Europ. J. Protistol. 38, 279–286 (2002)

http://www.urbanfischer.de/journals/ejp

European Journal of
PROTISTOLOGY

Molecular phylogenetic position of *Trichomitopsis termopsidis* (Parabasalia) and evidence for the Trichomitopsiinae

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Received: 6 March 2002; 8 July 2002. Accepted: 10 July 2002

The Parabasalia are one of the most morphologically diverse groups of flagellates. However, much of this diversity is restricted to organisms found only in the hindguts of wood-eating insects, primarily termites, making it difficult to acquire molecular data to study the phylogeny and evolution of diversity within this group. Here, the small subunit ribosomal RNA (SSU rRNA) has been characterised from one such parabasalian: the large flagellate *Trichomitopsis termopsidis*, which inhabits the hindgut of the North American termite, *Zootermopsis angusticollis*. With this new sequence the phylogenetic position of *Trichomitopsis* was determined in order to test the taxonomic validity of the Trichomitopsiinae, its position within the parabasalia, and to examine the evolution of cell size within the trichomonads. SSU rRNA phylogenies all yielded very strong statistical support for the Trichomitopsiinae (placing *Trichomitopsis* with *Pseudotrypanosoma* with 100% support). All phylogenies also showed Trichomitopsiinae branching within the Trichomonadidae, but not related to *Tritrichomonas*, as previously thought. In addition, the very large cell size characteristic of *Trichomitopsis* and *Pseudotrypanosoma* (ranging from 50 µm to nearly 300 µm) appears to be a homologous adaptation, and a single event in trichomonad evolution.

Key words: *Trichomitopsis*; *Pseudotrypanosoma*; Parabasalia; Termite hindgut symbiont; Evolution.

Introduction

The Parabasalia are a large and diverse assemblage of flagellate protozoa found in a number of low oxygen and anaerobic environments, very frequently as either symbionts or parasites of animals. Parabasalia are united by several characteristics, including the presence of a hydrogenosome, a Janicki-type parabasal apparatus, and a unique form of pleuromitosis (Kirby 1931; Kirby 1932; Honigberg 1963; Brugerolle 1975; Müller 1997). These organisms have generated a great deal of interest because of their medical importance as human pathogens, their unique biochemistry, their potentially pivotal position in various schemes of eukaryotic evolution, and their biodiversity.

The morphological diversity of parabasalia is impressive, encompassing small flagellates, amoeboflagellates, aflagellate amoebae, polymastigotes, and the hypermastigotes, which are large complex cells with hundreds or even thousands of flagella. Much of this diversity is restricted to a highly specialised niche found in the guts of wood-eating insects, namely those of lower termites and the cockroach genus *Cryptocercus* (Kirby 1931; Kirby 1932; Cleveland et al. 1934; Grassé 1952; Honig-

0932-4739/02/38/03-279 $ 15.00/0

berg 1963; Yamin 1979). This is the only known habitat of most described parabasalia, including all of the members of the order Hypermastigida (trichonymphids and spirotrichonymphids) and a significant fraction of the order Trichomonadida (monocercomonads, devescovinids, calonymphids, lophomonads, and "true" trichomonads). The hindgut environment is a rich source of parabasalia, but its inhabitants are difficult to study individually since each insect species contains numerous parabasalian species, and these hindgut symbionts are typically resistant to cultivation, presenting a serious obstacle for the study of parabasalian diversity and taxonomy using molecular techniques. Recently, however, molecular sequences have been generated from a large number of symbiotic parabasalia. In some cases the organisms have been cultivated (Berchtold et al. 1995), but most molecular data have been generated by "environmental" PCR, which allows the sampling of sequence diversity without identifying the organism (Gunderson et al. 1995; Keeling et al. 1998; Gerbod et al. 2000). This method has led to a great deal of small subunit ribosomal RNA (SSU rRNA) data from symbiotic parabasalia, but, without organismal identification, taxonomic questions cannot be addressed and little can be inferred about the evolution of characters in the group. This drawback has been avoided in some studies by coupling environmental sampling with *in situ* hybridisation, leading to a number of important insights (Ohkuma et al. 1998; Ohkuma et al. 2000). In a few instances, sequence data from uncultivated symbionts have been obtained by amplifying gene sequences directly from a small population of physically isolated cells (Dacks and Redfield 1998; Keeling et al. 1998). This method has also allowed detailed inferences about parabasalian evolution as it identifies the organismal source of the sequence.

