogenosomes, and mitosomes, respectively (Mai et al. 1999; Tovar, Fischer, and Clark 1999; Dyall and Johnson 2000; Rotte et al. 2000). Organelles resembling hydrogenosomes-mitosomes have now also been reported in several pelobionts (Andresen, Chapman-Andresen, and Nillson 1968; Chavez, Balamuth, and Gong 1986; Seravin and Goodkov 1987; Walker et al. 2001), and the most recent molecular phylogenetic analyses seem to place pelobionts as the sister to entamoebae (Silberman et al. 1999; Milyutina et al. 2001), indicating that they too are secondarily amitochondriate. Recently, the first molecular phylogenies placing oxymonads in a robust position (ssu rRNA) indicate a close ancestry with *Trimastix* (Dacks

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Table 1
***Retortamonas* Isolates, Their Hosts, Growth Conditions, and ssu rRNA Gene Sequence GenBank Accession Number**

Isolate	Host	Growth Temperature	Accession Number
Retortamonas sp.—Koza[a]	Goat, *Capra hircus*	37°C	AF439344
Retortamonas sp.—Los[b]	Elk, *Alces alces*	37°C	AF439345
Retortamonas sp.—Ovce[a]	Sheep, *Ovis aries*	37°C	AF439346
Retortamonas sp.—*caviae*[c]	Guinea pig, *Cavia porcellus*	37°C	AF439349[f]
Retortamonas sp. ATCC 50375[d]	Poison arrow frog, *Dendrobates auratus*	~20°C	AF439347
Retortamonas sp.—Vale[e]	Giant salamander, *Andrias davidianus*	~20°C	AF439348

[a] Organism isolated by Ivan Cepicka.
[b] Organism isolated by Vladimir Hampl.
[c] Organism isolated by C. Graham Clark.
[d] Organism isolated by Susan L. Poynton.
[e] Organism isolated by Jaroslav Kulda.
[f] Sequence previously reported by Amaral Zettler et al. (2000).

et al. 2001). Although no obvious double-membrane–bounded organelles have been described in oxymonads, the flagellated protist *Trimastix* does possess organelles that resemble mitochondria (Brugerolle and Patterson 1997). This *Trimastix*-oxymonad clade emerges nowhere near the base of the eukaryotic tree, which is also consistent with a mitochondriate ancestry.

The only original archezoan whose mitochondrial status remains unchallenged by molecular data is the retortamonad. Retortamonads are a small group of flagellates comprising two subtaxa (genera), *Chilomastix* and *Retortamonas*. Several ultrastructural synapomorphies (most notably the arched fiber form of the B fiber and the presence of the dorsal lapel structure) support the monophyly of the group (Simpson and Patterson 1999). Most retortamonads are obligate symbionts, commensals, or parasites inhabiting animal guts (Kulda and Nohynková 1978), with one free-living species also known, *Chilomastix cuspidata* (Bernard, Simpson, and Patterson 1997). In addition to lacking mitochondria, retortamonads also seem to lack Golgi, dictyosomes, and peroxisomes (Brugerolle and Müller 2000).

Retortamonads have often been considered together with the similarly cytologically depauperate diplomonads and oxymonads, collectively referred to by many as the metamonads (Cavalier-Smith 1987; Corliss 1994; Brugerolle and Müller 2000). However, there has never been strong morphological evidence uniting all three taxa (Brugerolle and Müller 2000), or even any two of them, prompting some workers to avoid the use this grouping (Patterson 1994, 1999). In fact, recent phylogenies based on molecular data do not group oxymonads and diplomonads (Moriya, Ohkuma, and Kudo 1998; Dacks and Roger 1999; Dacks et al. 2001; Moriya et al. 2001). More recently, ultrastructural data have emerged that link retortamonads to several mitochondrion-bearing flagellates, especially core jakobids (e.g. *Reclinomonas* and *Jakoba*) and *Malawimonas* (O'Kelly 1993, 1997; O'Kelly and Nerad 1999). The close structural similarities between retortamonads, core jakobids, *Malawimonas*, *Trimastix*, *Carpediemonas*, diplomonads, and Heterolobosea form the basis of the excavate hypothesis (Simpson and Patterson 1999) which argues that these taxa have descended from a common ancestor. Regardless of the validity of the excavate hypothesis, it remains unclear whether the common ancestor of these organisms had mitochondria or whether excavate organisms are a grade from which most other living eukaryotes have descended, possibly straddling the mitochondrial acquisition event (O'Kelly 1993; O'Kelly and Nerad 1999; Simpson and Patterson 1999). The latter possibility would allow retortamonads to be primitively amitochondriate, even if diplomonads were not. The relationships amongst excavate taxa remain poorly understood (O'Kelly and Nerad 1999; Simpson, Bernard, and Patterson 2000; Simpson and Patterson 2001), and there is no convincing structural evidence placing retortamonads or diplomonads any closer to each other than to any of the other excavate taxa.

