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Morphology, Phylogeny, and Diversity of *Trichonympha* (Parabasalia: Hypermastigida) of the Wood-Feeding Cockroach *Cryptocercus punctulatus*

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ABSTRACT. *Trichonympha* is one of the most complex and visually striking of the hypermastigote parabasalids—a group of anaerobic flagellates found exclusively in hindguts of lower termites and the wood-feeding cockroach *Cryptocercus*—but it is one of only two genera common to both groups of insects. We investigated *Trichonympha* of *Cryptocercus* using light and electron microscopy (scanning and transmission), as well as molecular phylogeny, to gain a better understanding of its morphology, diversity, and evolution. Microscopy reveals numerous new features, such as previously undetected bacterial surface symbionts, adhesion of post-rostral flagella, and a distinctive frilled operculum. We also sequenced small subunit rRNA gene from manually isolated species, and carried out an environmental polymerase chain reaction (PCR) survey of *Trichonympha* diversity, all of which strongly supports monophyly of *Trichonympha* from *Cryptocercus* to the exclusion of those sampled from termites. Bayesian and distance methods support a relationship between *Trichonympha* species from termites and *Cryptocercus*, although likelihood analysis allies the latter with Eucomonymphidae. A monophyletic *Trichonympha* is of great interest because recent evidence supports a sister relationship between *Cryptocercus* and termites, suggesting *Trichonympha* predates the *Cryptocercus*-termite divergence. The monophyly of symbiotic bacteria of *Trichonympha* raises the intriguing possibility of three-way co-speciation among bacteria, *Trichonympha*, and insect hosts.

Key Words. Anaerobic protists, bacterial symbionts, co-speciation, excavates, gut symbionts, lignocellulose recycling, protists, scanning electron microscopy, symbiosis, termites.

AMONG the most structurally complex and visually striking cells presently known are the hypermastigote parabasalids—flagellates that have undergone a spectacular adaptive radiation in the guts of wood-feeding insects, such as termites and the cockroach genus *Cryptocercus*, where they aid in the digestion of cellulose (Cleveland 1923, 1924; Ohtoko et al. 2000; Trager 1932; Yamin 1981). Like all parabasalids, the hypermastigotes are anaerobic or microaerophilic flagellates distinguished by the presence of hydrogenosomes, pleuromitosis with an external spindle, and a number of cytoskeletal synapomorphies (Brugerolle and Lee 2000). Traditionally recognized as Class Hypermastigida, the hypermastigotes differ from other parabasalids (Class Trichomonadida) by their generally larger size and a multiplication of flagella that arose without multiplication of the karyomastigont apparatus (i.e. they have a single nucleus). Hence, while some of the more complex Trichomonadida (e.g. calonymphids) are also large and structurally complex cells with many flagella, they have become so by multiplying the karyomastigont apparatus, and thus are multinucleate. Recent analyses of molecular data have called into question the monophyly of both Hypermastigida and Trichomonadida; it is possible that the trichonymphid and the spirotrichonymphid hypermastigotes arose independently from simpler trichomonad-like ancestors (Hampl et al. 2006; Ohkuma et al. 2005). Regardless of their origin however, their contribution to cellulose digestion in the guts of termites and *Cryptocercus* is of enormous ecological importance in terrestrial environments (Ohkuma 2003), and their morphological complexity and diversity make them equally interesting from an evolutionary perspective.

Trichonympha Leidy, (1877), a large, highly complex hypermastigote bearing hundreds or thousands of flagella, is notable among hypermastigote genera for its very wide distribution. Not only does *Trichonympha* have by far the widest distribution of any hypermastigote among termite hosts, occurring in numerous species of three of the four families that contain hypermastigotes (Kirby 1932, 1944), but it is also one of only two genera that is found in both termites and *Cryptocercus*, where there is considerable species diversity as well (Cleveland et al. 1934). Kirby (1932, 1944) believed it likely that the present-day distribution of *Trichonympha* is the result of its presence in the common ancestor of termites and *Cryptocercus*, from which it was inherited and transmitted vertically, with absence in some modern taxa explained as loss.

Various species of *Trichonympha* have been examined with light microscopy (Cleveland et al. 1934; Kirby 1932, 1944; Koidzumi 1921; Leidy 1877), but only a few species from termites have been examined at the ultrastructural level using transmission electron microscopy (TEM) (Grimstone and Gibbons 1966; Hollande and Carruette-Valentin 1971), and no *Trichonympha* species have been investigated with scanning electron microscopy (SEM). Similarly, the small subunit (SSU) rRNA gene has been sequenced from a handful of termite *Trichonympha* species, but only during the course of our study did a similar study report the first SSU rRNA sequences from one of the seven *Trichonympha* species proposed to exist in *Cryptocercus punctulatus* (Ohkuma et al. 2008). Here we have used SEM, TEM, and differential interference contrast (DIC) Nomarski light microscopy to examine the diversity of *Trichonympha* from several different populations of *C. punctulatus*. We have also sequenced the SSU rRNA gene from individually isolated cells of two species (*Trichonympha acuta* and *Trichonympha lata*), and conducted an intensive environmental PCR survey from several populations of the host to examine the monophyly and overall diversity of *Trichonympha* in *Cryptocercus*. Microscopy reveals a number of interesting new structural features of *Trichonympha*, and molecular data strongly support the monophyly of all *Trichonympha* species in *Cryptocercus* to the exclusion of *Trichonympha* species in termites—adding weight to Kirby's hypothesis (Kirby 1932, 1944) that *Trichonympha* was present in the ancestor of *Cryptocercus* and modern termites.

MATERIALS AND METHODS

Light and electron microscopy. *Cryptocercus punctulatus* collected from several sites in the Appalachian Mountain range of the eastern United States was generously provided by C. Nalepa (North Carolina State University). For light microscopy, live cells were diluted in Trager's Medium U (Trager 1934) and placed under cover glasses sealed with Vaseline. Cells were examined and

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photographed by DIC Nomarski microscopy using a Zeiss Axioscope II (Jena, Germany).

