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Phylogenetic Diversity of Parabasalian Symbionts from Termites, Including the Phylogenetic Position of *Pseudotrypanosoma* and *Trichonympha*

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ABSTRACT. The phylogenetic diversity of parabasalian flagellates from termite hindguts has been examined by small subunit ribosomal RNA (rRNA) amplification and sequencing. Two species of particular interest, the giant trichomonad *Pseudotrypanosoma giganteum* and the hypermastigote *Trichonympha magna,* were isolated from the gut of *Porotermes adamsoni* by micropipetting, and the rRNA genes from these small populations amplified and sequenced. rRNA genes representing Hypermastigida and the Trichomonadida families Devescovinidae and Trichomonadidae, were also recovered by amplification from whole hindguts of three termites, *P. adamsoni, Cryptotermes brevis,* and *Cryptotermes dudleyi.* The parabasalian rRNA genes from *C. brevis* were found to comprise a unique and extremely heterogeneous lineage with no clear affinities to any known parabasalian rRNAs. In addition, one of the sequences isolated from *P. adamsoni* was found to be similar to another uncharacterised rRNA gene from *Reticulitermes flavipes.* The phylogeny of all known parabasalian small subunit rRNAs was examined with these new sequences. We find many taxonomic groups to be supported by rRNA, but not all. We have found the root of parabasalia to be very difficult to discern accurately, but have nevertheless identified several possible positions.

Supplementary key words. Evolution, hypermastigote, small subunit rRNA, taxonomy, trichomonad.

THE Parabasalidea, commonly parabasalids or parabasalia, is a diverse assemblage of unicellular eukaryotes distinguished by the presence of Janicki-type parabasal apparatus, specialised metabolic organelles known as hydrogenosomes, and a particular style of closed mitosis, pleuromitosis [3, 16, 17, 23]. Since the description of the first parabasalian, *Trichomonas vaginalis* [10], the taxonomic diversity of parabasalia has expanded impressively to include trichomonad, monocercomonad and devescovinid flagellates, polymastigote calonymphids [23], the amoeboflagellate *Histomonas* [18], the amoeba *Dientamoeba* [5], and hypermastigotes [15, 17]. Hypermastigotes are large and complex cells with flagella that may number into the thousands and cover most of the cell surface. The striking and unique morphology of hypermastigotes led to a long-held belief that they were unrelated to flagellates altogether. However, some early investigators recognised features shared between hypermastigotes and trichomonads [23, 24], and subsequent ultrastructural comparisons led to the formal union of Trichomonadida and Hypermastigida in the Superorder Parabasalidea [17, 25]. Aspects of this classification have since been revised several times [4, 6, 7], but for consistency we will use the detailed and comprehensive classifications [16, 35].

Molecular studies have also begun to contribute to our understanding of the taxonomy of parabasalia [14, 29, 32–34]. Many molecular studies to date have concentrated specifically on the smaller trichomonads, particularly the human pathogen *T. vaginalis.* This stems in part from medical interest, but also reflects the difficulties in working with many other parabasalia. While trichomonads and monocercomonads are found in a variety of habitats, and many of these have been cultured, devescovinids, calonymphids and hypermastigotes are all found exclusively in the gut of termites and wood-eating cockroaches of the genus *Cryptocercus.* These gut-dwellers belong to an intimate symbiotic community from which it is difficult to extricate and study individual members. Hence, very few of these organisms have been cultured, prohibiting most molecular work.

We have sought to extend existing examinations of the molecular diversity of parabasalian termite symbionts by two means. First, small subunit rRNA (rRNA) genes were amplified specifically from isolated cells of the hypermastigote, *Trichonympha magna,* and also from the giant trichomonad, *Pseudotrypanosoma giganteum.* The phylogenetic place of these two species was examined and we found *P. giganteum* to be most closely related to the Trichomonadidae *Trichomonas, Trichomitus trypanoides* and *Pentatrichomonoides,* while *T. magna* was related to other newly reported Trichonymphidae. rRNA genes were also amplified from whole-hindguts of three termite species. This yielded a considerable diversity of parabasalian rRNA genes, including one lineage of four genes not closely related to any parabasalian previously characterised by molecular means. The phylogeny of the parabasalia was inferred using these new sequences, and the position of the root of the parabasalia was examined closely.

MATERIALS AND METHODS

Collection and manipulation of termites and hindgut symbionts. *Porotermes adamsoni* Froggatt was collected from a decomposing log on the floor of a eucalyptus forest in the Cathedral Ranges, Victoria, Australia. Large pieces of wood were collected and stored at room temperature in sealed 5 or 10 litre plastic drums lined with damp paper towels. *Cryptotermes brevis* Walker and *Cryptotermes dudleyi* Banks were cultured by J. W. Creffield, and six individuals of each were a gift from S. Tamm.