Here we report ssu rRNA gene sequences from several *Retortamonas* isolates. We demonstrate a strongly supported phylogenetic affinity between retortamonads and diplomonads. Because current evidence indicates that the ancestors of diplomonads once had mitochondria, a retortamonad + diplomonad clade suggests that retortamonads also descend from mitochondrion-bearing ancestors.

Materials and Methods

Organisms and Culture Conditions

Retortamonas sp. ATCC 50375 and the microaerophilic-anaerobic heterolobosei *Sawyeria marylandensis* (ATCC 50653) were purchased from the American Type Culture Collection and grown according to instructions. A cell pellet from the aerobic heterolobosei *Heteramoeba clara* (ATCC 30972) was kindly provided by Thomas Nerad of the American Type Culture Collection. The other retortamonads examined were isolated from the hosts indicated in table 1 and grown in Dobell and Laidlaw's biphasic medium (Dobell and Laidlaw 1926). Cultures were grown in 15 ml polypropylene screwcap tubes at either ~20°C or 37°C (table 1). Each culture tube contained a 3-ml slant of inspissated horse

serum (75–80°C for 2 h) covered by 3 ml of 10% egg white in Ringer's saline (6.5 g NaCl, 0.2 g $NaHCO_3$, 0.14 g KCl, 0.01 g NaH_2PO_3, 0.16 g $CaCl_2$ per liter H_2O). These cultures were maintained by serial transfer (twice weekly for cultures kept at ~20°C, 2–3 days for 37°C cultures).

Genomic DNA Isolation, ssu rDNA Amplification and Sequencing

Retortamonas and *Sawyeria* cells were harvested from approximately 6–12 ml of each culture. DNA from all organisms was isolated using the PureGene kit (Gentra systems, Inc., Minneapolis, Minn.) with a slight modification. After cell lysis and protein precipitation, the DNA-containing aqueous phase was extracted once with chloroform:isoamyl alcohol (24:1) before isopropanol precipitation. Except for the guinea pig *Retortamonas* isolate, eukaryotic-specific primers (Medlin et al. 1988) were used to amplify ssu rDNAs, as described elsewhere (Silberman et al. 1999). *Retortamonas* and *Sawyeria* ssu rDNAs were T-A cloned into the TOPO 2.1 vector (Invitrogen, Carlsbad, Calif.). *Heteramoeba* ssu rDNA was amplified and cloned into M13mp18 and M13mp19, and 10 clones in each orientation were pooled before manual sequencing using S^{35} dATP (Sanger, Nicklen, and Coulson 1977). Between 7 and 12 independent ssu rDNA clones from *Retortamonas* and *Sawyeria* were isolated (Holmes and Quigley 1981) and then pooled before sequencing on an ABI 377 using bigdye chemistry. Each gene was sequenced completely from both strands.

Because a mixed protist culture of *Retortamonas* and *Blastocystis* was recovered from the guinea pig (C. G. Clark, London School of Hygiene and Tropical Medicine, London, England), internal primers (based on the ssu rDNA sequences of diplomonads and *Retortamonas* sp. ATCC 50375) Dip-F (5′-GGGACAGGTGAAAY-AGGATGATCC-3′) and Dip-R (5′- GGATCATCCT RTTTCACCTGTCCC-3′) were used in conjunction with eukaryotic primers B and A, respectively, to specifically amplify the retortamonad ssu rRNA gene in two pieces. Each half of the ssu rRNA gene was T-A cloned and sequenced as described above. This *Retortamonas* sequence, labeled as *Retortamonas caviae*, has been previously used as an outgroup taxon in another study (Amaral Zettler et al. 2000). All sequence data have been deposited with GenBank with accession numbers AF439344–AF439351, and sequence alignments are available upon request (jsilber@ucla.edu).

Phylogenetic Analyses

Two data sets were constructed to ascertain the phylogenetic relationship of retortamonads among eukaryotes and to each other. A comprehensive data set, including ssu rDNA sequences from most major eukaryotic lineages plus an archaeal outgroup consisted of 40 taxa and 1,096 unambiguously aligned positions. Fine-scale relationships among the retortamonads were examined in a restricted data set that included all available retortamonad and diplomonad ssu rDNA sequences along with outgroup sequences from two separate excavate taxa (Heterolobosea and *Trimastix*) and the dictyostelid slime mold *Dictyostelium discoideum* (23 taxa, 1,177 positions).