For SEM, we prepared and examined gut contents of eight different individuals of *C. punctulatus* from the following populations: Bear Trap Gap, NC; Log Hollow, NC; Mount Collins, TN; Mountain Lake, VA; Mount Pisgah, NC, and South Mountains, NC. Global positioning system coordinates for these collection sites are given in Everaerts et al. (2008) and Nalepa et al. (2002). Material for SEM and TEM was prepared and examined as described previously (Carpenter and Keeling 2007; Carpenter, Waller, and Keeling 2008).

Single cell isolation, amplification of SSU rRNA, and phylogenetic analysis. The SSU rRNA gene was characterized from two species, *T. acuta* and *T. lata*, by amplification from manually isolated cells. Gut contents of *C. punctulatus* were re-suspended in Trager's Medium U in round-bottom cavity slides. Cells matching the description of these species (Cleveland et al. 1934) were individually extracted from the medium and washed 3 times in clean medium. DNA was extracted and SSU rRNA gene amplified as described previously (Carpenter and Keeling 2007). For *T. acuta* and *T. lata*, the SSU rRNA gene was amplified from a pool of 40 cells, and also from two single cells individually. In all cases, multiple clones from each amplified band were completely sequenced.

Total DNA was also purified from the whole-gut contents of *C. punctulatus* from Log Hollow (two insects), Mount Pisgah (two insects), Bear Trap Gap, and Mount Collins (one insect each). The SSU rRNA gene was amplified from these DNAs, and bands corresponding in size to parabasalid SSU rRNA were cloned. Clones from each library were screened by digestion with *Alu*I, and representatives for all unique restriction patterns (a total of 79 clones) were end-sequenced. One or more clones representing each unique end-sequence type found to be closely related to *Trichonympha* were completed on both strands.

All new sequences were deposited in GenBank under accessions (awaiting accession numbers), and added to an existing alignment of parabasalid SSU rRNA (Carpenter and Keeling 2007), resulting in a matrix of 56 sequences and 1,232 sites (alignment available on request). Phylogenetic trees were inferred using Bayesian, maximum likelihood, and distance methods. Maximum likelihood trees and 1,000 Ml bootstrap replicates were inferred by PHYML 2.4.4 (Guindon and Gascuel 2003) using the GTR substitution model and rate between sites modeled on a gamma distribution with eight variable categories and invariable sites. The proportion of invariable sites (0.11) and the α shape parameter (0.597) were estimated from the data. Bayesian analyses were carried out using MrBayes 3.1 (Ronquist and Huelsenbeck 2003) with the same substitution and rate between sites parameters. One million generations were run with three hot chains and one cold chain sampled every 10,000 generations with a burnin of 100,000 generations. Distances were calculated using TREE-PUZZLE 5.2 (Schmidt et al. 2002) with the settings described for ML analysis and 1,000 distance bootstraps calculating using the shell script puzzleboot (by M. Holder and A. Roger: http://www.tree-puzzle.de). Trees were inferred from distances using weighbour 1.2 (Bruno, Socci, and Halpern 2000). Approximately unbiased (AU) tests (Shimodaira 2002) were carried out using CONSEL 1.19 (Shimodaira and Hasegawa 2001). All possible topologies of the test data set were generated using PAUP 4.0 beta 10.

RESULTS

Light and electron microscopy. Cleveland et al. (1934) reported seven species of *Trichonympha* in *C. punctulatus*, with a range of features visible in light microscopy. Using SEM we observed a similar variety of *Trichonympha* exhibiting approximately the same range of shape and size variation (ranging from approximately 30 μm long × 21 μm wide to 110 μm long × 90 μm wide) as in LM (Fig. 1–11). In general, with SEM it proved difficult to match the *Trichonympha* individuals we observed to the any of six species described from the Appalachian *Cryptocercus* (Cleveland et al. 1934). An exception to this is *T. parva* (Fig. 6, 8), which is much smaller than the other species (approximate length to width measures of 30 × 21 μm to 47 × 30 μm).

With SEM, we discovered several new features. First, a dense population of rod-shaped bacterial symbionts is present on the posterior non-flagellated feeding zone of individuals where this structure is visible and not obscured by flagella (Fig. 12–15). Surface bacteria of *Trichonympha* are narrow, fusiform, generally ranging from 0.5 to 2.0 μm in length, and generally do not resemble other surface symbionts reported from this environment (Carpenter and Keeling 2007). In LM a few individuals were also observed to have a uniform coating of bacteria over the operculum (Fig. 16, 17). This was not observed in SEM.

Second, *T. acuta* (and perhaps other species—see below) displays numerous (approximately 200), closely spaced, elongate projections or ''frills'' (approximately 2 μm × 0.2 μm) along the circumference of the operculum, which are also visible in side view with LM (Fig. 18–23). In some views these frills are downturned (Fig. 18, 20), while in others they radiate laterally from the operculum (Fig. 22). At least one *Trichonympha* species lacks these frills (Fig. 24, 25), however, we cannot determine which one(s), because most of the features that have been used previously to distinguish *Trichonympha* species present in *Cryptocercus* (Cleveland et al. 1934) are internal (e.g. length or parabasals, size and location of the nucleus) and are not visible with SEM. Only in the case of *T. acuta* could the species identification and the presence of operculum frills be unambiguously correlated (e.g. see Fig. 23 where the upturned frills are visible in a cell with the cup-shaped nucleus distinctive of *T. acuta*). We do note that most opercula observed in SEM bore frills, so we assume they are likely present on species other than *T. acuta*. In one *T. acuta* individual that was osmotically unbalanced, we also observed the frills in a highly distended operculum (not shown), confirming they are not structurally associated with the flagella. A minority of *Trichonympha* individuals were observed with SEM to be missing the outer operculum entirely. This was likely lost during specimen preparation for SEM, as individuals without this structure are never seen in LM. Loss of this structure reveals the inner operculum where the parabasal fibers originate, and radiating folds containing bands of rostral flagella. The whole assembly of the inner operculum and folds bears striking resemblance to the compressor fan of a jet engine (Fig. 26–28).