One interesting characteristic that has arisen several times within parabasalia is the tendency towards very large cells. Although all are unicellular, some parabasalia reach enormous proportions: greater than 300 µm in some species (Kirby 1932). Within the Trichomonadidae (the "true" trichomonads), large cells are very rare, but not altogether absent. Two examples of gigantism in trichomonads are the genera *Trichomitopsis* (Kofoid and Swezy 1919) and *Pseudotrypanosoma* (Grassi 1919). Both genera are known only from termite guts, and their host range is restricted to a few species that are scattered worldwide. Two species of *Pseudotrypanosoma* are known only from southeastern Australia (*P. giganteum* and *P. minimum* in *Porotermes adamsonii*: Grassi 1919; Sutherland 1933). Four species of *Trichomitopsis* have been observed in termites from western North America (*T. termopsidis* in *Zootermopsis angusticollis* and *Z. nevadensis*: Kofoid and Swezy 1919; Cleveland 1925), the foothills of the Himalaya (*T. termitis* in *Archotermopsis wroughtoni*: Imms 1919; Cutler 1919), Panama (*T. barbouri* in *Glyptotermes angustus*: Kirby 1931), and Costa Rica (*T. cartagoensis* in *Glyptotermes contracticornis*: Kirby 1931). Both genera share the distinctive features that unite trichomonads (e.g., the presence of a costa and undulating membrane), but they are unusually large, some species are reported to reach 290 µm (Cleveland 1961). However, the gigantism in *Trichomitopsis* and *Pseudotrypanosoma* is fundamentally unlike that of other larger parabasalia: where the hypermastigotes, lophomonads, and calonymphids evolved by the repetition of structures, *Trichomitopsis* and *Pseudotrypanosoma* evolved by enlarging the existing body plan.

Because *Trichomitopsis* and *Pseudotrypanosoma* share several other morphological features, it is thought that this form of gigantism has evolved only once in the evolution of trichomonads. Both have four anterior flagella and one recurrent flagellum, a prominent costa, an undulating membrane that extends the whole length of the cell, and similarities in the morphology of the parabasal apparatus. These similarities were the basis for classifying these two genera in the Trichomitopsiinae (Brugerolle 1975), but it is not certain that they really are related or have evolved convergently (since some of these characteristics are found in other trichomonads). In addition, the evolutionary relationship of these taxa to other trichomonads is unclear. In most classification schemes, these genera are considered to be part of the Tritrichomonadinae (Honigberg 1963) or closely related to them (Brugerolle 1975; Brugerolle and Taylor 1979). Molecular phylogeny has not been used to address these issues, as only the SSU rRNA from *Pseudotrypanosoma giganteum* has been characterised and, in phylogenetic trees, this sequence does not show a strong affinity to any other trichomonad (Keeling et al. 1998). Here, the SSU rRNA has been characterised from *Trichomitopsis termopsidis*, a symbiont of the North American termite *Zootermopsis angusticollis* and the type species of the type genus for the Trichomitopsiinae. The va-

lidity of the Trichomitopsiinae was tested, and the relationships among these two unusual genera and other trichomonads was examined.

Materials and methods

Organisms, microscopy, single cell isolation, and amplification of SSU rRNA

Zootermopsis angusticollis Hagen was collected from decaying wood at Spanish Banks, Vancouver, BC, Canada, and maintained in the lab in portions of the logs from which they were collected. Hindgut material was extracted by removing the hindgut from live specimens and macerating it in Trager's medium (Trager 1934). This material was examined using differential interference contrast microscopy to confirm the presence of *Trichomitopsis termopsidis* and to record the morphology of the organism for comparison with published descriptions. Several individual cells were photographed to represent the various physical postures of *T. termopsidis*.