Phylogenetic analyses were performed with PAUP* 4.0b8 (Swofford 2000), using maximum likelihood (ML) distance (minimum evolution), and parsimony methods under a variety of evolutionary models of DNA substitution. The best available model, as determined by hierarchical nested likelihood ratio tests implemented in Modeltest version 3.06 (Posada and Crandall 1998), was a general time-reversible model of substitution, incorporating a gamma distribution for among-site rate variation (four discrete rate categories) plus an estimate of invariable sites (GTR + Γ + I). This model was employed in maximum likelihood and maximum likelihood distance tree reconstructions. Heuristic tree searches were conducted for each analysis with 20 and 100 random additions of taxa for maximum likelihood and parsimony analysis, respectively, each followed by tree bisection–reconnection topological rearrangements. Support for topological elements was assessed by tree reconstructions of bootstrap-resampled data sets with 200 (smaller-scale analysis) or 357 (large-scale analysis) replicates for maximum likelihood analyses and 1,000 replicates under distance and parsimony criteria. Because the ssu rRNA genes in some diplomonad species are highly biased toward G+C (up to 75% G+C in *Giardia intestinalis*), LogDet distance analyses incorporating an estimate of invariable sites (Gu and Li 1996) were also performed. No significant differences in branching topology were found between LogDet distance and trees estimated from the other methods or models employed. Shimodaira-Hasegawa and Kishino-Hasegawa tests (SH and KH tests, respectively, Kishino and Hasegawa 1989; Shimodaira and Hasegawa 1999) were used to test whether the difference between the log-likelihood (lnL) scores of the optimal ML trees (where retortamonads fall within the diplomonads) and the lnL of the ML trees in which the retortamonads were constrained not to branch within a monophyletic diplomonad clade ($\Delta\text{lnL}_{\text{observed}}$) was statistically significant.

For the smaller data set, we employed two additional tests: the parametric bootstrapping likelihood ratio test (described as the SOWH test by Goldman, Anderson, and Rodrigo 2000) and the expected likelihood weights method (ELW), recently described by Strimmer and Rambaut (2002). For the SOWH test, 1,000 data sets were simulated with Seq-Gen, Version 1.2.4 (Rambaut and Grassly 1997) using the optimal constrained monophyletic diplomonad tree and substitution model parameters (GTR + Γ + I) and branch lengths optimized for this topology, given the data. For each simulated data set, substitution model parameters were reestimated, followed by a heuristic ML tree search (one random stepwise addition replicate) to derive the optimal tree. The differences in lnL between this maximum likelihood topology and the monophyletic diplomonad tree used to simulate the data were then calculated (ΔlnL) to form the null distribution against which the $\Delta\text{lnL}_{\text{observed}}$ was compared. A more detailed exposition

of the SOWH test can be found in Goldman, Anderson, and Rodrigo (2000). To calculate confidence intervals for the optimal tree by the ELW method, two PERL scripts were created (elw.pl and calcwts.pl, available upon request from A.J.R., aroger@is.dal.ca) to automate the procedure using SEQBOOT (PHYLIP 3.57, Felsenstein 2000) and PAUP* 4.0b8. The best trees found in the 200 maximum likelihood bootstrap replicates (described previously) comprised a set of 66 unique trees. These included the maximum likelihood tree for the observed data as well as the monophyletic diplomonad topology. The 95% confidence interval, given this set of 66 likely trees, was then calculated with the ELW method using 1,000 bootstrap replications, with substitution model parameters reestimated for each replicate over a Jukes-Cantor distance–corrected Neighbor-Joining topology.

Results and Discussion

Small Subunit Ribosomal RNA Gene Attributes

All retortamonad ssu rRNA gene sequences are quite similar to each other. The identity among the mammalian isolates ranged from 99.5% to 99.8%, whereas the identity between the two amphibian isolates is slightly less (96.5%). The identity between the mammalian and the amphibian retortamonad ssu rRNA genes is 74.1% overall but is over 95% when gaps in pairwise alignments are not considered and known hypervariable regions are excluded. Unlike the compact ssu genes of diplomonads and trichomonads (1,500–1,650 bp), the retortamonad ssu rRNA genes are 2,031–2,050 bp in length, which is actually larger than the typical 1,800 bp ssu rRNA genes found in most eukaryotes. Length and sequence variations among the retortamonad ssu rRNA genes are largely confined to known hypervariable regions. The overall G+C base composition is 45% in the mammalian isolates and 54% in the amphibian isolates. These values are typical of most eukaryotes, in contrast to the G+C-rich composition characteristic of the ssu rRNA genes from *Giardia* (59% G+C in *G. muris*, 70%–75% G+C in other isolates).