Third, in many individuals we observed several patterns of adherence of the post-rostral flagella (seen in SEM and TEM). In some species, the flagella emerging from a single flagellar fold adhere to one another laterally, forming ribbonlike structures (Fig. 29, 30, 35). In other species, flagella adhere individually to the post-rostral area (Fig. 31, 32), or adhere to both adjacent flagella and the post-rostral area (Fig. 33–36). In some individuals, thin flanges of ectoplasm running parallel to the long axis of the cell separate adjacent rows of rostral flagella. These structures, which we have termed *ectoplasmic flanges*, originate just below the rostrum on the upper portion of the post-rostral region (Fig. 35, arrows; Fig. 37, arrowheads). Some individuals also show a pattern at the posterior end of the cell in which flagella seem to terminate in distinct whorls (Fig. 36).

Transmission EM of the upper post-rostral region, immediately posterior to the rostrum, reveals much of the volume is composed of flagella and the ectoplasmic flanges separating adjacent rows of flagella (Fig. 37). The interior endoplasm occupies a relatively

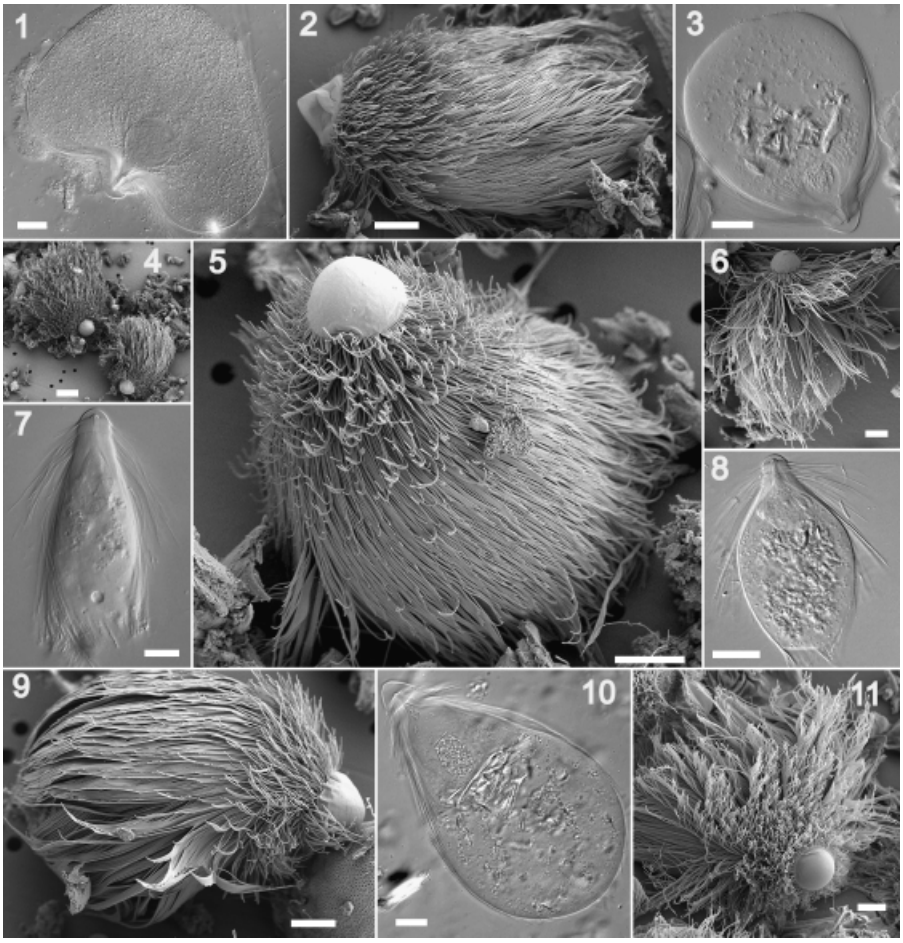

Fig. **1–11.** Scanning electron and light (differential interference contrast Nomarski illumination) micrographs of *Trichonympha* species from Appalachian populations of *Cryptocercus punctulatus*. **1**, **4**, **5**, **10**, **11.** *Trichonympha acuta*; **2.** likely *Trichonympha lata*; **3**, **7, 9.** *Trichonympha* spp.; **6, 8.** *Trichonympha parva*. Scale bars = 20 μm (Fig. 1, 4), 10 μm (Fig. 2, 3, 5, 7–11), and 5 μm (Fig. 6).

small volume at this level, and contains layers, from exterior to interior, of basal bodies, parabasal fibers, and a granular endoplasm filled with many hydrogenosomes (Fig. 37). The hydrogenosomes appear as irregularly circular to ovate or elliptical double membrane-bounded structures with densely staining granular interiors devoid of cristae or any other recognizable structure (Fig. 38).

Phylogenetic analysis of SSU rRNA. To determine the relationship between *Trichonympha* in termites and in *Cryptocercus*, we sequenced the SSU rRNA gene from two species (*T. acuta* and *T. lata*) that we could identify and manually isolate, and we also randomly sampled *Trichonympha*-like SSU sequences from many different populations of *Cryptocercus*. By far the most abundant *Trichonympha* species is *T. acuta*. It is also relatively large and easy to identify due to its distinctive cup-shaped nucleus (Cleveland et al. 1934) (Fig. 10). A second large species described by Cleveland et al. (1934) that is relatively distinctive due to its size and elongate shape is *T. lata* (Fig. 7). We also noted that *T. lata* also took on a swollen appearance in many cases (Fig. 1) and we also isolated such cells independently. For both species, we sequenced the SSU rRNA gene from three pools of isolated cells from three different hosts, one comprising 40 cells, and two comprising single cells. We sequenced six clones from each pool in the case of *T. acuta*, and from *T. lata* we completed six clones from the pool of 40 cells, and four and three clones from each of the single cells.