Thirty cells at the lower end of the scale of sizes previously noted for *T. termopsidis* were isolated from Trager's medium by micromanipulation and transferred to clean medium. Cells were re-isolated in this manner a total of three times until no contaminating protists could be seen. These thirty cells were lysed in 1.5 ml microfuge tubes using a disposable pestle, the lysate extracted once in chloroform:isoamyl alcohol (24:1), and the resulting DNA ethanol-precipitated. Amplification of SSU rRNA was carried out in a 25 ml reaction using the primers GCGCTACCTGGTTGATCCTGCC and TGATCCTTCTGCAGGTTCACCTAC. Amplification conditions consisted of 35 cycles of 30 seconds at 95 °C, 30 seconds at 45 °C, and 90 seconds at 72 °C with an initial denaturing step of 95 °C for 60 seconds and a terminal extension step of 72 °C for 90 seconds. Products were isolated by agarose gel electrophoresis, and products of the expected size (the only detectable product) were cloned using TOPO TA cloning (Invitrogen, Carlsbad, California). Four clones were sequenced on both strands and found to be identical.

Phylogenetic analysis

The new SSU rRNA sequence was added to an existing alignment of all currently known parabasalian SSU rRNA genes, including both identified species and samples generated from environmental PCR using termite hindgut material. Initial trees were inferred from an alignment containing all sequences, but a number of sequences from unidentified parabasalia that were similar to other sequences were excluded so as to limit the number of taxa. All sequences of Trichomonadidae that were closely related to *T. termopsidis* were retained. Phylogenetic trees of 49 taxa and 1298 unambiguously aligned positions were inferred using distance and maximum likelihood methods. Site-to-site rate variation was modeled on a gamma distribution approximated by eight rate categories, and the shape parameter and proportion of invariant sites estimated from the data using TREE-PUZZLE version 5.0 (Strimmer and von Haeseler 1996). Estimates were made under the HKY model of substitution, with the observed base frequencies and the transition-transversion ratio estimated from the data. The gamma shape parameter, proportion of invariant sites, and transition-transversion ratio estimated here were used in all subsequent analyses. Distance trees were inferred using PAUP 4.0b8 (Swofford 1993) with the general time reversible model corrected for rate variation using the gamma shape parameter and proportion of invariable sites estimated above (GTR+Γ+P_{inv}). Trees were searched using TBR branch swapping and 100 random sequence additions. Distance bootstraps were calculated in the same way, except that each data set was searched using 10 random addition replicates rather than 100. Gamma-corrected distance trees and bootstraps were also carried out using TREE-PUZZLE 5.0 and weighted neighbour joining, but no significant difference was observed between these trees and those above, and they are not shown. Gamma-corrected maximum likelihood trees were inferred using the HKY model of substitution with TBR and 10 random sequence additions. In addition, the GTR+Γ+P_{inv} distance tree was used as a starting point for TBR branch swapping using the GTR+Γ+P_{inv} model and maximum likelihood. Maximum likelihood bootstrapping was performed with the HKY+Γ+P_{inv} model (the GTR+Γ+P_{inv} model was too computationally intensive) using neighbour joining trees as a starting point and TBR branch swapping.

Results and discussion

Identification of *T. termopsidis* and sequencing of its SSU rRNA

Hindgut material from *Z. angusticollis* was examined by microscopy and a large population of *T. termopsidis* was consistently seen in all hosts examined. Cells ranged in size from 50–100 µm, although in rare individuals a distinct category of very large cells (over 200 µm) closely resembling *Trichomitopsis* could be seen. This has been observed previously and was suggested to represent cells about to undergo multiple fission events (Kofoid and Swezy 1919; Andrews 1925), although other studies suggest that there is an even distribution of sizes (Kirby 1931). Considering the possibility that these large cells may represent a separate population or undescribed species, only the more