Individual cells were isolated from *P. adamsoni* hindgut contents and collected in filter-sterilised buffer (0.1 M NaCl, 10 mM $NaPO_4$, pH 6.9). Aliquots of cell suspensions were placed in cavity slides and single cells collected by micropipetting. Individual cells were picked and washed three times, and the final suspension was examined closely for the presence of smaller cells, especially amongst the flagella. Approximately 30 cells were pooled in 0.1 ml of buffer and lysed by a single chloroform extraction. The partially purified DNA was ethanol-precipitated and dried, and the DNA from this isolation was resuspended in a pre-mixed PCR reaction (see below).

For whole-hindgut DNA isolations three to 10 hindguts were dissected and the contents released by dicing them on a glass slide in filter-sterilised buffer (as above). The solution was transferred to a chilled mortar and ground in liquid nitrogen until powdered. Ground material was resuspended in TE (10 mM TrisHCl, 1 mM EDTA, pH 8.0) and DNA was purified by repeated phenol-chloroform extractions and ethanol precipitations. Further purification using cetyltrimethylammonium bromide (CTAB) or Prep-a-gene (BioRad, Hercules, CA) was also used in certain cases to remove a residual contaminating pigment that co-purified with DNA.

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For micrographs, cells were extracted from the termite hindgut and transferred immediately to a solution of 1:1 of halocarbon oil series 700: halocarbon oil series 27 (Halocarbon Products Corporation, River Edge, NJ). A single drop of cells was placed on a microscope slide and sealed with VALAP (vaseline: lanolin: parafilm, 1:1:1). Cells were viewed with Nomarski oil immersion lenses, using a Zeiss Auxioplan 2 Research microscope, and video recordings were made in real time onto Panasonic MII metal tape. Single images were captured on a Targa 200 Pro video card.

Amplification, cloning and sequencing of small subunit rRNA genes. Small subunit rRNA genes were amplified using the primers GCGCTACCTGGTTGATCCTGCC and TGATCCTTCTGCAGGTTCACCTAC. Amplification reactions were 50 μl for isolated cells, and 100 μl for whole-hindgut preparations. Amplifications consisted of thirty five cycles of 1 min at 92° C, 1 min at 50° C, and 1.5 min at 72° C. A mixture of *Taq* and *Pfu* polymerases was used to lower the frequency of amplification errors.

Products of amplifications were separated by agarose electrophoresis, purified, and cloned in the T-vector, pGEM-T (Promega). Clones containing inserts of approximately the expected size were identified by PCR, and these were further screened by digesting the products of this PCR screen with *Sau*3AI. For each restriction pattern in whole-hindgut amplifications, 2–5 individual clones were picked and sequenced, and those which were not identical to another are reported here. All genes were sequenced on both strands using an ABI 373A sequencer with dye terminator chemistry and standard eukaryotic small subunit rRNA sequencing primers.

Phylogenetic analysis. Novel small subunit rRNA genes were aligned to a selection of other eukaryotic sequences from the Ribosomal Database Project [26] and all other parabasalian small subunit rRNA sequences from public databases. Two entries for *T. foetus* and *T. tenax* are virtually identical, so only one of each was used in the analysis. From this alignment phylogenetic trees were inferred, and the root of these trees examined by using other eukaryotic sequences as outgroups.

Unrooted parabasalian trees were inferred using 1401 positions from 36 sequences (*P. giganteum* and *T. magna* sequences amplified from whole-hindguts were excluded). Neighbor-joining and Fitch-Margoliash trees were constructed from distance matrices corrected according to both Kimura and F85 substitution models with programs from PHYLIP 3.57c [12] using a transition-transversion ratio estimated from the data by PUZZLE 3.1 [30]. Global rearrangements and ten random sequence additions were used in Fitch-Margoliash searches. Unweighted parsimony trees were inferred using PAUP test version 4.0d61 (written by David L. Swofford) using the estimated transition-transversion ratio and heuristically searching with 50 random addition replicates and tree bisection and reconnection. One hundred bootstrap resampling replicates were carried out with each of these methods. DNA maximum likelihood trees were calculated with fastDNAml 1.1 [11, 28] once more using the estimated transition-transversion ratio, random sequence addition order, local branch swapping over 1 branch and global branch swapping over 33 branches. Lastly, quartet puzzling trees were inferred using PUZZLE 3.1 [30] with 1,000 puzzling steps using both HKY and Kimura substitution models with an estimated transition-transversion ratio, and site to site substitution rate variation modeled on a gamma distribution with four categories and the shape parameter estimated from the data. These same methods were also applied to trees where *Dientamoeba* was excluded since it is highly divergent, and was identified by quartet puzzling analyses as having a base-composition significantly different from the rest of the data.

Testing the root of parabasalia. Trees of parabasalia were rooted with 30 eukaryotes from disparate groups, including diplomonads, heterolobosea, euglenozoa, *Entamoeba,* slime moulds, alveolates, rhodophytes, animals, plants and fungi aligned according to [26]. This dataset was analysed by the same methods described above.