Cleveland et al. (1934) also identified four other species of *Trichonympha* in Appalachian populations of *Cryptocercus* and one restricted to Pacific coastal populations. Some of these species were described as being rare and/or infrequently found in any given individual host, and most are more difficult to distinguish without fixation and staining, all of which make manual isolation and identification problematic. Therefore, as a means of broadly sampling *Trichonympha* diversity, we also carried out extensive environmental sampling from four geographically distinct populations of *Cryptocercus*. Total parabasalid SSU rRNA diversity was sampled from two individual hosts from each of Log Hollow and Mount Collins, and one host from each of Bear Trap Gap and Mount Pisgah. A total of 79 clones were identified as potentially distinct by restriction screening, and end sequenced, from which 34 clones were completed. Sequences from both *T. acuta* and *T. lata* were identified abundantly in all four populations, with the exception that *T. acuta* was not sampled in either insect from Mount Collins. In addition, five other distinct sequences were found, for a total of seven clearly distinguishable taxonomic units. This suggests that the taxonomic diversity of *Trichonympha* in *Cryptocercus* is greater than the six species suggested by Cleveland et al. (1934). However, we cannot say whether there is really a correlation between our data and the species descriptions based on LM, except to point out that 79 sequences characterized from six host insects from four distinct populations is a reasonably deep survey of *Trichonympha* diversity. Therefore, unless the survey

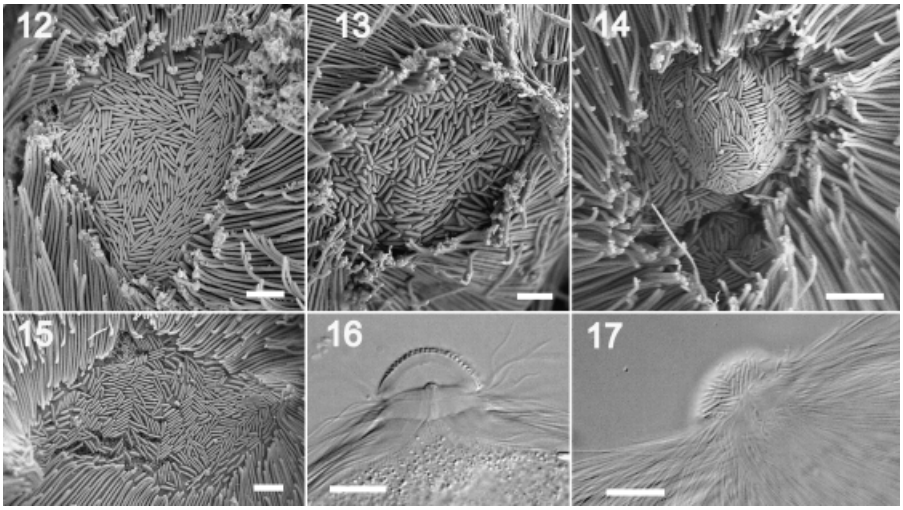

Fig. **12–17.** Scanning electron and light (differential interference contrast Nomarski illumination) micrographs of bacterial surface symbionts of *Trichonympha* from *Cryptocercus punctulatus* **12–15.** Scanning electron micrographs of rod-shaped surface bacterial symbionts on the non-flagellated posterior portion of the post-rostral area. **16, 17.** Light micrographs of rod-shaped surface bacterial symbionts on the outer operculum. Scale bars = 2 μm (Fig. 12, 13, 15), 3 μm (Fig. 14), and 10 μm (Fig. 17).