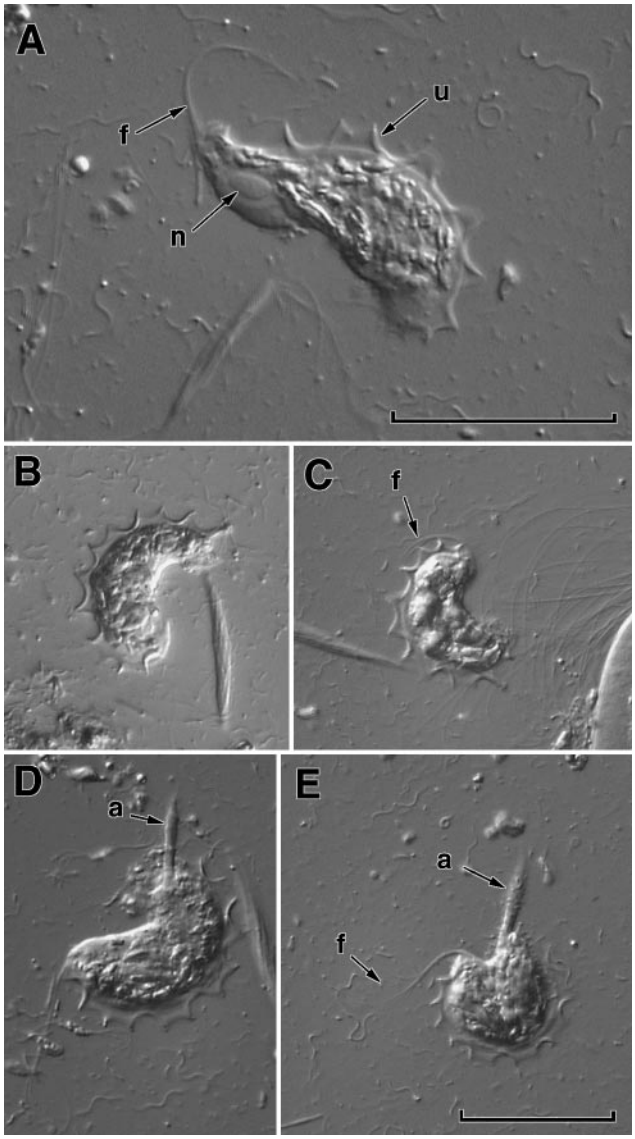

Fig. 1. Differential interference contrast light micrographs of *T. termopsidis* from *Z. angusticollis*. Abbreviations are: f, flagella; n, nucleus, u, undulating membrane, a, axostyle. (A) Elongate form showing anterior nucleus, flagellar bundle, and undulating membrane extending the length of the body. (B &C) Semi-contracted forms showing typical crescent shaped cells with the undulating membrane running the length of the outside edge of the crescent. (D & E) Highly contracted forms showing nearly rounded morphology with a peripheral undulating membrane and the exposed axostyle at the posterior end. Four anterior flagella can be seen emerging from the flagellar bundle in panel E. The smaller, cigar-shaped flagellate visible in A–D is *Streblomastix strix*. Superficially, *Streblomastix* can resemble the Trichomitopsis axostyle, but the identification of this structure as a protruding axostyle is based on observing living cells as well as transmission electron microscopy (P. J. K. and B. S. Leander, unpublished data). Scale bars represent 50 µm (parts B–E at same scale).

common cells at the lower end of the size range included in the original description of *T. termopsidis* were isolated. *Trichomitopsis* morphology can vary tremendously depending on the state of the costa. Light micrographs of cells in various common forms are shown in Figure 1 to confirm that the morphology and motile behavior of the cells isolated matches the description of *T. termopsidis*.

Thirty cells were manually isolated by micropipetting from the hindgut of a single host individual, and DNA from these cells was used to amplify SSU rRNA. A single product of the expected size (1574 bp) was amplified, cloned, and four clones completely sequenced. All four clones proved to be identical.

Phylogenetic position of *T. termopsidis* within parabasalia

Phylogenetic trees including the new *T. termopsidis* SSU rRNA sequence were inferred using distance and maximum likelihood (Fig. 2). In general, several aspects of the phylogeny were strongly supported and seen in all methods, while a number of relationships remain weakly supported or completely unresolved, which has been observed previously in parabasalian SSU rRNA phylogenies (Gunderson et al. 1995; Berchtold et al. 1995; Keeling et al. 1998; Ohkuma et al. 1998; Delgado-Viscogliosi et al. 2000; Ohkuma et al. 2000 Gerbod et al. 2001). Of the major groups recognised in parabasalian taxonomy, the Trichonymphidae, Spirotrichonymphidae, a group composed of Devescovinidae and Calonymphidae (now proposed to be classified together with a handful of other groups as the Cristamonadida: Brugerolle and Patterson 2001), and the Trichomonadinae are all consistently recovered and mostly well-supported statistically. In the case of the Trichomonadinae, the phylogeny shows the genus *Pentatrichomonoides* branching deep within Trichomonadinae, although this genus is typically classified as a member of the Pentatrichomonoidinae (Honigberg 1963). Based on the position of this genus and the support for this clade, this classification should perhaps be re-examined. Conversely, *Trichomitus batrachorum* has typically been included in the Trichomonadinae (Honigberg 1963), but this species shows no relationship to Trichomonadinae in SSU rRNA phylogeny, suggesting that this species is not a true Trichomonadinae (Delgado-Viscogliosi et al. 2000). The Monocercomonadidae and Tritri-

