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Phylogenetic Position and Morphology of Spirotrichosomidae (Parabasalia): New Evidence from *Leptospironympha* of *Cryptocercus punctulatus*

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Parabasalia are a large, diverse clade of anaerobic flagellates, many of which inhabit the guts of wood-feeding insects. Because most are uncultivable, molecular data representing the true diversity of Parabasalia only became possible with the application of single-cell techniques, but in the last decade molecular data have accumulated rapidly. Within the Trichonymphida, the most diverse lineage of hypermastigote parabasalids, molecular data are now available from five of the six families, however, one family, the Spirotrichosomidae, has not been sampled at the molecular level, and is very little studied with electron microscopy. Here we examine a representative of Spirotrichosomidae—*Leptospironympha* of the wood-feeding cockroach *Cryptocercus punctulatus*—with scanning and transmission electron microscopy, and analyze its small subunit rRNA gene to infer its phylogenetic position. Phylogenetic analyses place *Leptospironympha* as sister to a clade comprising Eucomonymphidae and Teranymphidae with moderate support. Examination with scanning and transmission electron microscopy reveals new classes of previously undetected symbiotic surface bacteria, a glycocalyx, granular particles on flagella, and putative phagocytosed bacteria. The range of flagellar patterns in Spirotrichosomidae is quite wide, and the possibility that some members may be more closely related to Eucomonymphidae or Teranymphidae is addressed.

Key words: bacterial symbionts; *Cryptocercus punctulatus*; *Leptospironympha*; Parabasalia; phylogeny; Spirotrichosomidae.

Introduction

Parabasalia are a large, diverse clade of microaerophilic and anaerobic flagellates that are most often found in symbiotic (mutualistic or parasitic) associations with animals. Most of the taxonomic and structural diversity of the group is restricted to the hindguts of wood-feeding insects, specifically lower termites and the cockroach *Cryptocercus*. Many parabasalids in these environments have evolved great structural complexity and increased size, especially the hypermastigotes, which may be covered with as many as tens of thousands of flagella organized in highly complex patterns. A well resolved phylogeny of Parabasalia would be

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useful because the evolution of these patterns and their other ultrastructural characters is of interest, however they are resistant to cultivation, so molecular data have only been acquired relatively recently using single-cell or hybridization techniques (Keeling 2002; Keeling et al. 1998; Ohkuma et al. 2000). These methods have greatly improved our knowledge of the evolution of Parabasalia, as small subunit ribosomal RNA (SSU rRNA) sequence data have become available for many of the major groups predicted from morphology (Brugerolle and Lee 2000). Some major revisions are suggested by the molecular data, in particular relating to the hypermastigotes. The three major lineages of hypermastigotes—Lophomonadida, Spirotrichonymphida, and Trichonymphida—are each supported as monophyletic, but as a whole they do not appear to form a monophyletic 'hypermastigote' clade (Carpenter et al. 2009; Hampl et al. 2006; Ohkuma et al. 2005).

Of the three hypermastigote lineages, order Trichonymphida is the most abundant and widespread, and also the best studied by molecular means. Indeed, at least one representative SSU rRNA sequence has been characterized for five of the six families of Trichonymphida, and in many cases several genera representing the taxonomic diversity of the family have been examined (Carpenter and Keeling 2007; Dacks and Redfield 1998; Keeling et al. 1998; Ohkuma et al. 1998, 2005, 2008). The one exception is the family Spirotrichosomidae. This family represents a significant gap in our knowledge of parabasalid phylogeny and morphology; it is the last major described group for which no sequence data exist whatsoever, and there have been no investigations with scanning electron microscopy, and only one with transmission electron microscopy (Hollande and Carruette-Valentin 1971).