The position of the root was also examined using a series of Kishino-Hasegawa tests using PAUP 4.0d61. These tests used both parsimony and maximum likelihood with the HKY model in maximum likelihood, and a transition-transversion ratio either estimated from the data (in the first two tests described below) or set to two (for the third test). For a very rough estimate of the stability of the root, the topology was constrained to that of the neighbor-joining tree, and the outgroup consisting of 30 other eukaryotes was grafted to 11 positions separating seven relatively robust clusters of sequences. Since the branching order of the parabasalian groups is not well resolved, it is likely better to perform such tests on a partially unresolved topology. The same 30 eukaryotes were therefore grafted to each of the seven sequence groups individually, and the branching order of the remaining groups left unresolved. In addition, an eighth root was also tested (based on the first test) that placed the clade consisting of *R. flavipes* symbiont 1 and *P. adamsoni* group 4 symbiont together with the trichonymphids at the base of the parabasalia. *Monocercomonas, Tritrichomonas* and *N. jouteli* symbiont Y08538 were treated as an unresolved group for rooting, but were not constrained when they were among the ingroup. To account for possible effects of the outgroup choice, the same eight topologies were also tested with 100 random pairs of outgroup taxa (chosen using a perl script written by W. Fischer) individually grafted to the eight positions used in the second test. This test gives a frequency with which a group is the deepest branch, as well as a frequency with which other possible roots are rejected.

RESULTS

Isolation of small subunit rRNA genes from the hypermastigote *Trichonympha magna,* and the trichomonad *Pseudotrypanosoma giganteum.* Hypermastigotes are a major constituent of the hindgut of *P. adamsoni* and many other lower-termites. Unfortunately these organisms are very rarely cultured, and at the time of this work no molecular data were known from this group. We observed large numbers of several types of hypermastigotes in *P. adamsoni,* and therefore sought to specifically identify the rRNA gene of a hypermastigote.

Although the exact composition of hindgut flora can change, numerous studies have identified five hypermastigotes as common constituents of the *P. adamsoni* hindgut: *T. magna, Joenina pulchella, Spirotrichonympha mirabilis, S. grandis,* and *Spirotrichonymphella pudibunda* [13, 19, 22, 31, 35]. We observed no discrepancies with these catalogues, and chose to isolate the hypermastigote, *T. magna* (as described by Kirby [ref. 22, plate 28]; Fig. 1, 3), because of its larger size and prevalence. Approximately 30 individuals were isolated and washed by micropipetting. The small subunit rRNA gene was amplified from this small population, and the product of this amplification was shown by restriction digestion to be composed of a single detectable sequence. Four clones were sequenced and over 1,526 nucleotides were found to differ by only 5 to 15 nucleotides, suggesting that they are all derived from the same species. The *T. magna* genes were also over 90% identical to rRNA genes recently characterised from two other trichonymphids, *T. agilis* from *Reticulitermes speratus* and an unidentified species of *Trichonympha* from *Zootermopsis angusticollis* [8, 27], further supporting the *T. magna* origin of these genes.