The extreme similarity among the ssu rRNA gene sequences from all the mammalian isolates is consistent with our inability to identify noteworthy morphological differences by light microscopy (J. D. Silberman, unpublished data). In fact, we are hard-pressed to differentiate these new isolates from the human commensal *Retortamonas intestinalis* based on morphological criteria. Considering morphological similarity along with ssu rDNA sequence conservation, the isolates from sheep, elk, goat, guinea pig, and human might provisionally be considered a single species. If conspecificity is accepted for all these mammalian isolates, the name *R. intestinalis* (Wenyon and O'Connor, 1917) Wenrich, 1932 has priority. On the other hand, even though the retortamonads isolated from the two amphibians are likewise morphologically indistinguishable from each other, the sequence dissimilarity between their ssu rRNA genes is somewhat greater than that found among the mammalian isolates, and it is unclear if they represent different species.

The Placement of Retortamonads in the Eukaryote Tree

All methods used here group retortamonads and diplomonads to the exclusion of all other eukaryotes (figs. 1 and 2). In our broad-scale phylogenetic analyses (fig. 1), this relationship is highly supported by bootstrap analyses using maximum likelihood, parsimony, and LogDet distance methods (maximum likelihood, 100%; parsimony, 89%; LogDet, 99%) and receives moderate support from maximum likelihood distance bootstrap analysis (66%). These analyses robustly recover many major eukaryotic clades, such as opisthokonts (Metazoa + Fungi + relatives), Viridiplantae, stramenopiles, alveolates, etc. and now *Retortamonas* + diplomonads. As with other rigorous analyses of ssu rDNA sequences, the relationships among major clades are often poorly resolved using models allowing among-site rate variation (Kumar and Rzhetsky 1996; Silberman et al. 1999).

The most puzzling aspect of this analysis concerns the relationships within the diplomonad + retortamonad clade. As in previous analyses (e.g., Cavalier-Smith and Chao 1996), the diplomonads comprise two monophyletic lineages, the *Giardia* branch and the *Trepomonas* + *Hexamita* + *Spironucleus* branch. Rather than forming the sister group to all diplomonads, the *Retortamonas* sequences branch within diplomonads, as the specific sister group to the *Giardia* sequences (fig. 1). Though bootstrap support for the *Retortamonas* + *Giardia* clade is weak (65%, 35%, 45%, 63% bootstrap support in maximum likelihood, parsimony, maximum likelihood distance, and LogDet distance analyses, respectively), this topology was recovered in the best trees of all the analyses, including the LogDet distance method which is expected to perform well even when base composition is strongly biased (Gu and Li 1996).

We further examined the relationships within the retortamonad + diplomonad clade with a second, more restricted analysis that included more aligned sites. This analysis included all the available retortamonad and diplomonad sequences, together with three eukaryotic outgroups. As with the broad-scale analysis, the retortamonads branch within the diplomonads (fig. 2). Support for the sisterhood of retortamonads and *Giardia* is actually slightly higher than in the broad-scale analyses.

Finally, we explicitly tested the plausibility of diplomonad monophyly within a likelihood framework. For both the broad and restricted analyses, we compared the likelihood scores of the optimal ML trees (in which the retortamonads group with *Giardia*) with the best trees when diplomonads were constrained to be monophyletic (i.e., to the exclusion of retortamonads). In the constrained ML trees the retortamonads branch as sister to the diplomonads (the monophyletic diplomonad tree). The difference in the likelihood of these trees was not significant as judged by both SH tests ($P = 0.409$ and $P = 0.238$ for the broad and restricted analyses, respectively) and KH tests ($P = 0.836$ and $P = 0.483$). We