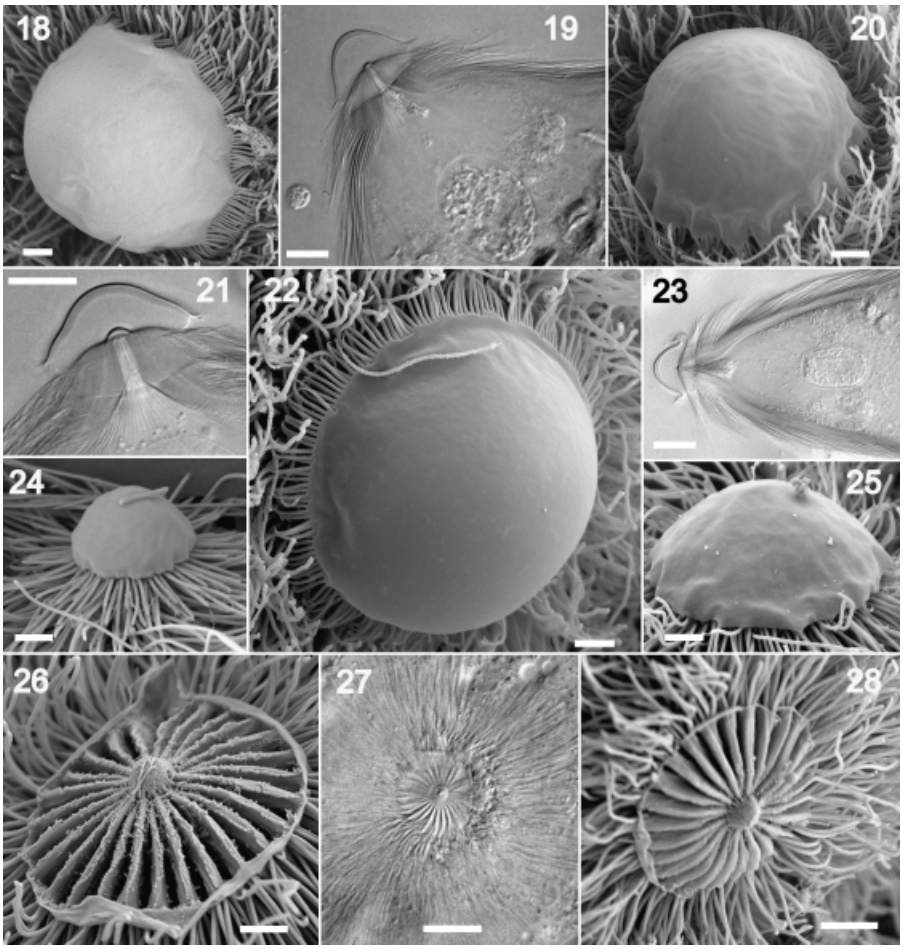

Fig. **18–28.** Scanning electron and light (differential interference contrast Nomarski illumination) micrographs of the inner and outer opercula of various *Trichonympha* species from *Cryptocercus punctulatus*. **18–23.** Scanning electron and light micrographs of individuals bearing frills along the circumference of the outer operculum. **24, 25.** Scanning electron micrographs of individuals lacking frills on the outer operculum. **26–28.** Scanning electron and light micrographs showing the inner operculum with radiating plates. Scale bars = 2 μm (Fig. 18, 22, 24–26), 10 μm (Fig. 19, 21, 23, 27), and 3 μm (Fig. 20, 28).

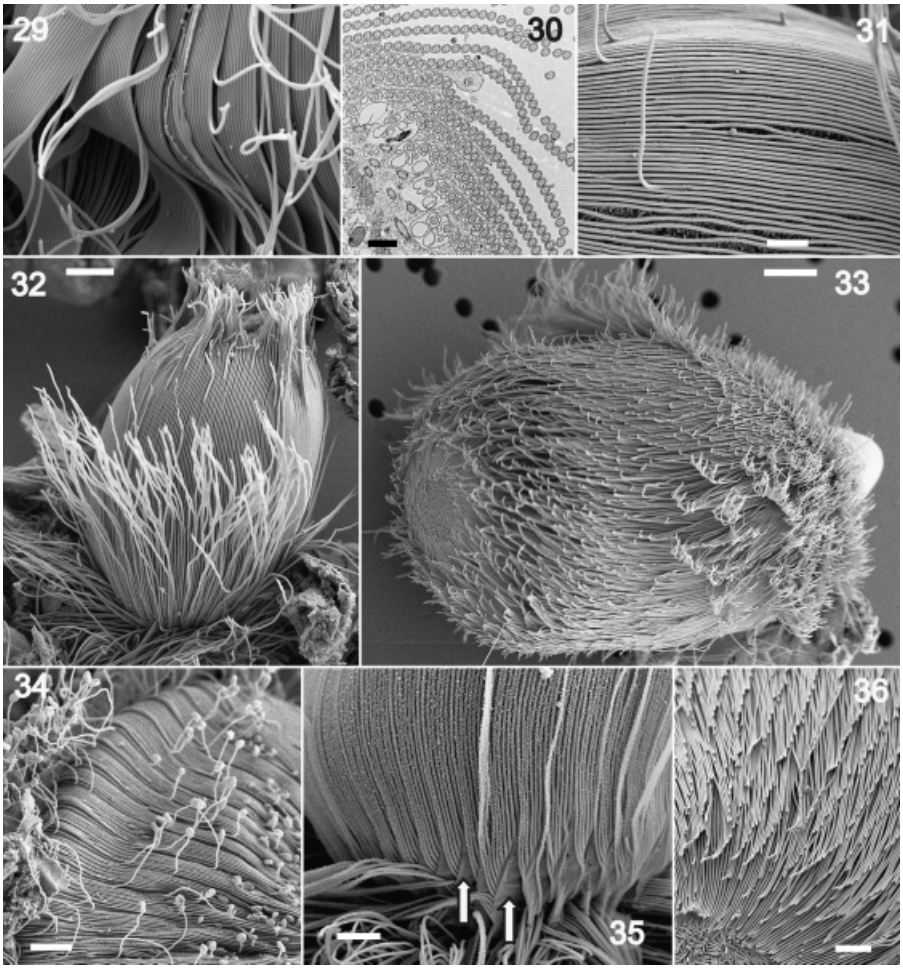

Fig. **29–36.** Scanning electron and transmission electron micrographs of flagella of *Trichonympha* from *Cryptocercus punctulatus*. **29.** Scanning electron micrograph showing bands of laterally adherent flagella in the post-rostral region. **30.** TEM micrograph showing bands of laterally adherent flagella. **31, 32.** Transmission electron micrographs showing flagella adherent to the post-rostral region. **33–36.** SEM micrographs showing post-rostral flagella adherent laterally to other flagella, and also adherent to the post-rostrum. Arrows on Fig. 35 point to ectoplasmic flanges. Swellings at the tips of flagella may be artifacts. Scale bars = 2 μm (Fig. 29, 31), 1 μm (Fig. 30), 5 μm (Fig. 32, 24), 10 μm (Fig. 33), and 3 μm (Fig. 35, 36).

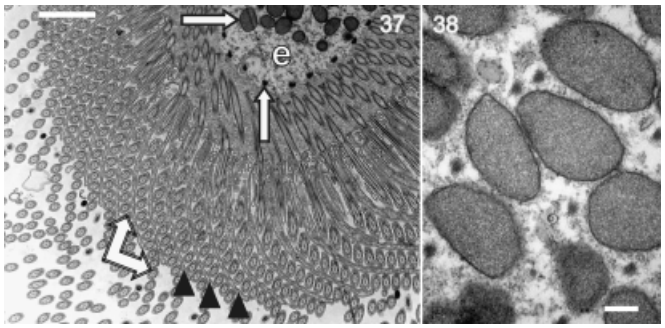

Fig. **37–38.** Transmission electron micrographs of various features of *Trichonympha* from *Cryptocercus punctulatus*. **37.** Transmission electron micrograph of approximately one half of a *Trichonympha* cell at a plane of section through the anterior portion of the post-rostral region. (Symbols: right-pointing arrow = hydrogenosomes; upward-pointing arrow = parabasal fiber; double-headed arrow = flagella; black arrowheads = ectoplasmic flanges; e = endoplasm.) **38.** Hydrogenosomes. Scale bars = 2 μm (Fig. 37) and 200 nm (Fig. 38).

was affected by strong biases, the likelihood of having missed a common species is low. In support of this, we noted all other groups of Parabasalia described to exist in *Cryptocercus* in our end-sequencing of over 100 non-*Trichonympha* like clones (data not shown).

