Oxymonads Are Closely Related to the Excavate Taxon Trimastix

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Despite intensive study in recent years, large-scale eukaryote phylogeny remains poorly resolved. This is particularly problematic among the groups considered to be potential early branches. In many recent systematic schemes for early eukaryotic evolution, the amitochondriate protists oxymonads and Trimastix have figured prominently, having been suggested as members of many of the putative deep-branching higher taxa. However, they have never before been proposed as close relatives of each other. We amplified, cloned, and sequenced small-subunit ribosomal RNA genes from the oxymonad Pyrsonympha and from several Trimastix isolates. Rigorous phylogenetic analyses indicate that these two protist groups are sister taxa and are not clearly related to any currently established eukaryotic lineages. This surprising result has important implications for our understanding of cellular evolution and high-level eukaryotic phylogeny. Given that Trimastix contains small, electron-dense bodies strongly suspected to be derived mitochondria, this study constitutes the best evidence to date that oxymonads are not primitively amitochondriate. Instead, Trimastix and oxymonads may be useful organisms for investigations into the evolution of the secondary amitochondriate condition. All higher taxa involving either oxymonads or Trimastix may require modification or abandonment. Affected groups include four contemporary taxa given the rank of phylum (Metamonada, Loukozoa, Trichozoa, Percolozoa), and the informal excavate taxa. A new ''phylum-level'' taxon may be warranted for oxymonads and Trimastix.

Introduction

Recent years have seen increasing uncertainty about the broadest-scale structure of the eukaryotic evolutionary tree, particularly the identity of the deepest extant branches. These difficulties have been revealed by the implementation of novel analysis methods (Stiller and Hall 1999), the use of different models of evolution (Silberman et al. 1999), and the use of genes giving conflicting results (Embley and Hirt 1998). Another major problem is the absence of many key protist groups from most or all molecular phylogenies. Oxymonads and Trimastix are two such key groups.

Oxymonads are a group of structurally distinct, obligately symbiotic flagellates (usually with four flagella per cell), most of which are cellulose digesters found in the hindgut of termites and wood-eating cockroaches. First described by Leidy in 1877, oxymonads are best known for their atypical sexual cycles, described in a long series of papers by Cleveland (summarized by Cleveland 1956). Some oxymonads, such as Saccinobaculus and Oxymonas, undergo self-fusion of gametes (autogamy). These taxa are thought to have a one-step meiosis, in which a single reductive division produces two daughter cells, instead of the two divisions and four daughter cells typical of meiosis in other organisms (but see Haig 1993). Other oxymonads, such as Pyrsonympha, do not undergo true sexual reproduction, but, rather, have a ploidy cycle in which their initially high ploidy is reduced by a series of apparently meiotic divisions and then restored by multiple rounds of DNA replication (Hollande and Carruette-Valentin 1970). Unlike most eukaryotes, oxymonads also lack mitochondria and Golgi dictyosomes (Brugerolle 1991). This cytological simplicity, especially the lack of mitochondria, led to oxymonads being advanced as one of the most primitive groups of eukaryotes (Cavalier-Smith 1981).

The relationships of oxymonads with other eukaryotes are uncertain and contentious. In the modern era, they have generally been allied with the other cytologically simple, amitochondriate, tetraflagellate protists, i.e., the retortamonads and diplomonads. These groups formed the widely accepted phylum Metamonada, united by their shared possession of four anterior basal bodies and lack of organelles (Cavalier-Smith 1981, 1998). However, the distinctive presence of a motile axostyle, a cytoskeletal backbone running the length of oxymonad cells, sets the oxymonads apart from the other metamonads. In his 1991 review, Brugerolle suggested that there was ''a probable long evolutionary distance between this group and the other two.'' Recent elongation factor (EF-1 alpha) phylogenies that include the first gene sequence data from oxymonads (Moriya, Ohkuma, and Kudo 1998; Dacks and Roger 1999) indicate that a

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Abbreviations: ML, maximum likelihood; sp., species; ssu rDNA, small-subunit ribosomal RNA gene.

Key words: ssu rDNA, eukaryote evolution, protist, phylogenetics, metamonad, mitochondria, sex.

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close relationship with diplomonads is unlikely. Newer accounts of eukaryotic diversity instead place oxymonads with Heterolobosea and Stephanopogon in the contentious phylum Percolozoa (Cavalier-Smith 1999, 2000) or simply describe them as ''eukaryotic taxa without known sister groups'' (Patterson 1999).

The genus Trimastix was first described by Kent in 1880 but has only recently become the subject of detailed study by evolutionary protistologists. Trimastix are free-living anaerobes/microaerophiles with four flagella and a broad ventral feeding groove. Ultrastructural examinations have revealed that Trimastix lack classical mitochondria, having instead small, membrane-bounded organelles resembling hydrogenosomes (O'Kelly 1993; Brugerolle and Patterson 1997; Simpson, Bernard, and Patterson 2000). The discovery of these organelles prompted Cavalier-Smith (1997) to group Trimastix with the hydrogenosome-bearing parabasalids in a new phylum, Trichozoa. However, detailed ultrastructural examinations also demonstrated that Trimastix shares a large number of cytoskeletal similarities with a seemingly diverse collection of mitochondriate and amitochondriate protists that also have feeding grooves: the retortamonads, core jakobids (Reclinomonas, Jakoba, and Histiona), Malawimonas, Carpediemonas, some diplomonads, and some Heterolobosea (O'Kelly, Farmer, and Nerad 1999; O'Kelly and Nerad 1999; Patterson 1999; Simpson and Patterson 1999). Trimastix has been included with these groups in the informal assemblage ''excavate taxa,'' envisaged as a monophyletic or paraphyletic group (Simpson and Patterson 1999). Cavalier-Smith (1999) recently rejected Trichozoa and instead erected a new phylum, Loukozoa, based on the shared presence of a ventral feeding groove (and referring to the presence of either mitochondria or mitochondrion homologs). Loukozoa, containing only Trimastix and the core jakobids (Cavalier-Smith 1999, 2000), is proposed as the most basal eukaryotic group. Until now, no published molecular sequence data have been available for Trimastix.