chomonadinae were not recovered here or in other reported phylogenies (Keeling et al. 1998; Ohkuma et al. 1998; Delgado-Viscogliosi et al. 2000; Ohkuma et al. 2000; Gerbod et al. 2001). Similarly, a number of unidentified sequences and a few taxa such as *Dientamoeba* and *Histomonas* are not strongly associated with any of the major taxonomic groups.

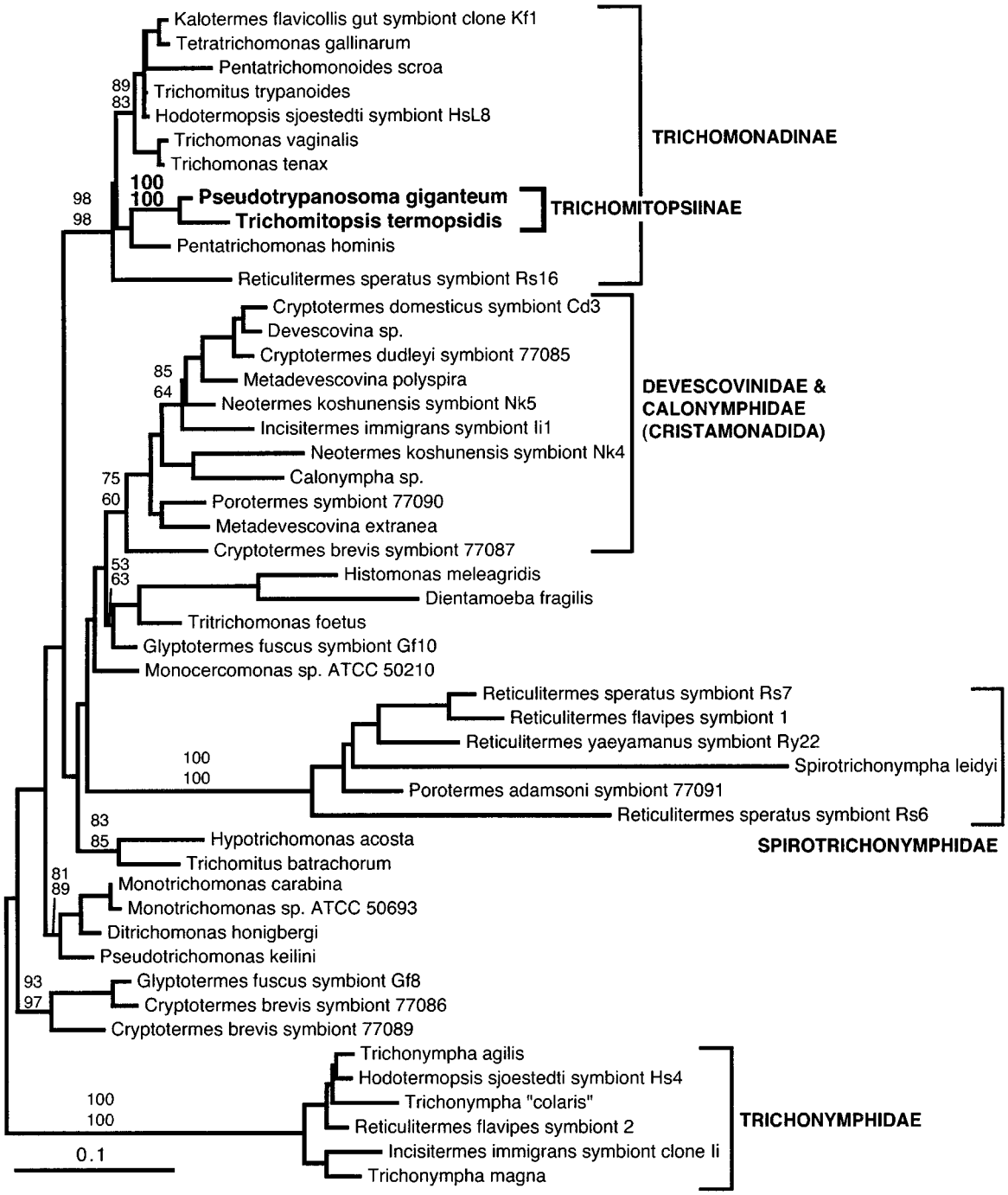

Fig. 2. Phylogeny of parabasalia. Maximum likelihood tree of selected parabasalia, including all known sequences from identified species and selected sequences from unidentified termite symbionts. Unidentified symbionts are designated by the name of the termite, followed by "symbiont", and a clone number corresponding to its title in GenBank. Numbers at nodes correspond to bootstrap support from maximum likelihood (top) and distance (bottom). Acknowledged taxonomic groups that are recovered in SSU rRNA phylogeny are represented as brackets to the right.

Pseudotrypanosoma and *Trichomitopsis* branch together with extremely strong support (100% in all methods), and consistently branch within the well supported Trichomonadinae clade (98% bootstrap support in ML and distance). A number of hyper-variable regions of the alignment that were not used to infer the phylogeny are also extremely similar or identical in *Pseudotrypanosoma* and *Trichomitopsis* (not shown), despite showing almost no conservation in other parabasalia. Altogether, these results provide very strong support for the Trichomitopsiinae (although perhaps at a different rank), and suggest that the group evolved from within, or perhaps as a sister group to the Trichomonadinae.

Interestingly, *Pseudotrypanosoma* and *Trichomitopsis* also showed a weakly supported (51% bootstrap in ML) but consistent affinity to *Pentatrichomonas hominis*, a human parasite. While this association is only weakly supported, it is found in all analyses, and a relationship between *Pseudotrypanosoma* and *Pentatrichomonas* has been observed with higher support in other phylogenetic studies containing fewer trichomonads (Delgado-Viscogliosi et al. 2000; Gerbod et al. 2001). *Pentatrichomonas* is a member of the Trichomonadinae that has been tentatively proposed to be derived from a *Tetratrichomonas*-like ancestor in some classifications (Honigberg 1963; Brugerolle 1975). While *Pentatrichomonas* more closely resembles *Tetratrichomonas* in morphology, *Trichomitopsis* and *Pentatrichomonas* do share some similarities in their basal body-cytoskeletal arrangement (*Pentatrichomonas* primarily