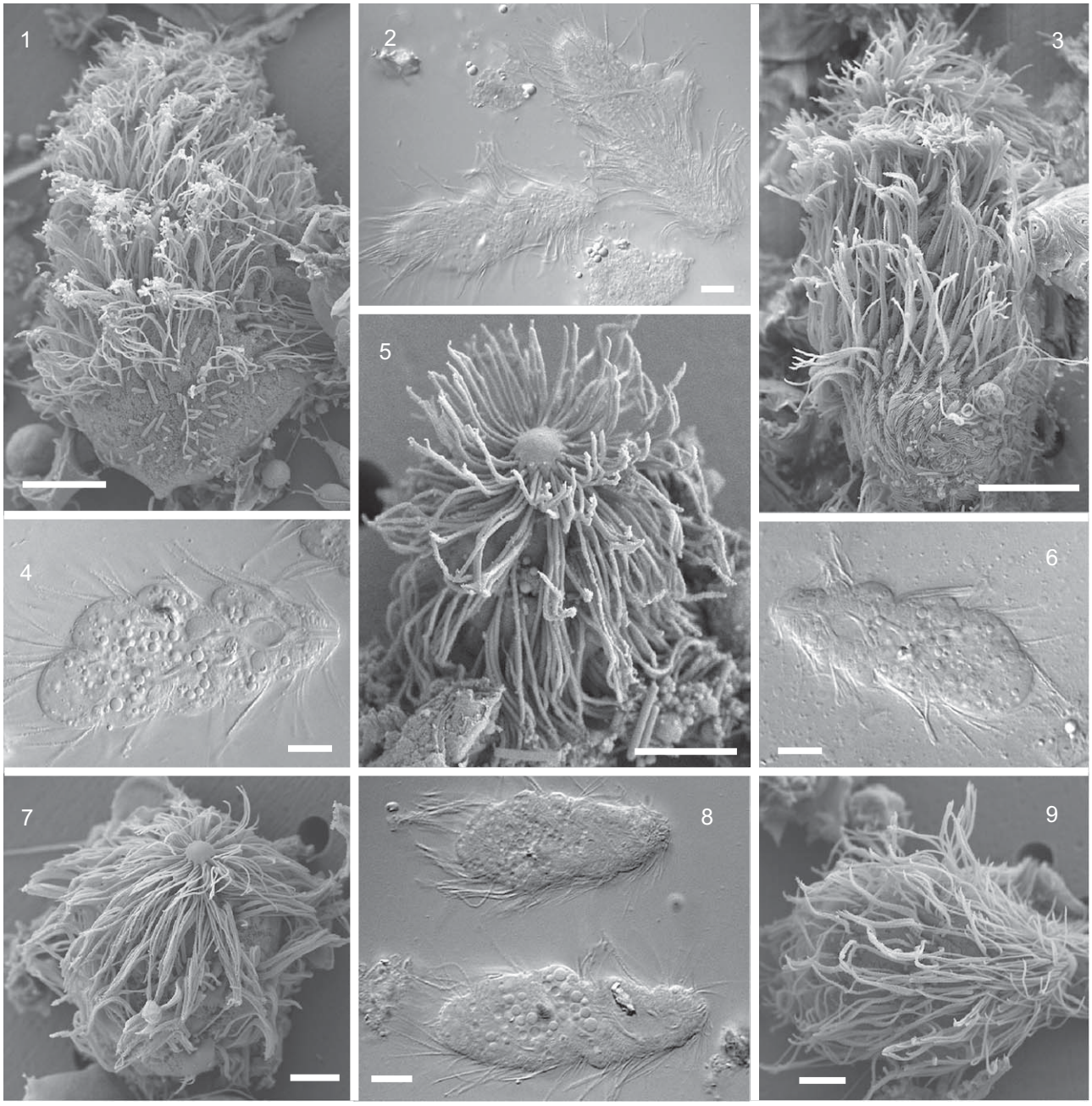
Spirotrichosomidae comprise 25 species in five genera: *Macrospironympha* from the North American wood-feeding cockroach *Cryptocercus puntculatus*, three genera—*Spirotrichosoma*, *Apospironympha*, and *Colospironympha*—from the termite *Stolotermes*, and *Leptospironympha*, which is one of the few genera of Parabasalia that is found in both *Cryptocercus* and termites (Cleveland and Day 1958; Cleveland et al. 1934). The family is characterized by flagellar bands that are straight in the rostrum but undergo relational coiling to form a left-handed helix (Brugerolle and Lee 2000). Superficially, this is similar to the Order Spirotrichonymphida (Hollande and Carruette-Valentin 1971), but in Spirotrichonymphida the flagellar bands form a right-handed helix (We define a left-handed helix as one that coils in a counter-clockwise fashion away from the viewer when viewed along the axis of the helix, and a right-handed helix as one that coils in a clockwise fashion). Based on other characters, such as the presence of a true rostrum in the Spirotrichosomidae, this has been treated as a convergent morphology, and Spirotrichosomidae are classified in the Trichonymphida (Brugerolle and Lee 2000). Like all hypermastigotes, Spirotrichosomidae occur exclusively in the guts of wood-feeding insects, where they likely play a role in enzymatic degradation of cellulose, as do other hypermastigotes (Cleveland 1923, 1924; Ohtoko et al. 2000; Trager 1932; Yamin 1981).

To determine the relationship of Spirotrichosomidae to other Trichonymphida, (if indeed they are members of this group), and to gain improved knowledge of their morphology and ultrastructure, we have examined *Leptospironympha* from Appalachian populations of *Cryptocercus punctulatus* with SEM, TEM, DIC Nomarski LM, and performed phylogenetic analysis of SSU rRNA gene sequences from manually isolated individual cells and environmental samples from a number of different host insects. We describe several new morphological characters of *Leptospironympha*, and confirm its position within the Trichonymphida, most likely as sister to a clade comprising Eucomonyphidae and Teranymphidae.

Results

Distribution

With SEM, we found *Leptospironympha* in *Cryptocercus* individuals from three of the five populations examined: Log Hollow, North Carolina, Mountain Lake Virginia, and South Mountains, North Carolina. With LM, *Leptospironympha* was present in low abundance in most, but not all *Cryptocercus* individuals examined. *Leptospironympha* was never as abundant as some other gut protists of *Cryptocercus*, such as the hypermastigote parabasalid *Trichonympha* (Carpenter et al. 2009) or the oxymonad *Saccinobaculus* (Carpenter et al. 2008). Rather, its somewhat sporadic occurrence was more similar to other hypermastigote parabasalids from *Cryptocercus*, such as *Eucomonympha imla* (Carpenter and Keeling 2007).