Without a reasonable phylogeny of eukaryotes, it is impossible to trace the origin and evolution of uniquely eukaryotic traits, be they ultrastructural traits, organizational traits, or aspects of life history. Given that both oxymonads and Trimastix have been independently proposed as deep-branching eukaryotes, their phylogenetic placement bears strongly on issues of deep eukaryote phylogeny. Furthermore, given the unusual sexual cycles of oxymonads and the absence of classical mitochondria from both oxymonads and Trimastix, resolving the placement of these lineages could improve our understanding of the evolutionary history of sex and of the acquisition, loss, and modification of mitochondria.

We sequenced the small-subunit ribosomal RNA genes (ssu rDNA) from three isolates of Trimastix and from an oxymonad (*Pyrsonympha* sp.). The Pyrsonympha sample had to be isolated by hand from the termite gut community using micromanipulation, and therefore in situ hybridization was employed to verify the source of the Pyrsonympha sequence. The relationship of these four rDNA sequences to each other and to other eukaryotic taxa was determined by phylogenetic analysis.

Materials and Methods

Protist Isolation and Gene Amplification

Pyrsonympha cells were obtained from specimens of the Western subterranean termite (*Reticulitermes hesperus*), a species known to harbor the oxymonads Pyrsonympha and Dinenympha (Grosovsky and Margulis 1982), collected from a natural colony near Kelowna, Canada. Termite gut contents were diluted into modified Trager's media (Buhse, Stamler, and Smith 1975). The largest cells with typical Pyrsonympha morphology were selected away from nonoxymonad flagellates by micromanipulation, washed, and reselected. Due to the difficulty of manipulation and identification, the cells were identifiable only as *Pyrsonympha* sp.

About 50–75 cells were pelleted by centrifugation at 3,000 rpm for 1 min, and DNA was extracted using standard techniques (Maniatis, Fritsch, and Sambrook 1982). The 3′ region of the *Pyrsonympha* sp. ssu rDNA gene (639 nt) was amplified by PCR, using eukaryotic specific primer 5′N (TGAAACTTAAAGGAATTGACGGA) and primer B from Medlin et al. (1988). Cycling parameters began with an initial denaturation of 95°C for 1 min, followed by 1 min at 45°C and 3 min at 72°C. This cycle was repeated an additional 29 times with the initial heating step at 94°C for 10 s, and was followed by a final cycle with extension time increased to 4 min to promote the complete extension of products. The resulting PCR products were cloned into a pGem-T vector (Promega BioTech, Madison, Wis.) and sequenced on an ABI sequencer.

Once the identity of this clone was verified by in situ hybridization (see below), its sequence was used to design the 3′ primer 3A (ACGCGTGCGGTTCAGATT). This was used with the universal 5′ primer 5A2 (CTGGTTGATCCTGCCAG) to amplify the remaining 5′ component of the oxymonad gene. The reaction was performed using *Taq* polymerase augmented with trace amounts of Pfu polymerase to discourage PCR-induced replication errors. Cycling parameters of 95°C for 1 min, 52°C for 1 min, and 72°C for 3 min were used for the first cycle. This was followed by 31 repetitions with the melting step at 94°C decreased to 30 s and one additional cycle with the final extension time at 72°C increased to 4 min. The resultant PCR products were cloned into a TopoTA vector 2.1 (InVitrogen, Carlsbad, Calif.).

Two independent 5′ ssu rDNA PCR clones, from separate PCR reactions, were sequenced on a LICOR sequencer. These 5′ ssu rDNA fragments of 1,553 unambiguously assigned bases overlapped the previous 3′ fragment by 182 positions. The consensus sequence was assembled based on two- to four-fold coverage of all regions (not always on both strands), with any discrepancies checked against gel traces and bases assigned manually.

The *Trimastix marina* isolate studied was the ''freshwater'' monoprotistan culture isolate detailed by

Bernard, Simpson, and Patterson (2000) and studied by Simpson, Bernard, and Patterson (2000). *Trimastix pyriformis* ATCC 50598 and *T. pyriformis* ATCC 50562 cell pellets were obtained from the American Type Culture Collection (Manassas, Va.). Genomic DNA was isolated using the PureGene DNA isolation kit (Gentra Systems, Inc., Minneapolis, Minn.). Following cell lysis, RNase treatment, protein precipitation, and precipitation of genomic DNA with isopropanol, the DNA was resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE), and subjected to chloroform : isoamyl alcohol extraction followed by precipitation with an equal volume of 13% PEG in 0.7 M NaCl. DNA was resuspended in TE. The ssu rDNA from each organism was amplified by PCR using eukaryotic specific primers A and B (Medlin et al. 1988) and cloned into pGem-T or pGem-T easy (Promega BioTech) as previously described (Silberman et al. 1999). Cycle conditions were 30 cycles of 10 s denaturation at 94°C, 1 min annealing at 37°C, and 3 min extension at 72°C, followed by a single ''polishing'' step of 10 min at 72°C. Multiple independent recombinant clones were used as sequencing templates (for *T. marina*, $n = 7$; for both *T. pyriformis* isolates, $n = 10$). The ssu rDNA clones of each species were independently pooled prior to sequencing on a LICOR 4200L apparatus using IR-labeled primers. Minimal heterogeneity was detected in the rDNA of *T. marina*, but this heterogeneity was confined to hypervariable regions. Additionally, the ssu rDNA PCR product of *T. pyriformis* ATCC 50562 was sequenced directly to confirm sequence homogeneity of the rRNA gene(s). All Trimastix genes were sequenced completely in both orientations.

In Situ Hybridization

To confirm the origin of the *Pyrsonympha* sp. sequence, in situ hybridization was performed on *Reticulitermes speratus* gut biota as described previously (Moriya, Ohkuma, and Kudo 1998). Fluorescence in situ hybridization (FISH) and enzymatic amplified immunohybridization studies used a probe specific to the Pyrsonympha-derived sequence (Oxy1270-FITC; TACGCGTGCGGTTCAGATTA), which differs from the eukaryote consensus at the six underlined positions. Probe Euk1379-Texas Red (TACAAAGGGCAGGGAC) was used as a positive control for the FISH analysis.