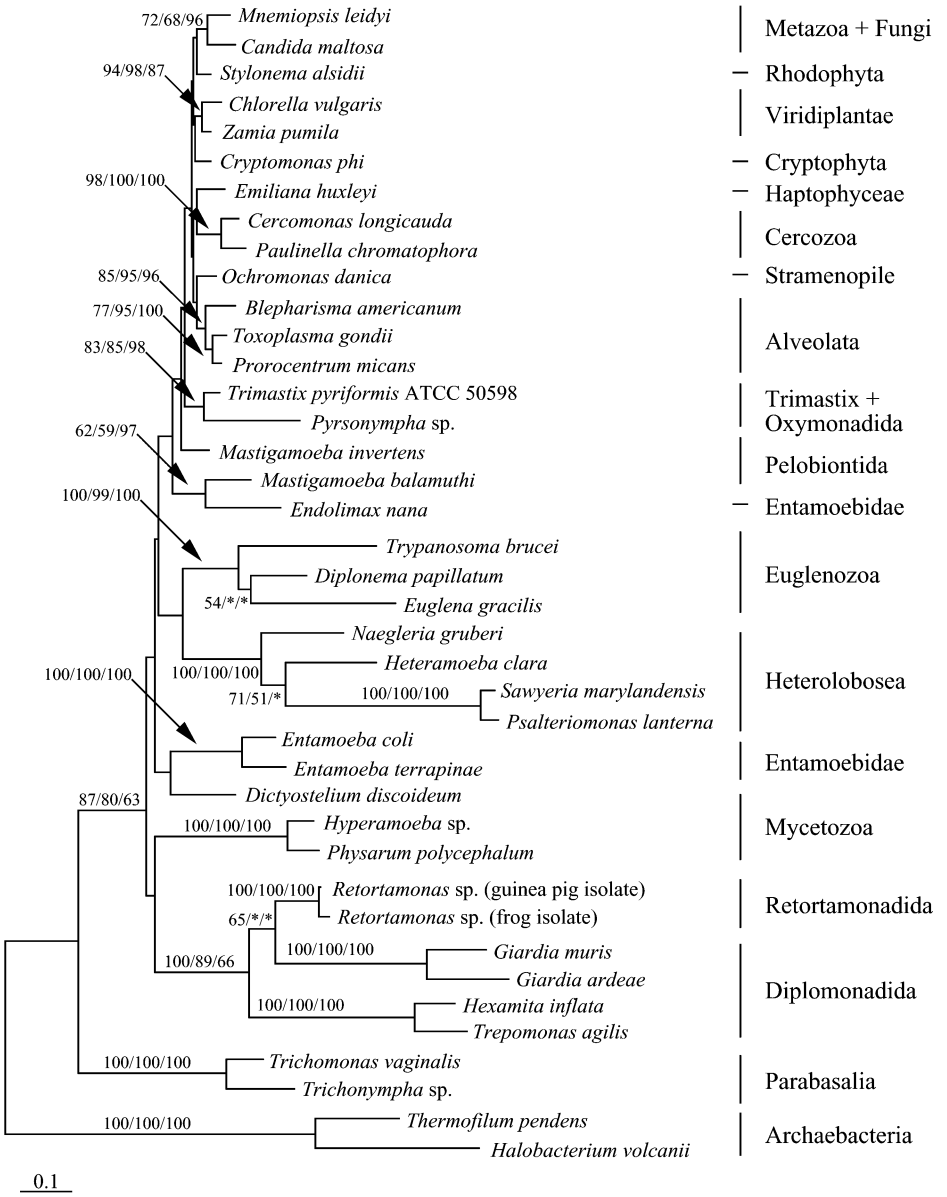

FIG. 1.—A global phylogeny of eukaryotes based on ssu rRNA sequences with 1,096 aligned nucleotide positions. The topology shown is the maximum likelihood tree under the GTR + Γ + I model with archaebacterial sequences rooting the eukaryotes. The estimated gamma-shape parameter α is equal to 0.897. The estimated proportion of invariable sites is 0.17. Bootstrap values from maximum likelihood (357 replicates), parsimony (10 random additions for each of 1,000 replicates), and ML-distance (1,000 replicates), respectively, are shown at the nodes. An asterisk (*) indicates less than 50% bootstrap support, and the scale bar represents 10 changes per 100 positions.

also used a parametric bootstrapping likelihood ratio test on the smaller data set (the SOWH test described by Goldman, Anderson, and Rodrigo 2000) to evaluate whether the optimal tree was significantly better supported than the monophyletic diplomonad tree. Results from the parametric bootstrapping procedure were borderline significant ($P = 0.007 \pm 0.003$) at an α-level of 0.01. This apparent conflict between the results of the SH, KH tests and the SOWH test is not surprising, given the parallel to conflicting results previously reported by others (Goldman, Anderson, and Rodrigo 2000; Andersson and Roger 2002). KH tests are widely accepted to be invalid in the case where one of the topologies is known in advance to be the ML tree (Shimodaira and Hasegawa 1999; Goldman, Anderson, and Rodrigo 2000). SH tests, although appropriate to our question, are thought to lack power and greatly overestimate the size of the confidence set of trees around the ML tree. In contrast, the SOWH parametric bootstrapping test, although powerful, is valid only if the model (e.g., at least one of the trees and the substitution model) is correctly specified. A recent study suggests that if the models are misspecified, the SOWH test may give an underestimate of the confidence set (Strimmer and Rambaut 2002). To address this Strimmer and Rambaut (2002) proposed a confidence interval estimation method of expected likelihood weights that is robust to model misspecification. We utilized this ELW method to derive