Figures 1–9. SEM and DIC Nomarski LM micrographs of *Leptospironympha* of *Cryptocercus punctulatus*. **1–4, 6, 8.** *Leptospironympha wachula* (Scale bars=10 μm). **5, 7, 9.** *Leptospironympha eupora* (Scale bars=5 μm). Note the spiraling flagellar bands, which can be seen to form a left-handed helix in Figures 1, 3 and 9. The two cells in Figure 8 appear to have a right handed helix because the plane of focus corresponds to the bottom of the cell.

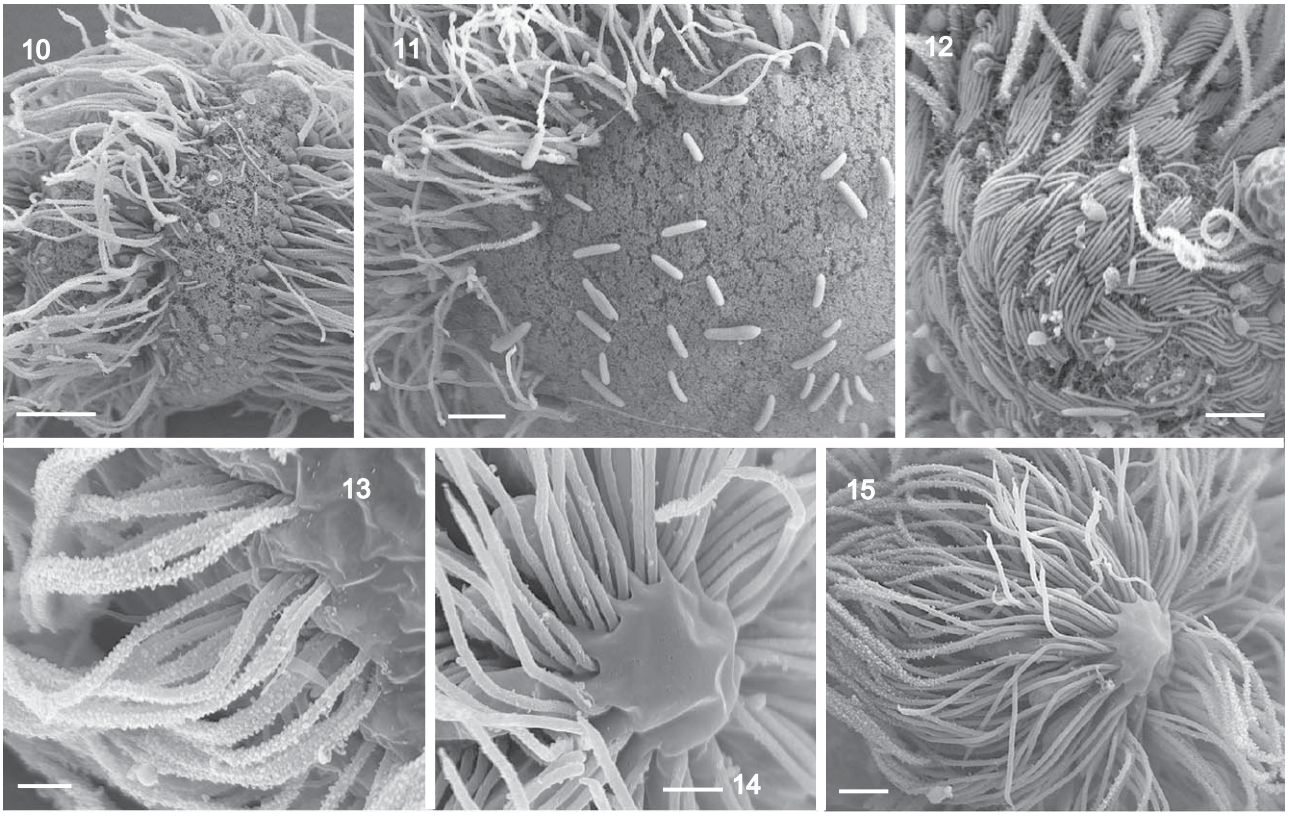
Morphology and Ultrastructure

A representative sampling of *Leptospironympha* cells as observed with LM and SEM is presented in Figures 1–9. The left-handed helical bands of flagella were observed in both SEM (e.g. Fig. 1) and LM (e.g. Fig. 2). Individuals are generally elongate, appearing roughly 1.5 to 3 times as long as wide—ranging from 27 μm long by 16 μm wide to 79 μm long by 33 μm wide, as measured from