Phylogenetic Analysis

Two distinct ssu rDNA data sets were analyzed to establish the phylogenetic affinities of the new sequences. DNA sequences were manually aligned using conserved primary and secondary structures. Only unambiguously aligned positions were considered in phylogenetic analyses. An evolutionary broad-scale data set containing species representing most major eukaryote lineages consisted of 45 taxa and 1,303 aligned positions (taxa and accession numbers are listed in table 1). To assess finer-scale relationships, we used a restricted subset (31 taxa, 1,447 aligned characters).

Hierarchical log likelihood ratio tests using the program MODELTEST, version 3.0b (Posada and Crandall 1998), showed that a general time-reversible model incorporating a correction for among-site rate variation and invariable sites (GTR+Γ+I) best described both data sets. The character state rate matrix, the base composition, the gamma shape parameter (α value), and the proportion of invariable sites (I) were similarly estimated by likelihood methods. This explicit model of nucleotide evolution was used in maximum-likelihood (ML) and distance analyses. For all analyses, gaps were treated as missing data and starting trees were obtained by 100 replicates of random stepwise taxon addition. Branching order and stability were assessed by analyses of 100 or more bootstrapped data sets. All phylogenetic analyses were performed using PAUP*, version 4.0b (Swofford 1998).

Kishino-Hasegawa tests (Kishino and Hasegawa 1989) using PAUP*, version 4.0b, were performed by constraining the backbone ML topology and removing the branch/clade of interest. All possible trees were then constructed by replacing the taxon/clade at each position on the constrained backbone. Significance between the likelihood scores of alternative tree topologies was tested under a GTR+Γ+I model of nucleotide evolution.

Assessment of phylogenetic signal content within the data sets and identification of taxa contributing excessive phylogenetic noise (i.e., putative long-branch taxa) were done by tree independent regression and variance analyses using the RASA computer package, version 2.3.7 (Lyons-Weiler, Hoelzer, and Tausch 1996), by implementing the analytical model for the estimation of null slope. Plotting the ratio of the variances of phylogenetic (cladistic) similarity to phenetic similarity (taxon variance ratio) identified those taxa which most contributed to branch length heterogeneity. The phylogenetic signal content of the data set was reassessed after systematic removal of long-branched taxa. The 31-taxon data set was also analyzed using the permutation model for the calculation of null slope provided by RASA, version 2.5 (10 permutations) (Lyons-Weiler and Hoelzer 1999).

Results

Physical Attributes of ssu rDNAs

The amplified regions contained all of each ssu rDNA gene except for the first and last ~25 nt. The sequences from *T. marina* and *T. pyriformis* ATCC 50562 and ATCC 50598 were typical in size for eukaryotes (1,850, 1,823, and 1,837 bp, respectively), while the hypervariable regions of the *Pyrsonympha* sp. sequence were slightly expanded, resulting in an ssu rDNA of 2,012 bp. The base composition of all four sequences was typical of eukaryote ssu rDNAs (44%–47% G+C).

In Situ Hybridization

The *Pyrsonympha* sp. cells were obtained from the hindgut of the subterranean termite *Reticulitermes hesperus*, which contains a heterogeneous protist commu-

Table 1
Taxa Included in the Broad-Scale Data Set

Taxon Name	Accession No.	Taxonomic Affiliation
Mnemiopsis leidyi	L10826	Ctenophore
Diaphanoeca grandis	L10824	Choanoflagellate
Corallochytrium limacisporum	L42528	Choanozoa
Apusomonas proboscidea	L37037	Apusomonad
Athelia bombacina	M55638	Basidiomycete
Candida maltosa	D14593	Deuteromycete
Chlorella vulgaris	X13688	Chlorophyte
Chlamydomonas reinhardtii	M32703	Chlorophyte
Oryza sativa	X00755	Streptophyte
Zamia pumila	M20017	Streptophyte
Acanthamoeba castellanii	M13435	Amoebae
Hartmannella vermiformis	M95168	Amoebae
Cryptomonas phi	X57162	Cryptomonad
Chroomonas sp.	X81328	Cryptomonad
Porphyra umbilicalis	L26202	Rhodophyte
Stylonema alsidii	L26204	Rhodophyte
Emiliana huxleyi	L04957	Haptophyte
Phaeocystis globosa	X77476	Haptophyte
Ochromonas danica	M32704	Stramenopile
Achlya bisexualis	M32705	Stramenopile
Oxytricha granulifera	X53486	Ciliate
Trimastix pyriformis ATCC 50562	AF244903	Trimastix
Trimastix pyriformis ATCC 50598	AF244904	Trimastix
Trimastix marina	AF244905	Trimastix
Pyrsonympha sp.	AF244906	Oxymonad
Blepharisma americanum	M97909	Ciliate
Symbiodinium pilosum	M88518	Dinoflagellate
Prorocentrum micans	M14649	Dinoflagellate
Toxoplasma gondi	M97703	Apicomplexan
Theileria annulata	M64243	Apicomplexan
Vannella anglica	AF099101	Amoebae
Endolimax nana	AF149916	Entamoebid
Entamoeba histolytica	X56991	Entamoebid
Mastigamoeba balamuthi	L23799	Pelobiont
Dictyostelium discoideum	K02641	Dictyostelid
Hyperamoeba sp.	AF093247	Myxogastrid
Physarum polycephalum	X13160	Myxogastrid
Euglena gracilis	M12677	Euglenozoan
Trypanosoma brucei	M12676	Euglenozoan
Naegleria gruberi	M18732	Heteroloboseid
Psalteriomonas lanterna	X94430	Heteroloboseid
Trichomonas vaginalis	U17510	Parabasalid
Trichonympha sp. (cf *collaris*)	AF023622	Parabasalid
Hexamita inflata	L07836	Diplomonad
Giardia muris	X65063	Diplomonad