The new sequences were aligned with known parabasalid SSU rRNAs, and the relationship of the *Trichonympha* species from *Cryptocercus* to *Trichonympha* from termites and other hypermastigotes in general was examined by phylogenetic reconstruction. Overall, the *Cryptocercus*-derived *Trichonympha* sequences formed a very strongly supported clade in all analyses (100% in all trees, Fig. 39), however different analyses disagree on its placement within Trichonymphida (represented here by members of Trichonymphidae, Hoplonymphidae, Staurojoeninidae, Eucomonymphidae). Bayesian and distance analyses unite the *Trichonympha* species from termites and *Cryptocercus*, but not with any support in either analysis. In contrast, maximum likelihood places the *Trichonympha* species from *Cryptocercus* within Eucomonymphidae with moderate support, and more specifically as sister to *Eucomonympha imla*, with no support (51%).

To compare the two topologies, we performed AU tests on all possible positions of *Trichonympha* species from *Cryptocercus*.

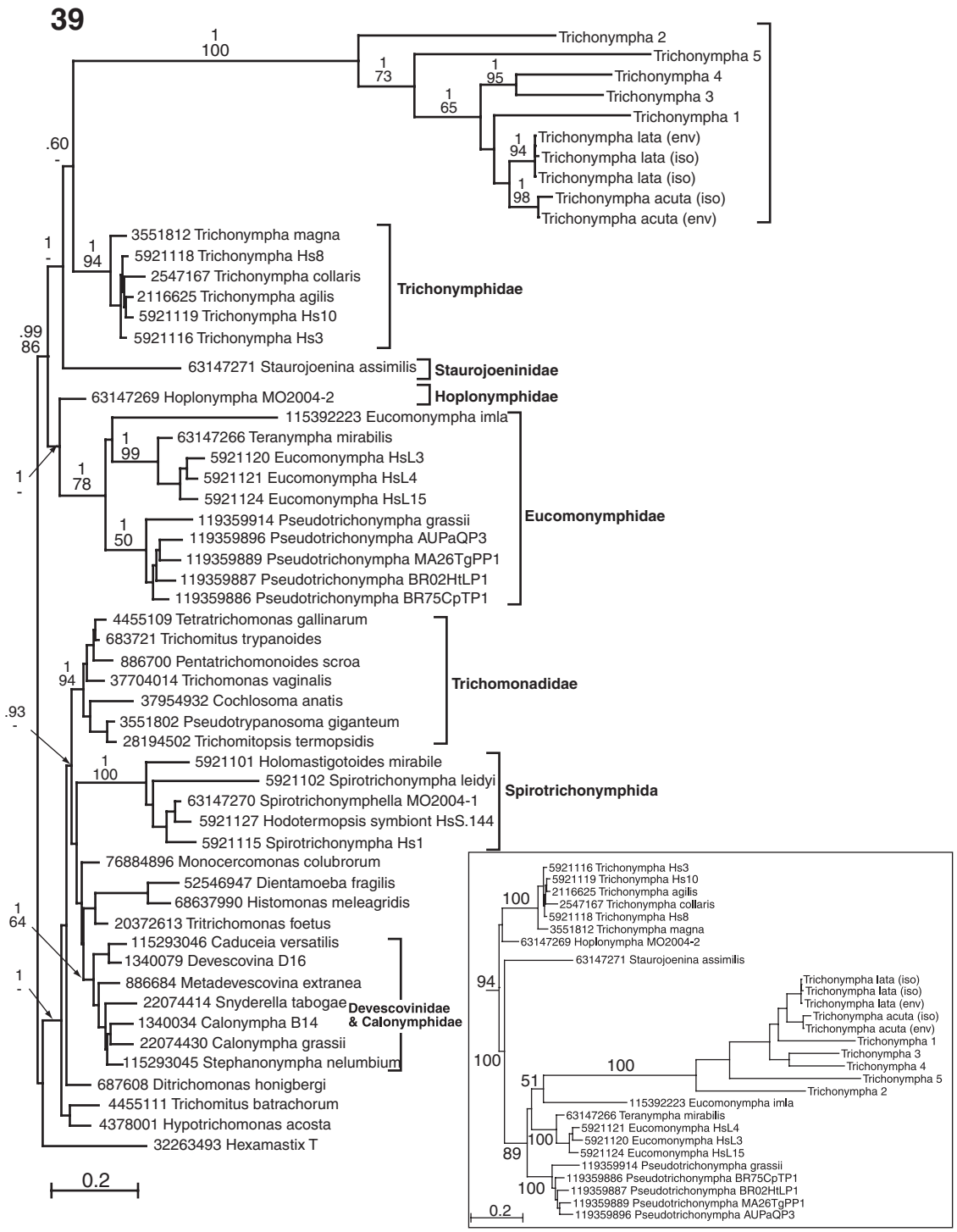

Fig. **39.** Phylogeny of the genus *Trichonympha* as inferred from Bayesian and distance analyses (see text). Inset: Topology obtained from maximum likelihood analysis. Species for which isolations were carried out are named and both the environmental sequence and one or two sequence derived from single cells are both shown (indicated by ''iso'' or ''env'' following the name). Sequences only known from environmental sampling are labeled *Trichonympha* followed by a number. The four environmental sequences recently reported by Ohkuma et al. (2008) correspond to our *Trichonympha acuta*, *Trichonympha lata* sequences, and environmental sequences 2 and 5. Numbers at nodes correspond to support over 50% from Bayesian posterior probabilities (top), distance bootstraps (bottom), or maximum likelihood bootstraps (inset).