A second interesting symbiont that we found to be abundant

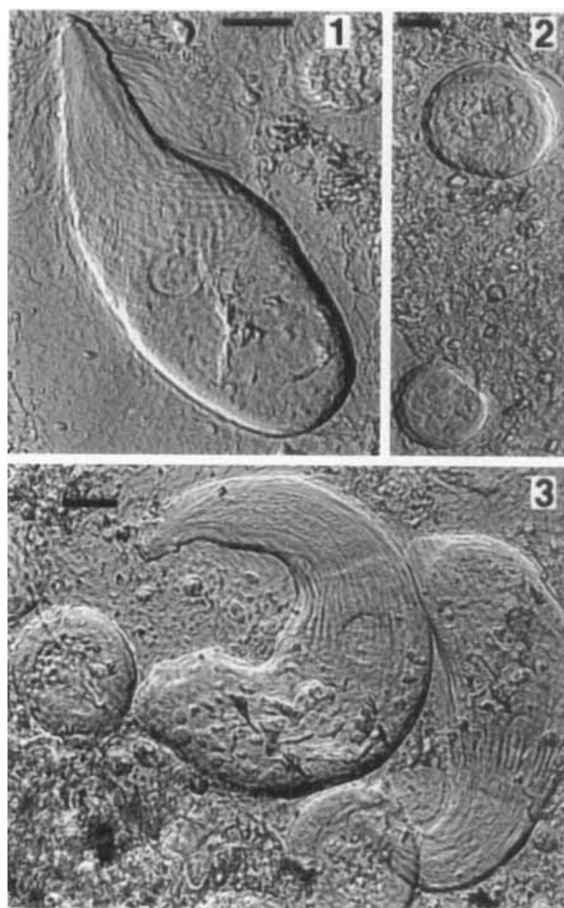


Fig. 1–3. *Trichonympha magna* and *Pseudotrypanosoma giganteum*. **1**. *T. magna*. Note the flagellated zone covering approximately half the body, and the large nucleus at about the transition point. The rostrum, upper left, is slightly thickened, and the cap or operculum is visible at the extreme anterior point of the cell. **2**. *P. giganetum* (top) and *P. minimum* (lower) showing the size variation. *Pseudotrypanosoma* cells contracted in the halocarbon suspension, and thus appear rounded with little definition. The undulating membrane and costa lie on the circumference of the contracted cell. They can barely be made out in *P. giganteum*, but are visible in the shadow of the *P. minimum* cell. **3**. Two individuals of *T. magna* with two individuals of *P. giganteum*. Scale bars each represent 20 µm.

in the hindgut of *P. adamsoni* was a very large trichomonad matching the descriptions of *P. giganteum*. *Pseudotrypanosoma* was first characterised in *P. adamsoni* and has not been recorded in any other termite [13, 21, 31]. These cells are striking because of their size, sometimes exceeding 100 µm, and also the presence of a distinctively robust costa, pronounced axostyle and a low-lying undulating membrane. The costa of *P. giganteum* is contractile [1], and when stressed the cells take on a spiral form. *Porotermes adamsoni* also contains a second species of *Pseudotrypanosoma*, *P. minimum* [31]. This species can be distinguished from *P. giganteum* in a number of characteristics, but the most obvious is size, since *P. giganteum* is two or even three times larger than *P. minimum* [31].

We isolated approximately thirty *P. giganteum* cells (Fig. 2, 3), and amplified small subunit rRNA genes from these. Again, a single restriction pattern was observed in all clones resulting from this amplification, and the two clones that were sequenced proved to be nearly identical to one another (5 bp different), and most similar to rRNAs from a variety of Trichomonadidae, in particular *Trichomonas*, *Trichomitus trypanoides* and *Pentatrichomonoides*.

Small subunit rRNA genes from whole-hindguts. For a broad view of parabasalian diversity in termite hindguts, we also amplified small subunit rRNA genes from whole-hindgut DNA preparations from three lower-termites: the damp-wood termite *Porotermes adamsoni*, and the dry-wood, or powder-post termites *Cryptotermes brevis* and *C. dudleyi*. The small subunit rRNA of parabasalia is much smaller than most eukaryotes, characteristically less than 1600 bp. Amplification reactions from whole-hindguts therefore yield two size classes, providing a way to specifically isolate genes of parabasalian origin.

Parabasalian-sized reaction products from *P. adamsoni* were found by restriction digestion to fall into four distinctly different groups, and three or four individual clones were sequenced from each group. Within each group, clones differed at only a few positions, but the four groups were quite distinct from one another. Interestingly, group 1 sequences were found to be nearly identical to those isolated specifically from *P. giganteum* (differing between 4 and 9 nucleotides out of 1,538), while group 3 sequences were found to be nearly identical to those of *T. magna* (differing between 3 and 11 nucleotides out of 1,526). Reamplifying the *P. giganteum* and *T. magna* genes in this way may reflect our observation that these two organisms were abundent in *P. adamsoni*. However, *P. adamsoni* harbours numerous other parabasalia, so it is also not surprising that groups 2 and 4 sequences are unique. Group 2 was found to be similar to devescovinids, and group 4 to the unidentified *Reticulitermes flavipes* symbiont 1 [14]. Reaction products from *C. brevis* were similarly found to fall into three restriction pattern types. Two of each were sequenced resulting in four unique sequences. These sequences were disparate, yet apparently most similar to one another. Only four products were obtained from *C. dudleyi*, and all belonged to a single restriction type. Two individual clones were sequenced and found to be identical to one another and similar to devescovinid rRNAs. Altogether these new sequences cover nearly the entire spectrum of parabasalian rRNA diversity.

Phylogeny of parabasalian small subunit rRNA. In total, 22 novel parabasalian small subunit rRNA genes were sequenced from specifically isolated *T. magna* and *P. giganteum*, or from whole-hindguts. These sequences were added to an existing database of 20 parabasalian rRNA genes, and the phylogeny inferred. In trees that included all sequences, the genes specifically isolated from *P. giganteum* and *T. magna* were interspersed among the nearly identical sequences amplified from whole-hindgut preparations (*P. adamsoni* group 1 and 3 sequences respectively). Therefore, to avoid cluttering the trees, the six *P. adamsoni* group 1 and 3 sequences were omitted and only the rRNAs isolated directly from *P. giganteum* and *T. magna* were used to represent these organisms. This had no observed effect on the rest of the tree.