differs in having one additional anterior flagella that originates and moves independently of the other four anterior flagella). In distance trees, this group also weakly includes the unidentified *Reticulitermes* symbiont Rs16 (no support from ML and 78% support from distance), which has previously been observed to branch with *Pseudotrypanosoma* (Ohkuma et al. 2000). However, with no information on the nature of this organism, it is impossible to say what significance this might have.

The close relationship between *Pseudotrypanosoma* and *Trichomitopsis* supports the view that their unusually large cell size originated once in the common ancestor of both genera. Both genera exhibit a great deal of size variation: in *Pseudotrypanosoma* a second smaller species has been described (Sutherland 1933) and past descriptions of *Trichomitopsis* seem to agree that large and small variants of this genus also exist (also observed here). Examining molecular sequences from these small cells could be very interesting as they might represent descendants of intermediate sized flagellates. However, if the large and small variants are distinct but closely related species, it suggests that the variants probably evolved relatively recently. Alternatively, the range of sizes could also represent growth stages of a single species if multiple fission indeed occurs, as suggested by Kofoid and Swezy (1919) and Andrews (1925). Nevertheless, the adaptation to large size in the Trichomitopsiinae is an interesting character since other parabasalia have evolved large cell size, but have done so in very different ways: where *Pseudotrypanosoma* and *Trichomitopsis* have simply grown larger, many trichonymphids, spirotrichonymphids, lophomonads, and calonymphids have increased cell size while replicating cell structures such as flagella and associated structures, or in some instances even nuclei. The numerous independent adaptations to large cell size in parabasalia are likely tied to life in the gut environment, since all of the most highly adapted and complex parabasalia are found in termites and the wood-eating cockroach, *Cryptocercus*. Perhaps the relatively static and mechanically protected environment of the gut allowed such alterations, or perhaps the need to ingest masticated wood particles, which are relatively large "prey", precipitated this adaptation. Despite the enormous morphological variety evident in parabasalia, the diversification of most known types must have proceeded relatively recently, probably after the origin of termites.