nity including three species of each of the two oxymonad genera Pyrsonympha and Dinenympha (Kirby 1932). The genera are readily distinguished by morphology and size (170 μm average and 25–80 μm, respectively), but the species of each are not readily distinguished, as their sizes and morphologies overlap (Grassé 1952). Because the DNA preparation was not from a pure culture, we used in situ hybridization studies to confirm the source of the ssu rDNA sequence. For convenience, these studies used the closely related Japanese termite *R. speratus* which contains the same two oxymonad genera. The positive control for FISH experiments was a Texas Red–labeled probe complementary to all eukaryotic ssu rDNA (Euk1379); it annealed to all of the protistan inhabitants of the *R. speratus* hindgut (fig. 1*A*-1). The FITC-labeled Pyrsonympha probe (Oxy1270; fig. 1) differed from the eukaryote consensus at six strongly conserved positions; it hybridized strongly to all cells with Pyrsonympha or Dinenympha size and morphology but not to nonoxymonad protists (fig. 1*A*-2). Similar results were obtained when termite gut contents stained with Oxy1270-FITC were examined with anti-FITC antibodies (fig. 1*B*). These results confirm that an oxymonad species was the source of the ssu rDNA sequence obtained. As the DNA was obtained from the largest cells with Pyrsonympha morphology, we assigned the sequence to *Pyrsonympha* sp.

Phylogenetic Analysis of the 45-Taxon Data Set

To test the relationships of the Pyrsonympha and Trimastix sequences to each other and to other eukaryotes, we initially performed phylogenetic analyses on a 45-taxon data set containing representatives of all major eukaryote groups. With this set, *Pyrsonympha* sp. and the Trimastix species formed a clade that was highly supported by bootstrap values under all models and methods of phylogenetic analyses (fig. 2*A*) and was re-

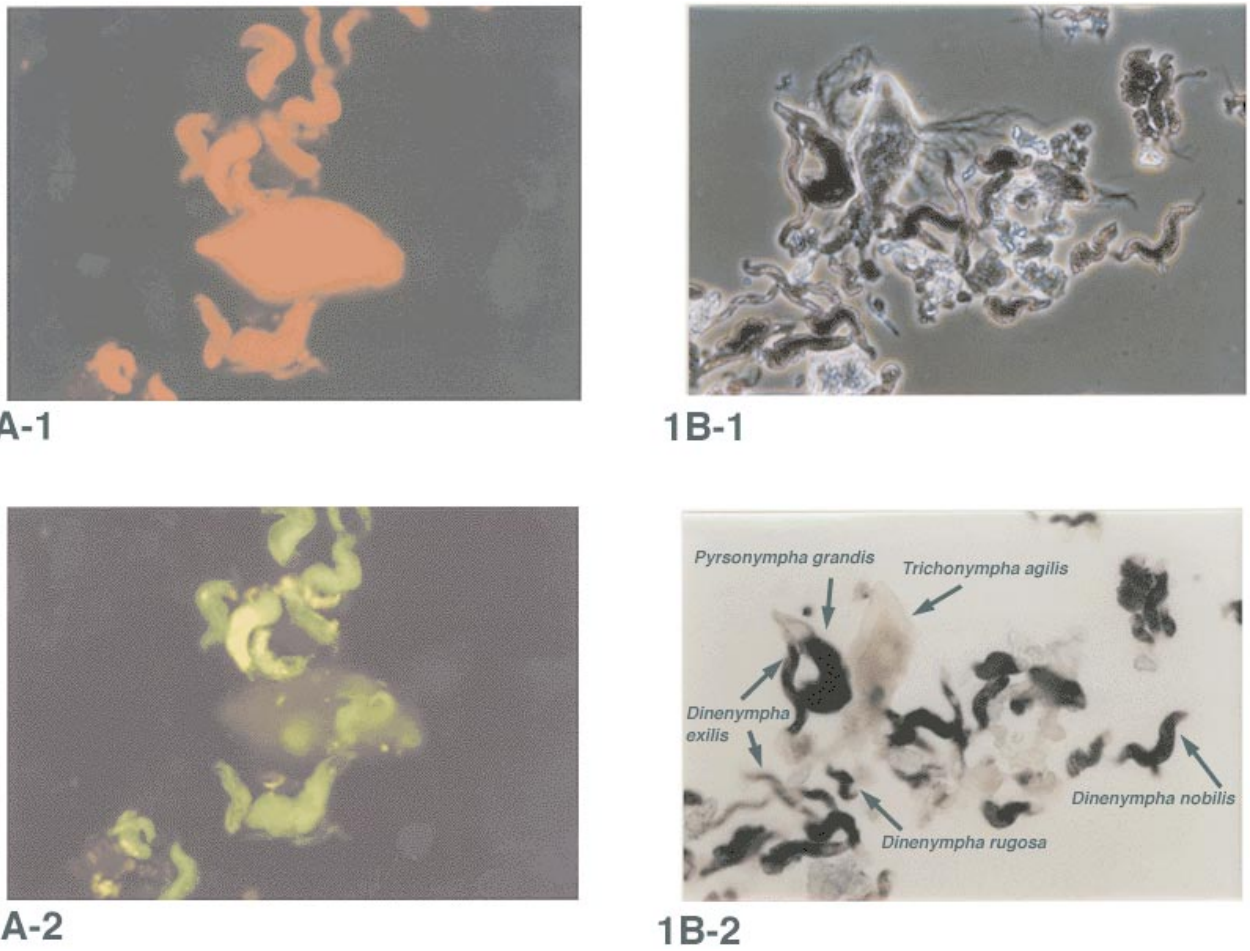

FIG. 1.—In situ micrographs of *Reticulitermes speratus* gut fauna. *A*, fluorescence in situ hybridization analysis with two probes: Euk1379 (eukaryote universal probe) labeled with Texas Red, and Oxy1270 (putative Pyrsonympha probe) labeled with fluorescein. *A*-1, Texas red fluorescence. *A*-2, fluorescein fluorescence. *B*, Anti-FITC antibody analysis using the Oxy1270 probe. *B*-1, Phase contrast illumination. *B*-2, Staining with anti-FITC antibody. Organisms are seen under 200× magnification.

covered in all optimum trees. Within this clade, the Trimastix sequences were monophyletic in ML and parsimony analyses, but *Pyrsonympha* sp. and *T. marina* were sister taxa under distance methods with the best available model of nucleotide evolution.