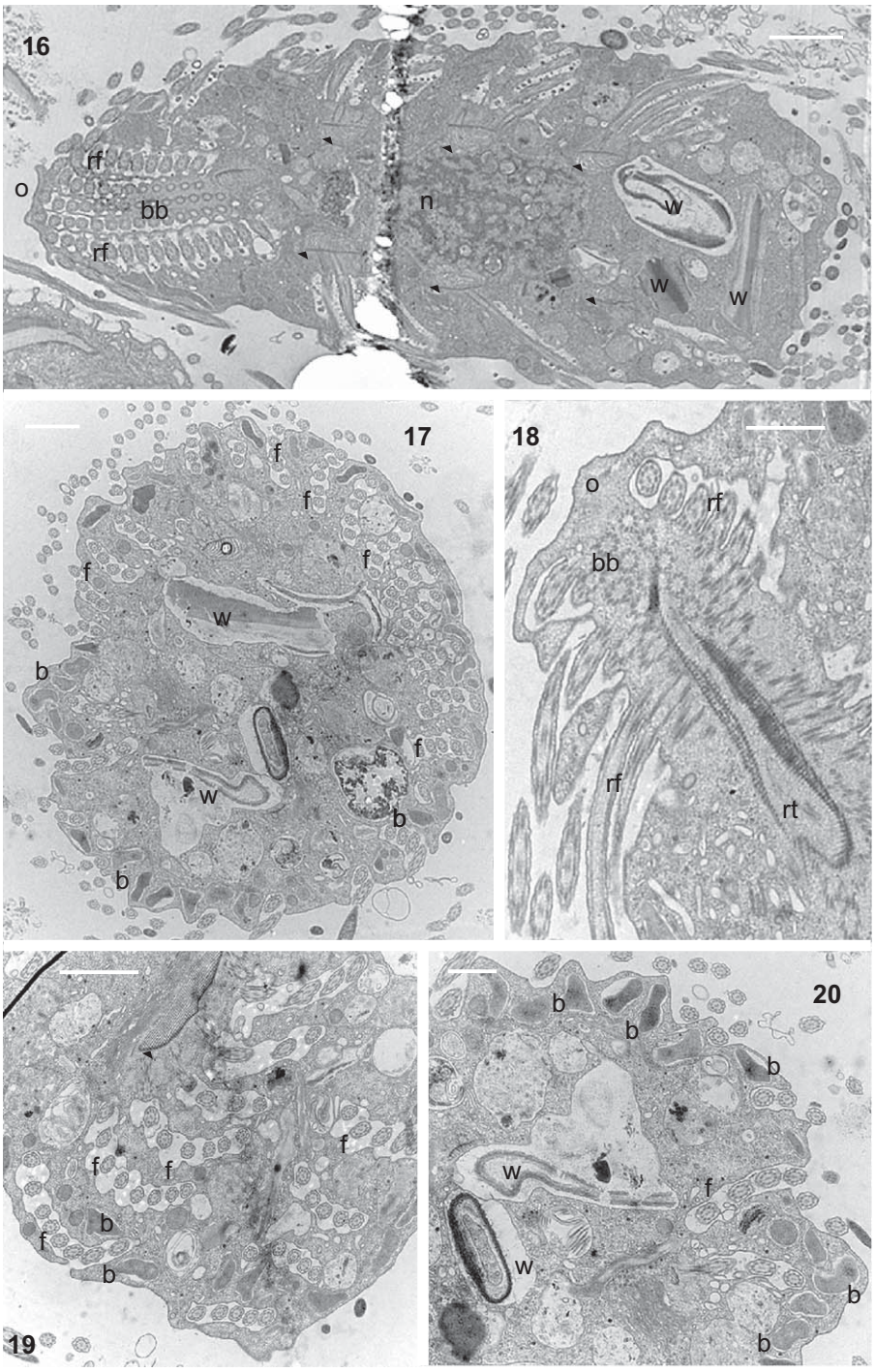
both LM and SEM micrographs. However, cells are somewhat plastic, and some may even appear roughly spherical at times (Fig. 7). Our measurements fell into two size classes that corresponded roughly to measurements of the two species described from Appalachian populations (Cleveland et al. 1934): the smaller size class, which ranges from 27 μm long by 16 μm wide to 43 μm long by 25 μm wide, roughly corresponds to *L. eupora*, but many individuals in our larger size class (ranging from 50 μm long by 25 μm wide to 79 μm long by 33 μm wide) are somewhat larger than reported measurements of *L. wachula*.

Several new features not visible with light microscopy were revealed with SEM and TEM. These include a few different types of bacterial surface symbionts, occurring mostly on the non-flagellated posterior-most region, and sometimes also between flagellar bands of *Leptospironympha* cells. The most commonly encountered type is rod-shaped, ranges from 1–3 μm long, has rounded ends (Figs 10–11) and a sporadic distribution. Another less common type is slightly longer but much narrower, and is often somewhat curved, appearing to follow the contour of the cell (Fig. 12). This latter type is found more densely covering the posterior end and between flagellar bands of some cells.