We constructed a dataset consisting of two representative sequences of termite-derived *Trichonympha*, *Cryptocercus*-derived *Trichonympha*, Hoplonymphidae, *Pseudotrichonympha*, Staurojoeninidae, and *E. imla*. We constructed all 105 possible topologies of these six taxa, and performed AU tests (Shimodaira and Hasegawa 2001). Altogether, 26 topologies were not rejected at the 95% confidence level, and these trees included several that showed a monophyletic *Trichonympha* clade, and several that

showed *Trichonympha* species from *Cryptocercus* as sisters to *E. imla*, suggesting neither possibility is rejected by the data.

DISCUSSION

Bacterial symbionts of *Trichonympha*. Scanning EM reveals that *Trichonympha* harbors a dense population of rod-shaped surface bacteria, but only in narrowly defined regions of the cell: commonly on the extreme posterior (Fig. 12–15), and more rarely on the operculum (Fig. 16, 17). This is in contrast to earlier reports that *Trichonympha* is free of such surface symbionts, but contains only endosymbionts (Bloodgood and Fitzharris 1976). The discovery of *Trichonympha* surface bacteria adds to those of other protist gut symbionts of wood-feeding insects, such as parabasalids, including *Eucomonympha* (Carpenter and Keeling 2007), *Barbulanympha* and *Urinympha* (Bloodgood and Fitzharris 1976), and oxymonads (Carpenter et al. 2008; Rother, Radek, and Hausmann 1999). In *Trichonympha*, the bacteria are likely excluded from the flagellated area because the flagella adhering to or emerging from the body are very closely spaced, and do not allow sufficient room between them for bacteria to attach (Fig. 31–33). Several studies have provided evidence that different gut protist species each harbor a unique lineage of symbiotic bacteria (Ikeda-Ohtsubo et al. 2007; Noda et al. 2005, 2006; Stingl et al. 2005). This, combined with our observations that *Trichonympha* surface bacteria do not resemble those of other protists from the *Cryptocercus* gut environment, suggests that *Trichonympha* surface bacteria represent distinct lineages from surface bacteria of other *Cryptocercus* gut protists, and perhaps different among the *Trichonympha* species themselves. Rod-shaped bacterial ectosymbionts of many gut protist genera belong to the bacterial order Bacteroidales (Noda et al. 2006; Stingl et al. 2004), so it is possible that those of *Trichonympha* belong to this order.

Given that the relationship between prokaryotes of various types (e.g. spirochetes, rod-shaped bacteria, and Archaea) and their protist hosts appears beneficial, if not vital to one or both partners in such gut environments (Bloodgood and Fitzharris 1976; Ikeda-Ohtsubo et al. 2007; Noda et al. 2006; Ohkuma 2008; Stingl et al. 2005), it seems possible that the need for bacterial attachment sites may have played some role in shaping the morphology, especially the distribution of flagella, of complex hypermastigotes such as *Trichonympha*. That this has occurred in some gut protists is evident in the hypermastigote *Hoplonympha*, which has numerous vanes that accommodate such bacteria radiating outward from the cell body (Noda et al. 2006), as well as the oxymonad *Streblomastix*, which has similar structures (Hollande and Carruette-Valentin 1970; Leander and Keeling 2004). Nitrogen fixation and/or provision of essential nitrogenous compounds not obtainable from cellulose digestion seems a likely role for at least some of the bacteria in this relationship (Hongoh et al. 2008; Noda et al. 2006; Ohkuma 2008; Stingl et al. 2005; Yamin 1981). It has also been suggested that different gut protist species may differ in their requirements for such nitrogenous nutrients, thus influencing their associations with prokaryote symbionts (Ohkuma 2008). Therefore it seems reasonable to hypothesize that if some species of protists (e.g. *Barbulanympha*, *Urinympha*, which have flagella limited to the anteriormost portion of the cell) have greater needs for such nitrogenous compounds than others (e.g. *Trichonympha*, *Eucomonympha*, which have most or all of the body covered by flagella), then the need for surface bacterial attachment sites may limit the distribution of their flagella.

Morphological features and functions. With SEM, many different individuals of *Trichonympha* are seen to have many, if not the majority of their flagella, especially those originating from the post-rostrum, laterally adherent along much of their lengths, and/or seemingly tightly adherent to the post-rostral cell surface (Fig. 29–36). That the free beating of flagella is largely restricted to the generally free anterior flagella originating from the rostrum is consistent with the behavior of *Trichonympha* as observed in LM: the predominant movement of the rostrum is a complex bending from side to side, accompanied by rotation that follows the beat pattern of the flagella (Kirby 1932). In contrast, beating of post-rostral flagella appears to result in undulations of the plasma membranes. Interestingly, some individuals with adherent flagella show a fairly well-defined pattern of flagella terminating in successive and periodic whorls surrounding the post-rostral nonflagellated region (Fig. 36), reminiscent of euglenid pellicle strip termination (Esson and Leander 2006). This pattern corresponds to the order of flagellar emergence from the ectoplasmic folds, and their regular, periodic termination indicates the flagella are of highly uniform length.

The frills occurring on the circumference of the outer operculum in at least *T. acuta*, and possibly also other species, were visible in both SEM and DIC (Fig. 18–23). They are clearly extensions of the operculum surface and not flagella. This has also been confirmed by LM in cells where the operculum was grossly swollen. Although the distribution of this character is not entirely clear, it seems likely they may be diagnostic for a subset of *Trichonympha* species within *Cryptocercus* because they are clearly present on some species but not others.