The strength of the oxymonad/Trimastix clade was further examined by performing a series of Kishino-Hasegawa (KH) log likelihood ratio tests under the optimum model of phylogenetic reconstruction (GTR+Γ+I). The best ML tree from the 45-taxon data set was used as a backbone constraint in the absence of Pyrsonympha, and Pyrsonympha was then added to each possible branching position. Log likelihood scores for each tree were then calculated. The most likely tree topology was that shown in figure 2*A*. Only five other topologies fell within the acceptable 95% confidence interval. Of these, the top four were simple permutations, with the Pyrsonympha branch connecting to all possible nodes within the Trimastix clade. Interestingly, the top *P* value for nonrejected trees was also the optimal topology recovered in distance analyses, a specific relationship of *Pyrsonympha* sp. with *T. marina* ($P = 0.2879$). Other support values ranged from $P = 0.23$ to $P = 0.07$. The least likely topology that failed to be rejected was that with Pyrsonympha branching as the sister taxon to *Vanella anglica*, but the value was marginal ($P = 0.06$). The KH tests were then repeated with the Pyrsonympha sequence retained and the Trimastix sequences removed, but no other topologies for the placement of the Trimastix sequences fell within the 95% confidence interval. Overall, these analyses strongly supported a specific relationship between oxymonads and Trimastix.

Our ssu rDNA analyses did not establish a specific relationship between the oxymonad-plus-Trimastix clade and the various groups to which either has been linked by morphological studies or recent classification schema, i.e., diplomonads, parabasalids, and heteroloboseids (Cavalier-Smith 1998, 1999; Simpson and Patterson 1999). In fact, there was no significant support for grouping this clade with any major eukaryotic lineage (fig. 2*A*). This was explicitly examined by constraining the oxymonad/Trimastix clade, rearranging it along a constrained ML backbone tree topology, and performing KH tests (GTR+Γ+I model of nucleotide evolution). Of the 79 potential branching positions for the oxymonad/Trimastix clade, 36 failed to be rejected. Notable among

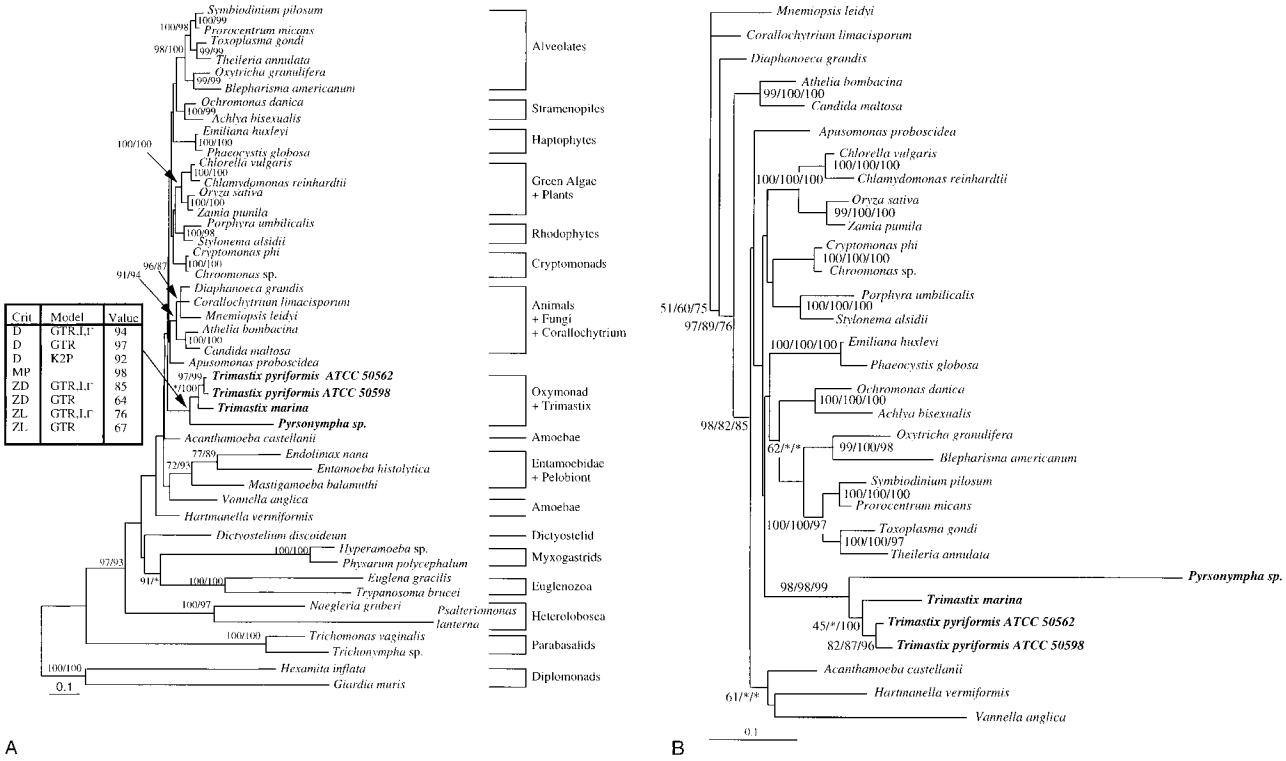

FIG. 2.—Phylogenetic analysis of ssu rDNA. New sequences are shown in bold. All other sequences were obtained from GenBank. *A,* The optimal maximum-likelihood (GTR+Γ+ I) tree is shown with bootstrap values for maximum-likelihood distances and maximum parsimony at nodes supported over 50%, with the exception of the Pyrsonympha/Trimastix node, for which values under a variety of methods and optimality criteria are listed. Crit = optimality criteria used in analysis; D = distance; K_2P = Kimura 2 parameters; L = likelihood; MP = maximum parsimony; Z = quartet puzzling. The tree is arbitrarily shown as rooted on diplomonads. *B,* The optimal maximum-likelihood topology is shown with maximum-likelihood, minimum evolution, and maximum-parsimony bootstrap values shown at nodes supported over 50%. An asterisk represents bootstrap values <50, and the scale bar shows the number of changes per site.

the rejected clades was sisterhood with parabasalids as proposed by Cavalier-Smith (1997).