From the remaining 36 sequences, trees were inferred by several methods: unweighted parsimony, corrected distance using both Fitch-Margoliash and neighbour-joining algorithms, and maximum likelihood by heuristically searching and by quartet puzzling. From these analyses a number of branches differ, but some relationships emerge consistently. The groups shown in Fig. 4 are those that are found fairly consistently with a variety of methods. Some of these groups support taxonomic divisions from intuitive evolutionary schemes [4, 16, 23], namely the Trichomonadidae (with the exception of *Tritrichomonas*), the Trichonymphidae, and a relationship between Devescovinidae and Calonymphidae. Of these, all are found in all of our analyses to branch together with the exception of *Ditrichomonas*

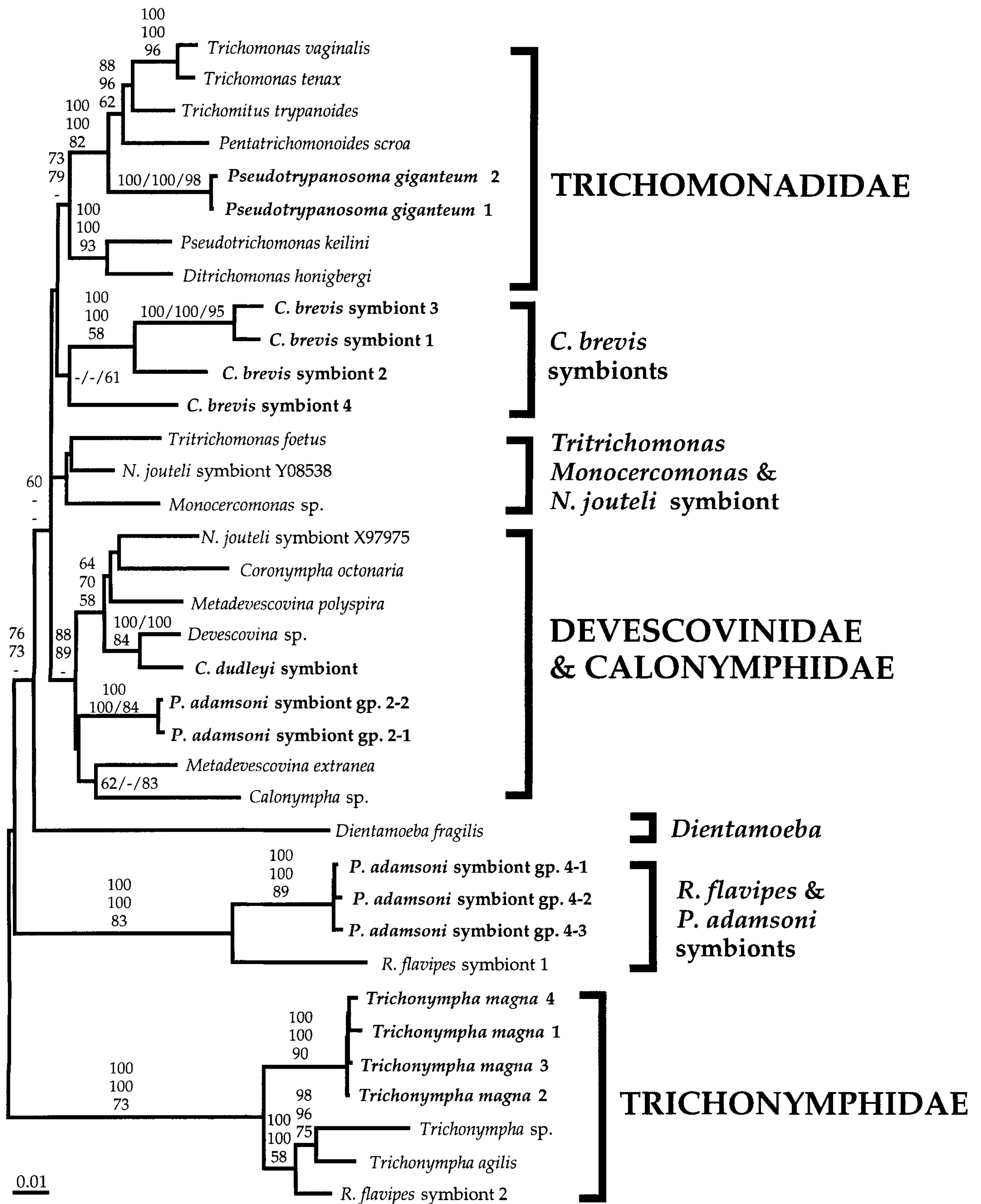


Fig. 4. Unrooted small subunit rRNA tree of parabasalia. Numbers at nodes indicate (from top to bottom or left to right) bootstrap proportions for neighbor-joining and Fitch-Margoliash, and the percent occurrence of a group in 1,000 quartet puzzling steps. Scale bar indicates 1 change

and *Pseudotrichomonas* which are paraphyletic with other Trichomonadidae in parsimony and DNA maximum likelihood, but are holophyletic in both distance trees, and are unresolved by quartet puzzling.