Taxonomic considerations

Pseudotrypanosoma giganteum was described from *Porotermes adamsoni* by Grassi in 1919, and was originally considered to be related to pyrsonymphids based on his observation of two anterior flagella and the nature of the axostyle. However, Kirby (1931) noted that the genus has four anterior flagella and an axostyle consistent with a closer relationship to trichomonads, and it has been considered a trichomonad since that time, although the exact nature of its relationship to other members of the group has not been clear (Cleveland 1961). A second species, *Pseudotrypanosoma minimum*, has also been proposed to live in *Porotermes* (Sutherland 1933). This species is reported to be highly similar to *P. giganteum*, but can be differentiated

based on being smaller in size, and distinct in its motility and the periodicity of its undulating membrane.

Trichomitopsis termopsidis was first described from *Zootermopsis* (then *Termopsis*) *angusticollis* by Kofoid and Swezy in 1919. It was recognised as a trichomonad and named *Trichomitus* (subgenus *Trichomitopsis*) *termitidis*. It was placed in the genus *Trichomitus* because an axostyle was not observed at that time, but the subgenus *Trichomitopsis* was given in recognition of the profound differences between this and other species of *Trichomitus*. Cleveland re-examined Z. *angusticollis* and Z. *nevadensis* and found the same flagellate, but was able to discern the presence of an axostyle and also four anterior flagella rather than the three originally reported, leading him to re-name the flagellate *Trichomonas termopsidis* (Cleveland 1925). Ultimately, Honigberg (1963) argued that this species belongs to neither genus, and elevated the subgenus *Trichomitopsis* Kofoid and Swezy 1919 to genus rank, which subsequently included *T. termopsidis* together with morphologically similar flagellates *Ditrichomonas termitis* (Imms 1919; Cutler 1919), *Trichomonas barbouri* (Kirby 1931), and *Trichomonas cartagoensis* (Kirby 1931).

Some relationship between the present members of *Trichomitopsis* and *Pseudotrypanosoma* has been entertained for over seventy years, but nothing was formally proposed until relatively recently. Kirby originally recognised that *T. termopsidis* was very similar to *Ditrichomonas termitis* as described by Cutler (1919) despite the great difference in size between the two (Kirby 1931). Sutherland also made a connection between *Pseudotrypanosoma minimum* and *Ditrichomonas termitis* (Sutherland 1933). One of the first large and detailed classifications of trichomonads indicated a relationship between *Trichomitopsis* and *Pseudotrypanosoma* (Honigberg 1963), and eventually the Trichomitopsiinae was erected by Brugerolle in 1975 to unite the two genera. The phylogeny of SSU rRNA presented here confirms this classification with very strong statistical support.

Although the two genera were proposed to be related, some unusual features of the Trichomitopsiinae has made it somewhat more difficult to determine their relationship to other trichomonads. In all classification schemes where this has been addressed, *Trichomitopsis* and *Pseudotrypanosoma* are placed within (Honigberg 1963) or close to (Brugerolle 1975; Brugerolle and Taylor 1977) the Tritrichomonadinae. This placement is not consistent with the SSU rRNA phylogeny, which shows the Trichomitopsiinae to be an early branch of the Trichomonadinae, and calls into question the existence of the Tritrichomonadinae (which explicitly encompasses *Tritrichomonas*, *Trichomitopsis*, and *Pseudotrypanosoma*: Honigberg 1963). Indeed, both molecular-phylogenetic and morphological studies in recent years (Keeling et al. 1998; Ohkuma et al. 1998; Delgado-Viscogliosi et al. 2000; Ohkuma et al. 2000; Brugerolle 2001; Brugerolle and Patterson 2001) have revealed that a number of serious revisions need to be made to the Parabasalia, and that the Trichomonadida is in particular need of re-classification.

Acknowledgements: This work was supported by an operating grant (227301-00) from the Natural Sciences and Engineering Research Council of Canada. PJK is a scholar of the Canadian Institute for Advanced Research and the Michael Smith Foundation for Heath Research. We would like to thank G. Brugerolle for providing the original description of the Trichomitopsiinae and for continuing helpful discussion. B. Leander, J. Archibald, and J. T. Harper are also thanked for critical reading of the manuscript.

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