RASA Analyses

Rapidly evolving gene sequences (''long-branch sequences'') in molecular data sets can produce enough phylogenetic noise to obscure biologically meaningful relationships (Lyons-Weiler, Hoelzer, and Tausch 1996; Stiller and Hall 1999). To determine whether Pyrsonympha and Trimastix constitute long-branch sequences, to assess the phylogenetic signal of our 45-taxon data set, and to aid in taxa selection for finer-scale analyses, we performed a series of regression analyses of signal content using the computer program RASA (Lyons-Weiler, Hoelzer, and Tausch 1996). The null hypothesis was that no relationship existed between cladistic signal and phenetic similarity among the sequences tested. For the 45-taxon broad-scale data set, this hypothesis could not be rejected, indicating that long-branch sequences may be obscuring some phylogenetic signal. Using variance analyses as a guide, ssu rDNA sequences were then removed from the data set until a statistically significant phylogenetic signal ($t_{RASA} \geq 1.65$) was achieved. Table 2 shows that to obtain significant signal content it was necessary to remove all diplomonads, parabasalids, heteroloboseids, euglenozoans, myxogastrids, and entamoebids and either *Mastigamoeba balamuthi* or *Dictyostelium discoideum*. Thus, any of the relationships in figure 2*A* involving these lineages may be due to long-branch attraction rather than phylogenetic signal. Importantly, Pyrsonympha and Trimastix do not branch among these taxa (fig. 2*A*). Consequently, their placement in trees is likely to be independent of long-branch artifacts.

To explore relationships involving Pyrsonympha and Trimastix, the diplomonads, parabasalids, heteroloboseids, euglenozoans, myxogastrids, and entamoebids were removed from the data set, leaving 33 taxa and 1,416 aligned characters, and RASA analyses were repeated. This set did not give a significant phylogenetic signal (table 2). However, when any one of the *M. balamuthi*, *D. discoideum*, or *Pyrsonympha* sp. sequences were also removed, significant phylogenetic signal was recovered. Thus, *M. balamuthi* and *D. discoideum* sequences were removed, yielding a data set of 31 taxa and 1,447 characters. RASA analysis under an analytical model then confirmed that this set produced significant phylogenetic signal (df = 431, $t_{RASA} = 4.03$). Two taxa in this set, *Pyrsonympha* sp. and the amoeba *V. anglica*, had relatively high taxon variance ratios and long branches. In fact, when the more stringent permutation model provided by RASA, version 2.5, was used for the calculation of the null slope (Lyons-Weiler and Hoelzer 1999), the presence of these two taxa in the data set

Table 2
Taxa Removed to Obtain r_{RASA} Values

Taxa Removed	df	t_{RASA}
Set of 45 taxa		
None	942	−2.43
A	857	−3.11
B	857	−2.60
C	857	−2.00
D	857	−2.97
E	857	−3.25
J	857	−2.62
A, B	779	−2.97
A, J	776	−3.27
A–C	699	−1.86
A–D	626	−1.06
A–D, I	557	−1.40
A–D, J	557	−1.62
A–E	557	0.19
A–D, I, J	524	−1.85
A–E, I	524	0.27
A–E, J	524	1.32
A–F	524	0.48
A–E, I, J	461	1.85*
A–F, I	461	0.05
A–F, J	461	2.21*
A–D, F, I, J	492	−1.66
A–F, I, J	431	3.30*
Set of 33 taxa		
None	492	0.96
F	461	1.92*
I	461	1.84*
L	461	2.72*
F, I	431	3.60*
F, L	431	4.84*
I, L	431	4.95*
F, I, L	402	11.04*
F, H, I, L	374	16.25*

NOTE.—A = diplomonads; B = parabasalids; C = Heterolobosea; D = Euglenozoa; E = myxogastrids; F = Dictyostelium; G = Hartmanella; H = Vanella; I = Mastigamoeba; J = Entamoebidae; K = Acanthamoeba; L = Pyrsonympha. Significant t_{RASA} scores (>1.65) are indicated by an asterisk.

caused t_{RASA} to be below the significance value. However, their wide separation in the phylogenetic trees in figure 2 suggests that long-branch attraction between them is not a problem. The removal of both Pyrsonympha and Vanella from the data set yielded a t_{RASA} value well above the significance value (df = 374, t_{RASA} = 7.39).

Phylogenetic Analysis of the 31-Taxon Data Set

Guided by the RASA results, we reduced the number of fast-evolving sequences in the data set to better resolve the phylogenetic relationship of Pyrsonympha to the different Trimastix species and that of the Pyrsonympha/Trimastix clade to other eukaryotic lineages. Use of only these 31 taxa allowed unambiguous alignment of 1,447 nucleotide positions, increasing the power of the phylogenetic analysis. The results from this reduced data set paralleled those of the previous analyses (fig. 2*B*). High bootstrap values under ML, distance, and parsimony strongly supported a Pyrsonympha-plus-Trimastix clade. Pyrsonympha was the earliest-diverging taxon within this clade in ML and parsimony analyses, while a weakly supported sister taxon relationship between *Pyrsonympha* sp. and *T. marina* was observed in distance analyses (bootstrap value of 43). Finally, as with previous phylogenetic analyses of ssu rDNA phylogenies, the major eukaryotic lineages were robustly monophyletic.