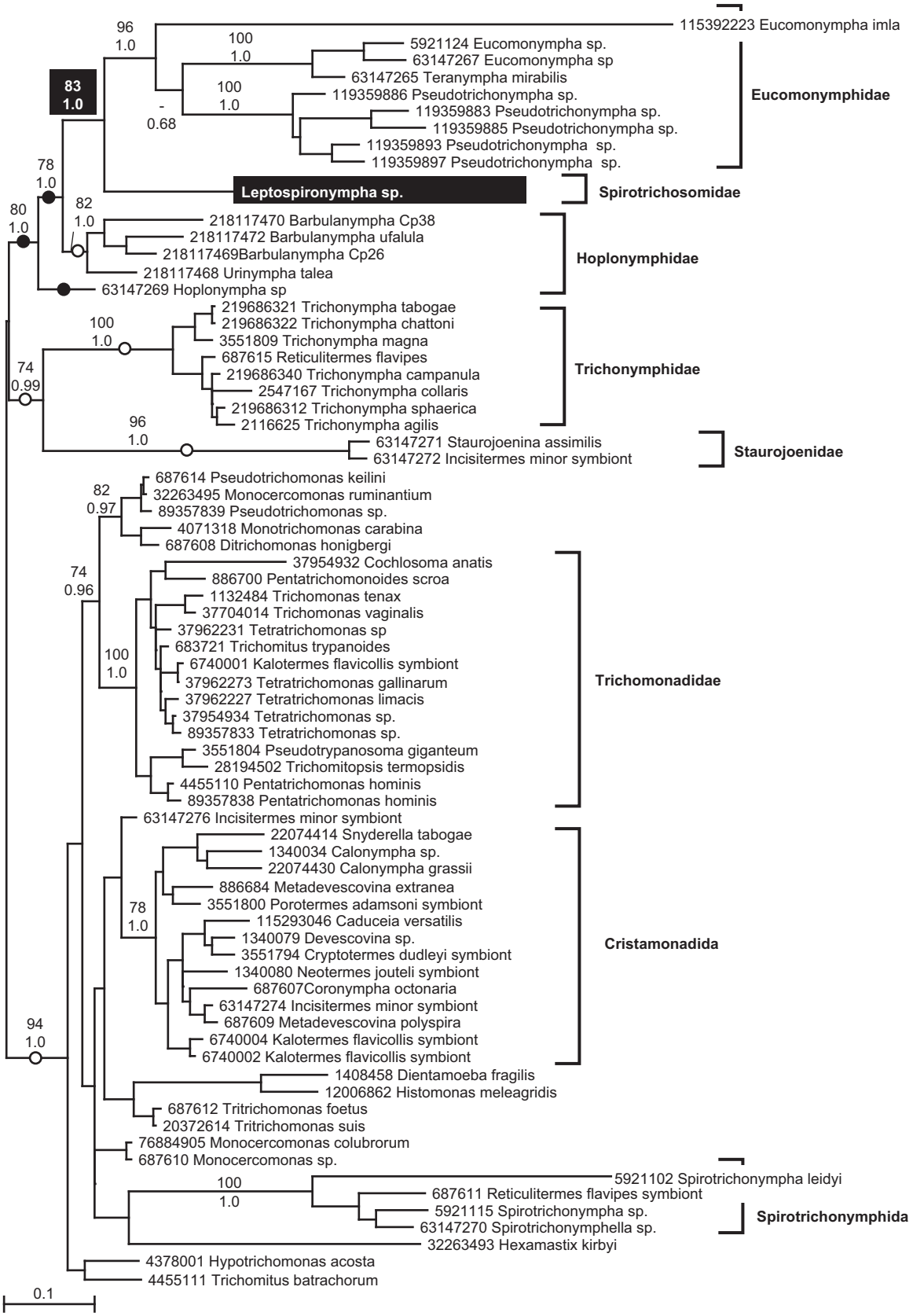
Several views of the parabasal fibers were observed in TEM. At the extreme anterior of the rostrum it was not observed, and only closely packed rows of basal bodies were seen (Fig. 16 arrowheads, Fig. 18). Slightly posterior to this in the rostral tube two rows of parabasal fibers were observed as striated bands, sharply bending at the point where the left-handed helix initiates (Fig. 18), from which point they spiral with regular spacing around the central nucleus (Fig. 16). Further to the posterior, tangential sections



Figures 10–15. SEM micrographs of *Leptospironympha* of *Cryptocercus punctulatus* showing symbiotic bacteria and surface details. **10.** Symbiotic surface bacteria between flagellar bands of *L. eupora* (Scale bar=5 μm). **11, 12.** Symbiotic surface bacteria on the posteriormost cell portion of *L. wachula* (Scale bars=3 μm and 2 μm respectively). **13.** Granular particles on the flagella of *L. eupora* (Scale bar=1 μm). **14.** Operculum of *L. eupora*, also showing flagella with a few granular particles (Scale bar=1 μm). **15.** Anterior view of *L. eupora* showing the operculum and flagella with granular particles (Scale bar=2 μm).