In addition to these relatively robust groups, there are also two well supported clusters composed entirely of rRNAs from unidentified organisms. The clade consisting of *P. adamsoni* group 3 symbionts and *R. flavipes* symbiont 1 is found with all analyses, and is strongly supported by bootstrap and by the presence of several unique signature sequences and two deletions unique to these genes. This group is also found in most analyses to be specifically related to the trichonymphids. Similarly, *C. brevis* symbionts 1–3 branch together quite consistently and strongly, and symbiont 4 branches weakly with the other *C. brevis* symbionts in neighbor-joining, quartet puzzling, and in parsimony when *Dientamoeba* is excluded. The four sequences also share a number of signature sequences, including a unique single nucleotide insertion (corresponding to position 719 of symbiont rRNA gene) in the otherwise highly conserved helix 25 [9], and a generally high degree of similarity over the highly variable regions of the gene. Throughout these variable regions the four *C. brevis* symbiont rRNAs are almost precisely collinear and their sequences are very similar. These portions of the gene are not included in the phylogenetic analyses, but if they were, the support for this clade would undoubtedly increase.

Lastly, in Fig. 4 there is a clade consisting of *Tritrichomonas, Monocercomonas,* and an unidentified symbiont of *Neotermes.* This grouping is very tenuous using small subunit rRNA (it is also found in parsimony trees, but not with Fitch-Margoliash, DNA maximum likelihood, or quartet puzzling). Nevertheless, the grouping of *Monocercomonas* with *Tritrichomonas* is found in phylogenies based on iron superoxide dismutase (SOD), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and large subunit rRNA [33–35], suggesting at least that these two genera may be related. Interestingly the representative *Trichomitus* in SOD and GAPDH trees is *T. batrachorum,* which is related to *Monocercomonas* in these trees [32, 33]. However, the small subunit rRNA of *T. batrachorum* is unknown and the representative *Trichomitus* rRNA is from *T. trypanoides,* which branches with Trichomonadidae (Fig. 4), suggesting that these two *Trichomitus* species are likely unrelated. Indeed, *T. trypanoides* was originally classified in the genus *Trichomonas* [see 2], which may be the more accurate classification based on Fig. 4.

In summary, the Trichomonadidae (excluding *Tritrichomonas*), the Devescovinidae and Calonymphidae, the Trichonymphidae, and two diverse groups of presently unidentified termite symbionts form relatively robust groups in rRNA trees. These groups are fairly consistent with other molecular phylogenies and morphology-based classifications insofar as they may be compared. The overall branching order of these groups and the phylogenetic positions of *Tritrichomonas, Monocercomonas,* an unidentified *Neotermes* symbiont, and *Dientamoeba,* all remain unresolved.

The root of the parabasalian small subunit rRNA tree. The root of a tree is often the most difficult aspect of a phylogeny to discern. This is in part because the outgroup is usually relatively divergent compared to members of the group itself. This problem is not unique to molecular phylogeny and applies equally to morphology, or any other characteristic. In the case of parabasalian rRNAs this is most certainly the case.

In previous analyses [8, 14, 29], the earliest branch of the parabasalia is *R. flavipes* symbiont 1, followed by *R. flavipes* symbiont 2 (now known to be *Trichonympha*). We also constructed rooted trees with a variety of other eukaryotes as outgroups and found that the deepest branch was inevitably the trichonymphids followed by the group of *R. flavipes* symbiont 1 and *P. adamsoni* group 4 symbionts (not shown). This root is well supported by bootstrap resampling, which places trichonymphids as the deepest branch in 82 and 86% of datasets in neighbor-joining and Fitch-Margoliash trees respectively. However, this root is inconsistent with the other published analyses, and they too have strong bootstrap support in some instances [8]. This could in part be due to the addition of the new sequences, but the root is still suspicious, and we have accordingly attempted a more thorough examination.

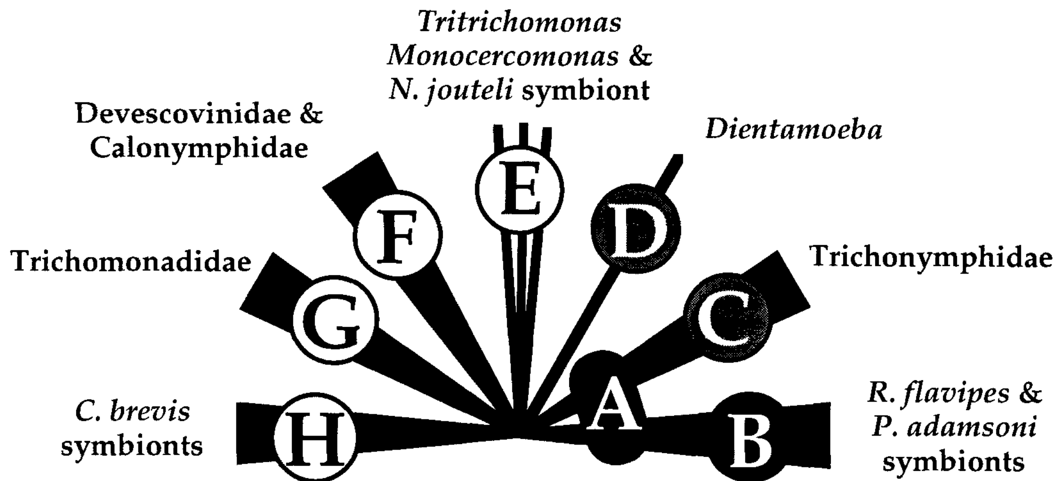
Kishino-Hasegawa tests were performed with a variety of conditions to estimate the significance of a root at any likely position. As a first approximation, the topology of the parabasalia was held constant as shown in Fig. 4, and the root grafted onto eleven branches that separate relatively well defined groups. The root position preferred by both parsimony and likelihood was in the branch leading to trichonymphids, however, the branch uniting the trichonymphids with *P. adamsoni* group 4 symbionts and *R. flavipes* symbiont 1 was not excluded at a 95% confidence limit, casting doubt on the position of the root.