RASA analyses of the 31-taxon data set (which included both the Pyrsonympha and the Vanella sequences) rejected the null hypothesis of no relationship between cladistic signal and phenetic similarity under an analytical RASA model, while analyses under a permutations model did not reject the null hypothesis. For this reason, phylogenetic analyses were performed to determine whether these ''long-branch taxa'' were masking any other affinities of the Trimastix sequences. In the absence of the Pyrsonympha sequence, with or without Vanella, the Trimastix sequences formed a clade with 100% support under parsimony and ML distance models and showed no strong affinity for any other lineage in the data set. Returning the Pyrsonympha sequence to the data set in the absence of Vanella produced a robust Pyrsonympha/Trimastix clade with 100% support in phylogenetic analyses with both parsimony and ML distance models (data not shown). Therefore, the presence of the ''long-branch sequences'' of Pyrsonympha and Vanella do not appear to obscure any relationships relevant to this study.

Unfortunately, inclusion of more characters did not improve resolution of the relationships among the major eukaryote lineages. The oxymonad/Trimastix clade seems to represent yet another eukaryotic lineage without obvious close relatives in ssu rDNA phylogenies. However, since KH tests of the 45-taxon data set failed to reject sister relationships of this clade with many of the major eukaryotic clades, this interpretation should be viewed as tentative, to be further tested as new sequences and methods become available.

Discussion

Our study showed a specific relationship between the representative oxymonad Pyrsonympha and Trimastix. The result was robust under a variety of phylogenetic reconstruction methods, optimality criteria, and models of nucleotide evolution and was confirmed by rigorous statistical tests. This relationship suggests new lines of research into the evolution of some important eukaryotic traits and has a variety of implications for broad-scale eukaryote systematics.

Relationship Between Pyrsonympha and Dinenympha

We assigned the name *Pyrsonympha* sp. to our oxymonad sequence because it was derived from a pool of the largest cells with distinctive Pyrsonympha morphology. However, despite this selection during the initial micromanipulation and cell isolation, the Oxy1270 probe hybridized to both Pyrsonympha and Dinenympha specimens. Both *R. hesperus* and *R. speratus* are reported to harbor multiple morphologically distinguishable species of both Pyrsonympha and Dinenympha (Yamin 1979). However, Hollande and Carruette-Valentin (1970) concluded from microscopic observations and

DNA content measurements that Pyrsonympha and Dinenympha are probably morphs of the same organism, with the reduction in size from Pyrsonympha to Dinenympha being due to successive rounds of meiotic division. The hybridization of Oxy1270 to both Dinenympha and Pyrsonympha cells may indicate that these are the same organism or that the probe (to a fairly conserved region of the ssu rDNA) was unable to distinguish these two genera. Further molecular phylogeny and in situ microscopy work will be necessary to better address this question.

Evolution of Mitochondria, Commensalism, and Sex

Under the archezoa hypothesis, amitochondriate protists without obvious relationships to mitochondrion-bearing taxa are viewed as potentially primitive eukaryotes that diverged prior to the acquisition of the mitochondrial symbiont (Cavalier-Smith 1983; Roger 1999). However, wherever examined, amitochondriate taxa have been found to possess nuclear-encoded genes that appear to be of mitochondrial origin (see Roger 1999). In the cases of two such amitochondriate taxa, parabasalids and Entamoeba, diverse evidence suggests that certain membrane-bounded organelles within the cell (hydrogenosomes in parabasalids; mitosomes/cryptons in Entamoeba) are the physical relics of mitochondria (Clark and Roger 1995; Roger, Clark, and Doolittle 1996; Mai et al. 1999; Tovar, Fischer, and Clark 1999; Rotte et al. 2000). These findings have bolstered the position that double-membrane-bounded organelles of other amitochondriate taxa, where present, are also likely to be modified mitochondria (e.g., Roger 1999). Conversely, the apparent lack of any similar double-membrane-bounded organelle in diplomonads has allowed some researchers the freedom to continue to propose a (form of) primitively amitochondriate status for this group. For example, it has been suggested that the diplomonad ''mitochondrial genes'' were all acquired by separate lateral transfers from other prokaryotes or were transferred prior to the complete and/or permanent incorporation of the mitochondrial symbiont, with the protomitochondrial form discarded by ancestors of diplomonads (Sogin 1997; Chihade et al. 2000).

Like diplomonads, oxymonads are widely held to completely lack mitochondria-like organelles (Brugerolle 1991; but see Bloodgood et al. 1974; Roger 1999). No potentially mitochondrial genes have yet been reported and, viewed by themselves, oxymonads have remained candidates for true ''archezoa,'' that is, primitively amitochondriate eukaryotes. In contrast, Trimastix cells contain small organelles bounded by two membranes. The cellular and molecular biology of these organelles have not been examined; however, most authors have argued that they are modified mitochondria of some form (Brugerolle and Patterson 1997). This argument is consistent with ultrastructural evidence suggesting a close relationship between Trimastix and the mitochondrion-bearing excavate taxa, such as Malawimonas (O'Kelly, Farmer, and Nerad 1999; O'Kelly and Nerad 1999; Simpson, Bernard, and Patterson 2000). If either or both of the propositions are accepted, the close relationship between Trimastix and oxymonads demonstrated here is the strongest (and arguably the only) positive evidence to date that oxymonads are secondarily amitochondriate. Thus, while the arguments for primarily amitochondriate diplomonads are weak in our opinion, whatever their merit they cannot be generalized or transplanted to oxymonads without provision of an alternative explanation for the organelles in Trimastix.

The relationship uncovered in this study may instead help illuminate the evolutionary process of mitochondrial loss. Our phylogenies are consistent with Trimastix and oxymonads sharing a recent common ancestor that lacked classical mitochondria, with further evolutionary divergence then generating the different amitochondriate conditions seen in extant Trimastix and oxymonads. The most intriguing possibility is that Trimastix retains an intermediate stage in the same process of mitochondrion loss that culminated in the condition now found in oxymonads. If this were even approximately true, oxymonads and Trimastix together could provide a particularly useful system for the study of the evolution of amitochondriate states.