Figures 16–20. TEM micrographs of *Leptospironympha* of *Cryptocercus puntculatus*. **16.** Median longitudinal section of *L. eupora* showing operculum (o), rostral flagellar bands (rf), basal bodies (bb), nucleus (n), phagocytosed wood fragments (w) (Scale bar=2 μm). **17.** Transverse section of *Leptospironympha* sp. (likely *L. eupora*) showing post-rostral flagellar fascicles from spiraling flagellar bands (f), phagocytosed wood fragments (w), putative phagocytosed bacteria (b) (Scale bar=2 μm). **18.** Oblique, near medial longitudinal section of *Leptospironympha* sp. showing operculum (o), rostral flagellar bands (rf), basal bodies (bb), and rostral tube (rt) (Scale bar=1 μm). **19.** Transverse section of *Leptospironympha* sp. showing rostral flagellar fascicles from spiraling flagellar bands (f), and putative phagocytosed bacteria (b) (Scale bar=1 μm). **20.** Detail of Figure 17 showing post-rostral flagellar fascicles from spiraling flagellar bands (f), phagocytosed wood fragments (w), putative phagocytosed bacteria (b) (Scale bar=2 μm).



Figures 21. Maximum likelihood phylogeny of Parabasalia with an emphasis on Trichonymphida. Numbers at nodes correspond to ML bootstrap support (top) and Bayesian posterior probabilities (bottom). Circles at nodes correspond to alternative positions of *Leptospironympha* tested by AU-tests with filled circles being rejected at the 5% level, and open circles failing to be rejected (one alternative position, plus the position recovered in ML and Bayesian trees). Major groups are bracketed and labeled to the right.

through the parabasal fiber give the appearance of a tight mesh-like grid of fibrils (Fig. 19, arrowhead). In cross section, they form an array of stacked semi-circular fibrils terminating in an electron-dense plate situated on the flat side of the semi-circle (Figs 16, 17). The circular side of the parabasal fiber faces the interior of the cell and the nucleus, while the plate faces the exterior of the cell, and the rows of basal bodies form adjacent to the plate (Fig. 16). Basal bodies (9+0) transition to flagella (9+2) as they emerge from the cytoplasm, but they emerge into deep invaginations of the plasma membrane into the cell, so that the flagella are arranged in rows that sit within deep pockets or slots in the cell surface. The flagella themselves are often covered with small granular particles, with the distal ends often bearing a denser coating than the proximal ends (Figs 7, 9, 12, 13, and 15). All individuals were observed to have these granules on at least some flagella, however some had denser coatings than others. These granules were not observed on all other flagellates in the same preparations, but were also common on *Trichonympha* (Carpenter et al. 2009).

The cell body also contains numerous large and irregularly shaped objects (presumably wood fragments) within vacuoles (Figs 16, 17, 20); and irregularly shaped, darkly staining objects that resemble bacteria enclosed in vacuoles near the plasma membrane (Figs 17, 19, 20). A glycocalyx is evident covering the cell surface of nearly all individuals observed with SEM (Figs 1, 10–12), and is absent only on the operculum Figs 14, 15).

Phylogenetic Analysis

The criteria distinguishing *L. eupora* and *L. wachula* (mainly differences in the pattern of helical gyres, parabasals, and an overlapping range of cell size) are not sufficient to permit their separation under the conditions used for manually isolating cells. Accordingly, we manually isolated several live *Leptosprionympha* cells from *Cryptocercus*, but made no identification to the species level. Altogether SSU rRNA was sequenced from isolations consisting of five and one cell, and from environmental samples from cockroach hosts from Log Hollow. All sequences were nearly identical (sequences from the two manual isolations were 0.56% different, and the environmental sequence was 0.62% different from those of isolated cells), and there is no evidence that sequences from two distinct species were characterized, so the sequence from the single-cell manual identification was used for phylogenetic analyses (this sequence has been deposited in GenBank under accession GQ168515). The remaining sequences from the environmental surveys (the great majority of them) corresponded to other parabasalian genera known to exist in this cockroach and whose SSU rRNA has already been characterized (Carpenter and Keeling 2007; Carpenter et al. 2009; Ohkuma et al. 2008).

Likelihood and Bayesian phylogenetic methods consistently placed *Leptospironympha* as sister to a clade comprising the hypermastigote families Eucomonymphidae plus Teranymphidae (Fig. 21). This position was supported by 83% ML bootstrap and a posterior probability of 1. More broadly, this group falls within the strongly supported Trichonymphida (94% and PP of 1), as predicted by morphology-based classification schemes (Brugerolle and Lee 2000).

To test alternative positions, we carried out approximately unbiased tests where *Leptospironympha* was re-positioned at ten branches corresponding to the remaining major dichotomies within the Trichonymphida and two positions within the Eucomonymphidae. Overall, all these alternatives were rejected at the 5% level (filled circles in Fig. 21), except one position where *Leptospironympha* was the sister group to the genera *Barbulanympha* and *Urinympha* (open circle in Fig. 21).