As we have already indicated, the branching order between the groups of parabasalia is not well resolved, so we sought to test the root without interference from possibly erroneous branching patterns in the ingroup. The branching order between the parabasalian lineages was therefore left unconstrained, and the position of the root tested once more. In this case the rooting position most favoured by parsimony was again the branch leading to trichonymphids, but the best root according to maximum likelihood changed to the branch uniting the trichonymphids with *P. adamsoni* group 4 symbionts and *R. flavipes* symbiont 1. All other alternatives are rejected at a 95% confidence limit except where the root falls in the branches leading specifically to either of these two clades, or to *Dientamoeba.*

A final test was performed to take into account the possible effects of the topology of the outgroup. One hundred random pairs of taxa from the outgroup were chosen and used as the outgroup in 100 Kishino-Hasegawa tests. Since there is only one topology of each outgroup, the only effect will be the choice of taxa. The results of these tests are tabulated in Fig. 5 to show the frequency of both the preferred root, and also the frequency with which an alternative was not rejected at a confidence level of 95%. The best root was most often in the branch uniting trichonymphids with *P. adamsoni* group 4 symbionts and *R. flavipes* symbiont 1 (Fig. 5, root A). The only other root that was ever preferred was in the branch leading to trichonymphids (root B), but in every one of these cases root

←

per 100 positions. Two unidentified *Neotermes* symbionts are named according to their GenBank Accession Numbers. Unidentified *P. adamsoni* symbionts are numbered according to the group and the clone number, and groups 1 and 3 have been excluded since they are nearly identical to *P. giganteum* and *T. magna* respectively. This is a neighbor-joining tree, and the clusters indicated by brackets at the right are groups that were found by other methods with some consistency (see descriptions in text). When these correspond closely to taxonomically defined groups the name is given in upper case. Groups of sequences that are not defined taxonomically or are composed entirely of sequences from unidentified organisms are named in lower case for reference.



ROOT	PARSIMONY		MAXIMUM LIKELIHOOD	
	Best	Not Rejected	Best	Not Rejected
A	92	100	94	100
B	14	84	6	92
C	-	69	-	58
D	-	70	-	76
E	-	-	-	65
F	-	-	-	4
G	-	-	-	-
H	-	-	-	-

Fig. 5. Testing the root of the parabasalian small subunit rRNA tree. The schematic (**A**) shows the root positions tested, and the results of these tests are tabulated below (**B**). The values in the table are the number of times in 100 Kishino-Hasegawa tests that a root was either the best, or not rejected at a confidence limit of 95%. Dashes indicate never observed. In each case except A and E, the root was positioned in one lineage, and the ingroup was a polytomy consisting of the remaining groups. In tree A, the outgroup consisted of the trichonymphids and the unidentified *R. flavipes* and *P. adamsoni* symbionts, and in tree E the outgroup consisted of *Tritrichomonas*, *Monocercomonas* and the unidentified *Neotermes* symbiont. The filled circles in **A** and **B** indicate they were the best root in some replicates, while shaded circles in **C** and **D** indicate that they were not rejected in more than half the replicates in both analyses.

A was not rejected, and in many cases the two were equally parsimonious.

Altogether there seems to be very little confidence in the exact position of the root of the parabasalian rRNA tree. However, there does seem to be a degree of consistency supporting the root somewhere among the branches leading to the trichonymphids and the lineage of still uncharacterised *R. flavipes* and *P. adamsoni* symbionts.