The morphological simplicity of many obligately symbiotic or parasitic taxa is routinely interpreted as a ''reduction'' in consequence of their adoption of the commensal habit. However, if the common ancestor of Trimastix and oxymonads was amitochondriate and was free-living like its Trimastix descendants, the ''reduced'' amitochondriate state in oxymonads would in fact predate their adoption of a commensal habit and might be viewed instead as a preadaptation to life in metazoan digestive tracts. Alternatively, Trimastix may be secondarily free-living, a scenario which seems less parsimonious with present data, and, intuitively, less likely. However, we note that reversions to a free-living habit from commensalism appear to have occurred within some other amitochondriate taxa, including parabasalids (Gunderson et al. 1995; Edgcomb et al. 1998) and possibly diplomonads and retortamonads (Siddall, Hong, and Desser 1992; Bernard, Simpson, and Patterson 1997).

Given the potential interest to evolutionary and cell biologists, it is important to establish whether the oxymonad/Trimastix clade includes other extant organisms. Assuming a single origin for the mitochondrion, the hypothesis of a shared amitochondriate history for Trimastix and oxymonads would be falsified by the discovery of any mitochondriate group that branched within the oxymonad-Trimastix clade. Given the current poor sampling of mitochondriate excavate taxa for nuclear genes (see below), it would appear unwise to discount this possibility at present. A better understanding of the immediate relations of the oxymonad-Trimastix clade may also help polarize the evolutionary history of commensalism for the group. Molecular phylogenies are required that incorporate a broad sample of mitochondriate excavate taxa in addition to Trimastix and oxymonads. The contention that oxymonads lack any cellular mitochondrial homolog is also worth testing. Unusual densely staining bodies have been recorded in Pyr-

sonympha (Bloodgood et al. 1974), although it is unclear whether these are double-membrane-bound or have any equivalent in other oxymonads.

The identification of a definite relative of oxymonads also provides a new opportunity to understand the evolutionary significance of their unusual sexual cycles. To date, sexuality has not been reported in Trimastix, although this is unsurprising given the limited contemporary examination of the group. If subsequent studies demonstrate that the unusual features of oxymonad sex, such as one-step meiosis, autogamy, or ploidy cycles, are shared by Trimastix, then these traits may be traced back to the common oxymonad/Trimastix ancestor. On the other hand, finding that Trimastix mirrors standard eukaryotic sexual cycles would suggest that the atypical features of oxymonad sex are derived. An asexual Trimastix lineage would shed no light on the matter, as sex could easily have been lost. Since ploidy cycles, in particular, have been proposed as an intermediate evolutionary step to the origin of sex (Kondrashov 1994), it is important to examine both the overall placement of the oxymonad/Trimastix clade and the possibility of a sexual cycle in Trimastix.

Implications for Broad-Scale Eukaryotic Systematics

The close relationship between oxymonads and Trimastix has important implications for ''phylum-level'' eukaryotic systematics. In the modern era, oxymonads have been considered to be allied with diplomonads and retortamonads in the phylum Metamonada (Cavalier-Smith 1981, 1998) or with Heterolobosea (sensu lato) and Stephanopogon in the phylum Percolozoa (Cavalier-Smith 1999). Trimastix has been allied with parabasalids in the phylum Trichozoa (Cavalier-Smith 1997) and with jakobids in the phylum Loukozoa (Cavalier-Smith 1999). All of these phyla were or are primarily diagnosed by circumscription (a summary of important attributes of the organisms is contained therein) rather than in an explicitly ''phylogenetic'' manner. However, the circumscriptions of Metamonada and Percolozoa would not accommodate Trimastix, which has both mitochondrion-like organelles and Golgi dictyosomes, nor would the circumscriptions of Trichozoa and Loukozoa accommodate oxymonads, which lack Golgi dictyosomes and ventral feeding grooves. It may be most expedient to create a new taxon to encompass Trimastix and oxymonads. Given that each has generally been given its own class or subphylum (e.g., Cavalier-Smith 1997, 1998, 1999, 2000), this new taxon would arguably deserve the rank of phylum if Linnean ranking were continued.

Should the ''excavate taxa'' grouping be abandoned? Although the excavate taxa are remote from one another in our ssu rDNA analysis (fig. 2*A*), it may be difficult to establish phylogenetic ties among sequences possessing very different rates of evolutionary change, i.e., Trimastix versus diplomonads and heteroloboseids. In fact, we demonstrate the poor quality of the ssu rDNA data set for phylogenetic reconstruction if diplomonads and heteroloboseids are included in the analysis. In light of the extensive morphological data supporting a common origin for excavate taxa (Simpson and Patterson 1999; Simpson, Bernard, and Patterson 2000), it would be most premature to abandon the excavate hypothesis on the basis of our phylogenies. Additional molecular and morphological studies may help clarify the issue. Small-subunit rDNA sequences from additional excavate taxa that do not fall into the fast-evolving category would be particularly useful. The close relationship between Trimastix and oxymonads is, of itself, consistent with the excavate hypothesis, provided it is assumed that oxymonads have lost an ancestral ''excavate-type'' feeding groove and that the excavate taxa are a paraphyletic assemblage (Simpson, Bernard, and Patterson 2000).

The lack of signal in the small-subunit rDNA data set when long-branch taxa are included highlights the current uncertainty as to the identity of the most basal branches in the eukaryotic tree. It has been suggested that the bacteria-like mitochondrial genome of the core jakobid *Reclinomonas americana* most closely resembles the ancestral state (Lang et al. 1997), leading to arguments for a rooting of the eukaryotic tree with this group (Cavalier-Smith 1999). This possibility could suggest an early-diverging status for oxymonads independent of previous arguments of cellular simplicity, since ultrastructural data strongly link Trimastix and core jakobids as typical excavate taxa (O'Kelly, Farmer, and Nerad 1999).

It was originally thought that the eukaryotic tree could be rooted using single-gene sequences from single representatives of key eukaryotic groups. We now know that this strategy is very unlikely to be successful. The best approach may be to first establish solid within-group phylogenies for each of the major eukaryote groups using multiple genes as well as ultrastructural data. Should this result in a well-resolved and taxonomically diverse eukaryotic phylogeny, then rooting experiments could be performed to identify the earliest-branching eukaryotic lineages.

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