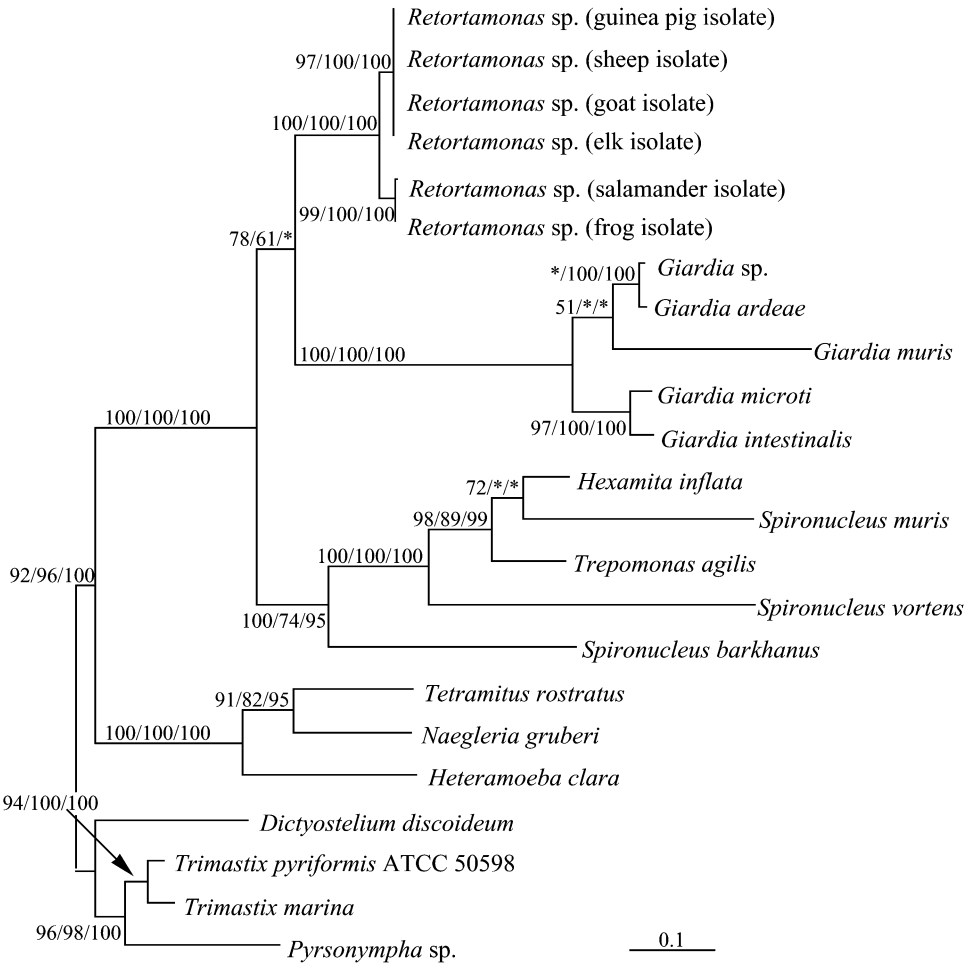

FIG. 2.—Phylogenetic relationships between retortamonads and diplomonads based on ssu rRNA sequences. These analyses were based on 1,177 aligned nucleotide positions. The unrooted tree shown is the optimal maximum likelihood topology obtained under the GTR + Γ + I model for a taxonomically restricted data set that included all available diplomonad and retortamonad (this study) sequences. The estimated gamma-shape parameter α is equal to 1.090. The estimated proportion of invariable sites is 0.224. Bootstrap values from maximum likelihood (200 replicates), parsimony (10 random additions for each of 1,000 replicates), and ML-distance (1,000 replicates), respectively, are shown at the nodes. An asterisk (*) indicates less than 50% bootstrap support, and the scale bar represents 10 changes per 100 positions.

a confidence interval from within 66 trees of high likelihood. By this method, the monophyletic diplomonad tree fell within the 55% confidence interval (i.e., well within the 95% confidence interval) of the ML tree; therefore, it cannot be rejected. In the light of the strengths and weakness of the various tests, the balance of evidence suggests that the monophyletic diplomonad tree cannot be rejected as an alternative explanation of the data.

To our knowledge, a placement of retortamonads cladistically within diplomonads has not been seriously entertained before, other than in the now disfavored hypotheses in which diplomonads are the stem group for almost all extant eukaryotes (Cavalier-Smith 1992). We are suspicious of this placement in our trees for three reasons: (1) the particularly unusual nature of *Giardia* ssu rDNA sequences (i.e., in terms of base composition), (2) the low bootstrap support for the *Giardia* + retortamonad clade, and (3) the failure of SH, KH, and ELW tests to reject the intuitive hypothesis that retortamonads and diplomonads are each monophlyetic. However, it is interesting to note that *Retortamonas* and *Giardia* ssu rRNA gene sequences share a single guanosine nucleotide insertion that is not present in any other sequenced eukaryote ssu rRNA gene (position 544, using the guinea pig *Retortamonas* sequence as reference).