Discussion

Morphology

Examination of protist gut symbionts with SEM often reveals the presence of previously undetected bacterial surface symbionts. Such associations have been found with other genera of order Trichonymphida from *Cryptocercus*, specifically *Eucomonympha* (Carpenter and Keeling 2007) and *Trichonympha* (Carpenter et al. 2009), with Trichonymphida in termites such as *Hoplonympha* (Brugerolle and Bordereau 2004; Noda et al. 2006) and with oxymonads (Hollande and Carruette-Valentin 1970; Leander and Keeling 2004; Rother et al. 1999). Likewise, in *Leptospironympha* we observed rod-shaped surface bacteria (Figs 10, 11) of sporadic distribution, which differ somewhat in appearance from those associated with other hypermastigotes in *Cryptocercus*. In having rounded ends, they differ from those on *Trichonympha*, which have tapered ends (Carpenter et al. 2009), from those on *Barbulanympha*, which

have sharply truncate ends (Carpenter and Keeling, unpubl. data), and from those on *Eucomonympha* by their larger observed size range (Carpenter and Keeling 2007). The less commonly occurring thin, curved bacteria are unlike anything we have previously observed in association with a protist in the *Cryptocercus* gut environment. These morphological differences among surface symbiotic bacteria may be significant, especially in light of evidence that many gut protist species each harbor a unique lineage of bacterial symbiont (Ikeda-Ohtsubo et al. 2007; Noda et al. 2005, 2006; Ohkuma et al. 2007; Stingl et al. 2005). It seems possible that at least one of the observed surface symbionts may represent a novel bacterial lineage specifically adapted to association with *Leptospironympha*.

With SEM, a thick, spongy surface texture—likely a glycocalyx—was observed on 39 of 41 *Leptospironympha* cells from four different *Cryptocercus* individuals representing three different populations (e.g., Figs 1, 10–12). However, at least one, or possibly two of the smallest individuals appeared to lack this (Figs 5, 13), and it was not observed in TEM (Figs 16–20). We have not observed a glycocalyx on other hypermastigote parabasalids of *Cryptocercus* (Carpenter and Keeling 2007; Carpenter et al. 2009) or any Trichonymphida in termites, nor do we know of any published reports of this in other hypermastigote parabasalids. It is possible that the glycocalyx was not preserved for one or two individuals we examined with SEM. However, the two individuals we observed that appeared to lack the glycocalyx were small (both less than 25 μm in length), so it may simply be absent in some or perhaps even all individuals of a smaller species, e.g., *L. eupora*. Further investigation of other representatives of Spirotrichosomidae with SEM will be necessary to determine whether this feature may have any phylogenetic significance.

We consistently observed small, darkly staining, irregularly shaped objects in vacuoles near the plasma membrane of all specimens. These are clearly distinct from the hydrogenosomes that are visible deeper inside the cell, but are within the right size range (1–2 μm) to be bacteria. If they are indeed bacteria, they may be endosymbionts, or they may be in the process of being enzymatically degraded, i.e., being utilized as food. The fact that endosymbiotic bacteria strongly resemble, and in fact are thought to be derived from ectosymbiotic bacteria in many cases (Bloodgood and Fitzharris 1976; Ohkuma 2008) argues against the former interpretation because the putative bacteria in vacuoles of *Leptospironympha* differ markedly in shape from their surface bacteria. The former have irregular, sometimes somewhat polygonal shapes, while the surface bacteria would appear to have uniformly circular cross sections, as suggested by SEM images. Thus, two explanations seem possible: that these bacteria are endosymbionts that have been greatly modified in appearance, or, perhaps more likely, they are simply being utilized as food.

Phylogeny

The placement of Spirotrichosomidae within a clade corresponding to Order Trichonymphida (Fig. 21) is in agreement with classifications based on morphology (Brugerolle and Lee 2000). It also supports the conclusion that the left-handed helical symmetry of Spirotrichosomidae evolved independently of the right-handed helical symmetry of the Spirotrichonymphida (Brugerolle and Lee 2000; Hollande and Carruette-Valentin 1971), which is in contrast to an earlier classification (Cleveland and Day 1958). Moreover, the association between Spirotrichosomidae and the Eucomonymphidae and Teranymphidae has not been predicted based on morphology or any other criteria. This association is relatively robust in our trees, and if these organisms are related, the group encompasses a wide range of morphologies—much greater than those of the other trichonymphid families Trichonymphidae, Staurojoeniidae, and Hoplonymphidae, whose members have fairly uniform plans of flagellar distribution and underlying cytoskeletal configurations (e.g., arrangements of basal bodies and parabasal fibers: Brugerolle and Lee 2000). All members of the putative Spirotrichosomidae-Eucomonymphidae-Teranymphidae clade are ultimately bilaterally symmetric, but there are three major subtypes of flagellar distribution: flagellar bands that extend in more or less straight lines from the rostrum to the post-rostral region, with an equal number of bands in both regions, as in Eucomonymphidae (Cleveland et al. 1934); flagellar bands that are straight in the rostrum but undergo helical coiling in the post-rostral region, as in Spirotrichosomidae (Cleveland and Day 1958); and flagellar bands that are separate and encircle the cell perpendicular to its anterior-posterior axis, as in Teranymphidae (Brugerolle and Lee 2000; Koidzumi 1921).