DISCUSSION

Identifying new parabasalian rRNAs. Grassi first described *P. giganteum* in *P. adamsoni* and classified it with the Pyrsonymphidae [13]. Kirby emended this description, noting several similarities to trichomonads, reclassifying it as a member of the Trichomonadinae [21, plate 22]. Since then, Kirby's Trichomonadinae has been elevated in rank to Trichomonadida and subdivided extensively [4, 16]. In these subdivided schemes, *Pseudotrypanosoma* has been proposed to be sister to the genus *Trichomitopsis,* for which no molecular data are known, and together these have been classified as Trichomonadidae, either within the Tritrichomonadinae by Honigberg [16] or representing a unique subfamily, the Trichomitopsiinae, by Brugerolle [4]. The small subunit rRNA of *P. giganteum* branches strongly with the clade of *Trichomonas, Trichomitus trypanoides,* and *Pentatrichomonoides,* which are all members of the Trichomonadidae (noting the possible mis-classification of *Trichomitus trypanoides*). This confirms the classification of *Pseudotrypanosoma* within the Trichomonadidae; however, neither *Pseudotrypanosoma* nor indeed any of the Trichomonadidae show any specific relationship with *Tritrichomonas,* so it clearly should not be classified in the Tritrichomonadinae. As for its possible inclusion in a subfamily Trichomitopsiinae, this will have to wait for molecular data from *Trichomitopsis.*

T. magna was also first described by Grassi and its relationship with other trichonymphids has never been in question. However, the phylogenetic relationship between the hypermastigote trichonymphids and other parabasalia had not been tested with molecular data until very recently. We have found the *T. magna* sequence to be closely related to other parabasalian rRNAs. In support of this, two other trichonymphid rRNAs have also recently been reported, that of *T. agilis* from *R. speratus* [27] and another *Trichonympha* of an uncertain species from *Z. angusticollis* [8]. These sequences are also closely related to *R. flavipes* symbiont 2 [14], which likely corresponds to *T. agilis,* the only trichonymphid characterised in *R. flavipes* [19, 22, 35].

Interestingly, we also found *R. flavipes* symbiont 1 rRNA to be closely related to *P. adamsoni* group 4 sequences. It is unfortunate that the source of these has not been identified since the nature of this clade is of interest to our understanding of the ancestral state of parabasalia. Nevertheless, with two related sequences one can cautiously speculate on their origin by comparing the contents of the two termites looking for parabasalia common to both. In this case, besides *Trichonympha,* the only closely related parabasalia ever documented in both termites are hypermastigotes of the family Spirotrichonymphidae [13, 19, 35]. Both termites have been reported to contain a variety of spirotrichonymphid genera, any of which could be the sources of unidentified *P. adamsoni* or *R. flavipes* symbiont rRNAs.

Applying the same rationale to other unidentified parabasalian rRNAs is more difficult. *P. adamsoni* group 2 sequences, for instance, branch with devescovinids and calonymphids, but no such parabasalia have been characterised in *P. adamsoni* [19, 35]. Conversely, *C. dudleyi* has been reported to contain three species of *Devescovina,* so the devescovinid-like sequence from this termite could come from any one of these. The identity of the four related sequences from *C. brevis* is also unfortunately unclear, but of interest since they appear to represent a unique clade. One possibility is that they come from the monocercomonad genera *Hexamastix* and *Tricercomitus* [20] since the only other characterised *C. brevis* parabasalia are all devescovinids and calonymphids [35], and these are clearly unrelated to the *C. brevis* symbionts in the rRNA tree.

Phylogeny and root of parabasalia. The rRNA tree of parabasalia shown in Fig. 4 is not as well resolved as one would like, but it does nonetheless identify a handful of apparently related groups of sequences. These groups are, for the most part, consistent with classifications based on morphology, but there are a few exceptions. Some of the more serious prevailing questions include the phylogenetic place and cohesiveness of the Monocercomonadidae, the phylogenetic place of *Tritrichomonas,* the relatedness of various *Trichomitus* species, and the branching order among the major groups of parabasalia. Perhaps the most important problems at present are the identification of the remaining uncharacterised termite symbionts, and the molecular characterisation of several as yet untouched groups. Undoubtedly fulfilling the first of these necessities will obviate many of the second.

Many of the overall features of the rRNA tree are important independent confirmations of relationships proposed based on morphology. These congruencies are encouraging, however, one of the main features of the rRNA tree is not at all in agreement with morphology-based evolutionary schemes, and this is the root. Hypermastigotes have traditionally been considered to have arisen from the devescovinids, and the most ancient lineage of parabasalia was reasoned to be the monocercomonads due to their cytoskeletal simplicity [4, 16]. The rRNA tree is apparently at odds with this scheme, as it is the hypermastigotes that diverge early, and they are not specifically related to devescovinids. However, we should point out that the molecular data provided here and by others have only identified part of the hypermastigote lineage, *Trichonympha.* The lineage presently represented only by uncharacterised sequences from *R. flavipes* and *P. adamsoni* may also be hypermastigotes, but this is in need of confirmation.

Although it is obviously not without weaknesses, the rRNA tree of parabasalia is providing some important and sometimes unexpected insights into the evolution of the group. In the future, a reclassification of the parabasalia should likely take much of this evidence into account along with morphological characters.

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