Retortamonads have a conventional monomonad cell structure rather than the distinctive doubled-cell organization of well-known diplomonads (fig. 3). A placement of retortamonads specifically with *Giardia* would therefore imply either a reversal to the monomonad condition or multiple origins of the doubled-cell phenotype. Both these alternatives seem unlikely. However, a collection of diplomonads called the enteromonads are usually observed in a monomonad state (Brugerolle and Müller 2000). The placement of enteromonads with respect to other diplomonads is unclear (they lack the morphological synapomorphies for retortamonads but may or may not be basal to other diplomonads), and it is therefore possible that they too have reverted to a monomonad state. The only published analysis that includes both retortamonads and a wide diversity of di-

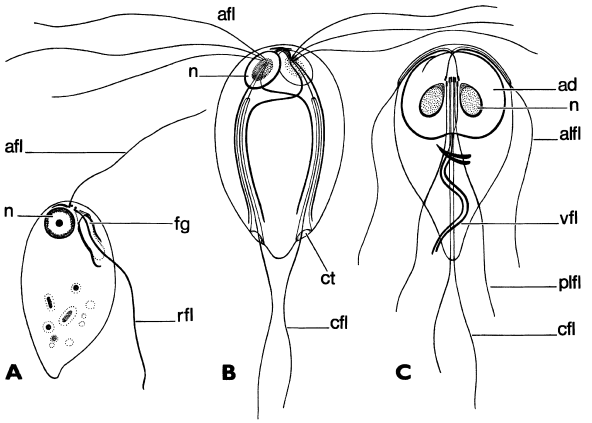

FIG. 3.—Schematic representations of (*A*) *Retortamonas*, (*B*) *Hexamita*, and (*C*) *Giardia*. *Retortamonas* cells have a single nucleus, two emergent flagella, and a single ventral feeding groove; (n) nucleus, (afl) anterior flagellum, (rfl) recurrent flagellum, (fg) feeding groove. *Hexamita* and *Giardia* are diplomonads possessing two nuclei and eight flagella arranged as two identical clusters of four flagella. Each nucleus is associated with a flagellar cluster and a set of kinetosome-associated fibrils. *Hexamita* has two cytostomes that open at the posterior end of the cell; (afl) anterior flagella, (cfl) caudal (cytostomal) flagella, (ct) cytostome, (n) nucleus. *Giardia* has a ventrally flattened body equipped with an adhesive disk (ad) supported by a complex cytoskeleton. No cytostomes are present. The two clusters of flagella lie close to each other in the center of the cell, between the nuclei (n). The four flagella from each cluster are termed: anterolateral (alfl), posterolateral (plfl), caudal (cfl), and ventral (vfl).

plomonads is the parsimony analysis of morphological data by Siddall, Hong, and Desser (1992). This analysis actually employs retortamonads as the outgroup to diplomonads. Nonetheless, there is no way of rerooting their maximum parsimony tree to recover a clade of retortamonads plus *Giardia* to the exclusion of *Hexamita*, *Spironucleus*, and *Trepomonas*; therefore, it is incompatible with our trees. However, their tree is also not reconcilable with the contemporary molecular-phylogenetic understanding of diplomonad phylogeny (Cavalier-Smith and Chao 1996; Rozario et al. 1996; Keeling and Doolittle 1997*b*) and is not recovered in parsimony analyses of an updated morphological data set (A. G. B. Simpson, unpublished data). Thus, although we cannot nominate any morphological evidence to support a placement of retortamonads close to *Giardia*, we cannot exclude it as a possibility. Examination with more taxa and additional phylogenetic markers will be required to resolve the phylogeny within the diplomonad + retortamonad clade. It has been noted that *Giardia* proteins are translated with the universal genetic code, whereas proteins are translated with an alternative code in *Hexamita*, *Trepomonas*, and *Spironucleus* (TAA and TAG encode glutamine rather than termination; Rozario et al. 1996; Keeling and Doolittle 1997*b;* Horner, Hirt, and Embley 1999). Characterization of protein-encoding genes from retortamonads, in conjunction with phylogenic reconstruction, may prove especially informative for interpreting both retortamonad and diplomonad evolutionary history.