Interestingly, the variability in plans of flagellar distribution within Spirotrichosomidae itself is almost as wide as among the three families of this putative clade. Examining the drawings of

Cleveland and Day (1958), it is not hard to imagine how some of these configurations could have been transformed into those seen in Eucomonymphidae and Teranymphidae. The coiling of post-rostral flagellar bands is highly variable in the family, ranging from very tight coiling with a few dozen or more relational gyres (turns), to only a couple (as in the *Leptospironympha* of *Cryptocercus*). In two genera, *Apospironympha* and *Colospironympha*, short spiraling flagellar bands are only present in the anterior-most portion of the post-rostral region, and secondary bands, along which most flagella are organized, emerge from the anterior of these spirals and run parallel to the anterior-posterior axis of the cell (Cleveland and Day 1958). It is not difficult to imagine the transformation of such a body plan into that of *Eucomonympha* by the loss of the post-rostral spirals and retention of the secondary flagellar bands. Conversely, in species with tight coiling and many gyres, where the flagellar bands have the overall appearance of a tightly compressed spring, the flagella are arrayed largely perpendicular to the anterior-posterior axis (e.g., *Spirotrichosoma capitatum*, which has only two helical turns in their primary flagellar bands, before secondary bands such as those of *Apospironympha* and *Colospironympha* emerge from these, and these form tightly backed helical coils). If these flagellar bands were to disconnect, then a series of separate encircling flagellar bands might result, as in *Teranympha*. Thus, it seems quite possible that Spirotrichosomidae may turn out to be paraphyletic, with one or more described members more closely related to Eucomonymphidae or Teranymphidae than to other Spirotrichosomidae. Further sampling of SSU rRNA from other representatives of this family will be needed to test this hypothesis.

Methods

Location and source of *Leptospironympha*: We examined hindgut contents from individuals of *Cryptocercus punctulatus* collected from several different sites in the Appalachian mountains of the eastern US, generously provided by Christine Nalepa (North Carolina State University): Bear Trap Gap, North Carolina; Log Hollow, North Carolina; Mount Collins, Tennessee; Mountain Lake, Virginia; and South Mountains, North Carolina. GPS coordinates for these collection sites are given in (Everaerts et al. 2008; Nalepa et al. 2002).

Light and electron microscopy: Living material extracted from *Cryptocercus* hindguts was placed in Trager's Medium U (Trager 1934), and examined and photographed with a Zeiss Axioscope II using DIC Nomarski illumination. Material was fixed, prepared, and examined with SEM and TEM as previously described (Carpenter and Keeling 2007; Carpenter et al. 2008).

Single cell isolation, amplification of SSU rRNA, and phylogenetic analysis: Live cells were prepared as described for light microscopy. Individual cells matching the characteristics of *Leptospironympha* were identified using a Zeiss Axiovert 2 microscope, and manually isolated using a micropipette. A single lot of five cells and a single lot of one cell were isolated and DNA prepared as described previously (Keeling 2002). SSU rRNA was amplified using primers GCGCTACCTGGTTGATCCTGCC and TGATCCTTCTGCAGGTTCACCTAC (Keeling et al. 1998). Amplification conditions were 35 cycles of 30 sec at 95 °C, 30 sec at 45 °C, and 90 sec at 72 °C with an initial denaturing step of 95 °C for 60 sec and a terminal extension step of 72 °C for 90 sec. Products were isolated on agarose gels and those of the expected size (the only product) were cloned using TOPO TA cloning (Invitrogen) and sequenced on both strands, as described previously (Keeling 2002). The sequence from the single cell isolation has been deposited in GenBank under accession GQ168515. For environmental sequencing, DNA was prepared from whole gut contents, and PCR, cloning, and sequencing were carried out as described above.

Phylogenetic analysis: New sequences were added to an existing alignment (Carpenter and Keeling 2007; alignment available upon request), and phylogenetic relationships inferred using several methods. The maximum likelihood (ML) topology was inferred with RAxML 7.04 software (Stamakis 2006) using GTR+GAMMA model of evolution. To insure the search algorithm did not find a local optimum, we performed one hundred independent runs, each starting with different randomized maximum parsimony tree, and chose the topology with highest likelihood score. The branching support was assessed using ML bootstrap analysis (RAxML, GTR+GAMMA, 1000 replications) and Bayesian posterior probability values based on 1,000,000 generations and priors set to default using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Approximately unbiased (AU) tests were also performed to determine if other positions of *Leptospironympha* were not rejected by the data. For each alternative, we constrained the position of *Leptospironympha* to a node (shown in Fig. 21), reoptimized trees using ML under the conditions described above, and performed AU tests on all these alternatives using CONSEL (Shimodaira 2002; Shimodaira and Hasegawa 2001).

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