Diversity, phylogeny, and host/symbiont co-speciation. Analysis of environmental SSU rRNA sequences from *Cryptocercus* shows considerable diversity: seven distinct environmental sequence types were identified in the Appalachian populations we examined, two of which corresponded to the two manually isolated species (*T. acuta* and *T. lata*). Previous estimates identified six species in these populations of *Cryptocercus* (Cleveland et al. 1934). The other recent report of *Trichonympha* SSU sequences from *Cryptocercus* identified four distinct sequences (Ohkuma et al. 2008), each of which corresponds to one of our sequences. The sequence shown by Ohkuma et al. (2008) to hybridize to *T. acuta* is virtually identical to our *T. acuta* sequence. Of the three unidentified sequences, Unidentified *Trichonympha* 05 is virtually identical to our *T. lata* sequence; Unidentified *Trichonympha* 07 is virtually identical to our *Trichonympha* 2; and Unidentified *Trichonympha* 21 is virtually identical to our *Trichonympha* 5. Overall, the environmental samples from a variety of host populations consistently suggest that the majority of *Trichonympha* SSU diversity has been sampled. This in turn suggests there are seven species in total in Appalachian populations of *Cryptocercus*—one more than the original estimate (Cleveland et al. 1934). Species delineation with SEM proved difficult, largely because the features used previously by Cleveland et al. (1934) to characterize species, such as length of parabasals and size and position of the nucleus, were not visible. Exceptions included *T. parva*, which is distinctly smaller than the other species (Fig. 6, 8), and *T. acuta*, which at its largest, is larger than any other species (Fig. 5), and for which operculum frills were identified in both LM and SEM.

Phylogenetic analyses of *Trichonympha* SSU rRNAs yielded two important results. First, Bayesian and distance methods recover a monophyletic *Trichonympha* (Fig. 39), a result also found in the other recent molecular study of the *Trichonympha* species of *Cryptocercus* (Ohkuma et al. 2008). In contrast, maximum likelihood trees consistently show *Trichonympha* from *Cryptocercus* to branch within Eucomonymphidae (Fig. 39, inset). Although this latter result cannot be ruled out at this point (several topologies with this relationship are not rejected by AU tests), we believe that the weight of evidence favors a monophyletic *Trichonympha*. First, *Trichonympha* from *Cryptocercus* has a very different body plan than the described members of

Eucomonymphidae. *Trichonympha* species from *Cryptocercus*, like those from termites, do not bear flagella on the entire surface of the body, and sometimes have a sizeable non-flagellated posterior portion—in some cases, over half of the length of the cell (Cleveland et al. 1934; Kirby 1932). In contrast, members of Eucomonymphidae (e.g. *Eucomonympha* and *Pseudotrichonympha*) bear flagella over the entire body surface, except for the tiny non-flagellated area represented by the operculum (Brugerolle and Lee 2000; Carpenter and Keeling 2007). Also, the rows of flagella and the parabasal fibers that underlie them are arrayed differently in Eucomonymphidae than in any of the *Trichonympha* species; the eucomonymphids have as many rows of flagella on the rostrum as on the post-rostrum (Brugerolle and Lee 2000), while *Trichonympha* species have half as many on the rostrum as the post-rostral area (Cleveland et al. 1934; Kirby 1932). Second, the maximum likelihood result unites two very long branches, *Eucomonympha imla* and *Trichonympha* from *Cryptocercus*, suggesting that it should be viewed with skepticism, especially given that such a result would require a massive degree of morphological convergence to explain the overall similarity of *Trichonympha* species.

The second important result of phylogenetic analysis is that, despite extensive environmental sampling and direct isolation, no *Trichonympha* SSU rRNA sequence from *Cryptocercus* falls within the clade of termite-derived *Trichonympha* in any analysis. Instead, all SSU rRNA analyses show the *Cryptocercus*-derived *Trichonympha* to be a strongly supported clade. Indeed, the sequences from this clade are quite divergent compared with other parabasalid SSU rRNAs, and share a number of distinguishing features at the level of the alignment. If the current sampling is representative of the natural diversity of *Trichonympha* in *Cryptocercus*, then the *Trichonympha* species in *Cryptocercus* and termites represent two independent lineages separated by a long period of isolation. This is important when viewed in light of recent phylogenetic analyses that place termites as the sister group to *Cryptocercus*, nested within a paraphyletic grade of cockroaches (Inward, Beccaloni, and Eggleton 2007; Ware et al. 2008); it leads to the intriguing hypothesis that the ancestor of modern *Trichonympha* was present in the common ancestor of termites and *Cryptocercus*, both of which have subsequently inherited *Trichonympha* through vertical transmission. This possibility was addressed by Ohkuma et al. (2008), but such a conclusion requires that the molecular sampling of *Trichonympha* in *Cryptocercus* is as close as possible to saturated. Given that different symbionts are differentially represented in different populations and individuals of *Cryptocercus*, both deep and broad sampling were necessary to recover the seven distinct sequence types we identified. It remains possible that the *Trichonympha* species of *Cryptocercus* are derived from within the termite *Trichonympha* lineage and that this result is not recovered because of the high level of divergence in *Cryptocercus*-derived *Trichonympha* species, or lack of sampling from termites, but so far there is no direct evidence to support this. Altogether, the deep divergence between the two clades of *Trichonympha* seems most likely due to an ancient split and subsequent independent evolution (i.e. no exchange of *Trichonympha* symbionts between termites and *Cryptocercus*). Because *Trichonympha* is one of the few genera that is shared between termites and *Cryptocercus*, and the only one that is diverse and widely distributed in both, this seems to be a realistic interpretation.

One last intriguing possibility that is emerging from various data is that a third group of organisms—specifically the bacterial symbionts of *Trichonympha*—may also share a congruent topology. Four different species of endosymbiotic bacteria of *Trichonympha* from four different termites have been shown to form a clade to the exclusion of all other symbiotic gut bacteria (Ikeda-Ohtsubo et al. 2007). If, as seems likely, the evolutionary history of *Trichonympha* mirrors that of its insect hosts to some degree, a similar co-diversification of the bacterial symbionts within *Trichonympha* would imply a pattern co-speciation permeating through three layers of symbiotic interactions. This type of complex co-speciation has been inferred for termites of the family Rhinotermidae with gut protists of the hypermastigote genus *Pseudotrichonympha* and their bacterial endosymbionts (Ikeda-Ohtsubo and Brune 2009; Noda et al. 2007). Whether this can be said to be a general phenomenon within the termite/*Cryptocercus* clade will depend in part on future investigations of the monophyly of the bacterial symbionts of *Trichonympha* from *Cryptocercus*.

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