Retortamonads, Excavate Taxa, and the Evolution of Amitochondriate Protists

The group of eukaryotes known as excavate taxa is well represented in these analyses with the inclusion of *Trimastix*, heteroloboseids, diplomonads, and the retortamonads. In spite of an entirely new lineage being represented (the retortamonads) and two additional heterloboseid sequences, ssu rDNA analyses do not unite these lineages into either a clade or a plausible grade. This pattern has been observed previously in both ssu rDNA and tubulin analyses (Dacks et al. 2001; Edgcomb et al. 2001) and is difficult to reconcile with the excavate hypothesis. Ongoing research is exploring the apparent incongruence between molecular and morphological data at the level of relatedness of excavate taxa.

Two ancillary observations from the broad-scale phylogenetic analysis deserve further comment: (1) *Sawyeria marylandensis*, an anaerobic-microaerophilic heteroloboseid branches with 100% bootstrap support with the other anaerobic amoeboflagellate, *Psalteriomonas lanterna*, thus forming an apparent clade of anaerobes embedded within a predominantly aerobic lineage; (2) As noted in previous analyses of *Entamoeba* ssu rRNA gene sequences (Silberman et al. 1999), *Endolimax nana* tends to branch with the pelobiont *Mastigamoeba balamuthi* in phylogenies based on evolutionary distances. The present analysis also groups *Endolimax* with the pelobiont *Mastigamoeba balamuthi* to the exclusion of the other *Entamoeba*. In addition to a phylogenetic affinity, these organisms possess ssu rRNA genes that are similar in size to one another and significantly larger than those reported for *Entamoeba* (Silberman et al. 1999). These observations suggest that *Endolimax* may really be more closely related to pelobionts than to *Entamoeba* or any other taxon.

Retortamonads and the Archezoa Hypothesis

Regardless of the internal phylogeny of the diplomonad-retortamonad clade, it now seems reasonable to consider diplomonads and retortamonads as one group with respect to mitochondrial history. The status of mitochondrial genes in diplomonads has been somewhat controversial in recent literature and is worth restating briefly here. Five genes, chaperonin 60 (cpn60), heat shock protein 70 (hsp70), valyl tRNA synthetase (ValtRS), triose phosphate isomerase (TPI), and, most recently, pyridoxal-5′-phosphate–dependent cysteine desulfurase (IscS) have been claimed as potential mitochondrial relics from the diplomonad *Giardia* (Keeling and Doolittle 1997*a;* Hashimoto et al. 1998; Roger et al. 1998; Morrison et al. 2001; Tachezy, Sánchez, and Müller 2001). A mitochondrial relict cpn60 has also recently been reported from a second diplomonad, *Spironucleus* (Horner and Embley 2001). In each case, the claims are based on phylogenetic analyses which place the diplomonad gene(s) with mitochondrial forms from other eukaryotes (cpn60, hsp70, and IscS) or with sequences from proteobacteria (or both), the bacterial clade from which the mitochondrial symbiont arose

(Val-tRS, TPI). Two of these five cases now appear problematic: TPI and Val-tRS. For TPI, additional α-proteobacterial sequences have been obtained, and they do not clearly form a clade with the eukaryote sequences (B. Canbäck, S. G. E. Andersson, and C. G. Kurland, personal communication), casting doubt on earlier claims (Keeling and Doolittle 1997*a*). Similarly, Val-tRS genes are now available from α-proteobacteria that appear to lack the γ-proteobacterial + mitochondrial insertion, questioning the endosymbiotic origin of all eukaryote homologs (T. Hashimoto, personal communication). Furthermore, in hsp70 phylogenies, the close proximity of the highly divergent *Giardia* sequence to mitochondrial and α-proteobacterial forms is indicative of a mitochondrial origin, but the overall topology is poorly supported (Morrison et al. 2001). On the other hand, the cpn60 and IscS phylogenies consistently and robustly place the *Giardia* sequence with, and often within, mitochondrial forms to the exclusion of all sequences from bacteria (Roger et al. 1998; Horner and Embley 2001; Tachezy, Sánchez, and Müller 2001). Although additional α-proteobacterial IscS gene sequence data are desirable to confirm that they share a common ancestry with mitochondrial eukaryotic homologs, the case for cpn60 is most convincing. Although it is important to establish the presence of genes of mitochondrial origin in retortamonads and other diplomonads, the most straightforward interpretation of available evidence is that ancestors of both diplomonads and retortamonads once had mitochondria.

With the strong confirmation that retortamonads and diplomonads do form a clade, all of the taxa suggested as primitively amitochondriate under the archezoa hypothesis appear to have physical or genetic (or both) mitochondrial relics or form a strongly supported clade with a group bearing such a relic. The perpetuation of the original archezoa hypothesis requires the refuting of all the mitochondrial relics from one of the amitochondriate taxa, demolition of one of the clades, or the discovery of new amitochondriate taxa.

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