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Morphology and Molecular Phylogeny of *Pseudotrichonympha hertwigi* and *Pseudotrichonympha paulistana* (Trichonymphea, Parabasalia) from Neotropical Rhinotermitids

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ABSTRACT. *Pseudotrichonympha* is a large hypermastigote parabasalian found in the hindgut of several species of rhinotermitid termites. The genus was discovered more than 100 years ago, and although over a dozen species have since been described, this represents only a small fraction of its likely diversity: the termite genera from which *Pseudotrichonympha* is known are all species rich, and in most cases their hindgut symbionts have not been examined. Even formally described species are mostly lacking in detailed microscopic data and/or sequence data. Using small subunit ribosomal RNA gene sequences and light and scanning electron microscopy we describe here the morphology and molecular phylogenetic position of two *Pseudotrichonympha* species: the type species for the genus, *Pseudotrichonympha hertwigi* from *Coptotermes testaceus* (described previously in line drawing only), and *Pseudotrichonympha paulistana* from *Heterotermes tenuis* (described previously based on light microscopy only).

Key Words. Diversity, DNA barcoding, intestinal symbionts, Isoptera, lower termites, Parabasalia, Rhinotermitidae, Teranymphidae, Trichonymphida.

PSEUDOTRICHONYMPHA is a large hypermastigote parabasalian belonging to the Teranymphidae (Cepicka, Hampl, and Kulda 2010) and found in the hindgut of several species of rhinotermitid termites. In 1910, the German biologist Max Hartmann investigated the protozoan symbionts of a then unnamed species of the termite genus *Coptotermes* (Isoptera, Rhinotermitidae) collected in Manguinhos, Rio de Janeiro, Brazil. Having been interested throughout his career in mechanisms of sexuality, Hartmann (1910) described three organisms inside that termite that he interpreted as the sexual stages and juveniles of a single species of protozoan, his *Trichonympha hertwigi*. The Italian zoologists Giovanni Battista Grassi and Anna Foà (1911) objected to Hartmann's classification of those protozoans as members of the genus *Trichonympha*. They also disagreed with his interpretation of the three morphotypes as sexual stages of a single species of protist, and redescribed the ''male'' and ''female'' forms of *T. hertwigi* as two new genera: *Pseudotrichonympha* and *Holomastigotoides*, respectively, though they provided no further morphological observations. Confusingly, in the original paper the correspondence of *Pseudotrichonympha* and *Holomastigotoides* to male and female forms was misprinted and given in reverse, a mistake that was corrected later (Grassi 1917). As for the ''juvenile'' forms, Grassi and Foà (1911) assigned them to a third new genus, *Spirotrichonympha*, but did not select the form seen by Hartmann as its type species. The *Coptotermes* host of *Pseudotrichonympha hertwigi* was named *Coptotermes hartmanni* by Holmgren (1911), but he failed to provide a formal description and as a consequence this name is now considered invalid (Snyder 1949). Subsequent detailed surveys of neotropical termite diversity have shown that all native species of *Coptotermes* in Brazil correspond to *Coptotermes testaceus* (RHS, unpubl. data), so the type species of *Pseudotrichonympha* is therefore most reasonably concluded to be a symbiont of *C. testaceus*.

Since the description of *P. hertwigi* many other species of *Pseudotrichonympha* have been described. Almost all are symbionts in rhinotermitid termites of eight genera: *Coptotermes*, *Heterotermes*, *Parrhinotermes*, *Prorhinotermes*, *Psammotermes*, *Termitogeton*, *Rhinotermes*, and *Schedorhinotermes* (Kitade and Matsumoto 1998; Noda et al. 2005; Yamin 1979), although *Pseudotrichonympha* has also been reported from the serritermitid genera *Serritermes* and *Glossotermes* (Cancello and DeSouza 2004; Costa-Leonardo and Kitayama 1991), and there is also one report of *Pseudotrichonympha* in *Rugitermes*, a kalotermitid termite (De Mello 1954a). Recent studies have made it clear that the diversity of *Pseudotrichonympha* is likely underdocumented, like that of many other genera of protists. Noda et al. (2007) revealed that the evolutionary history of at least some members of the genus *Pseudotrichonympha*, based on molecular phylogenies, reflects very closely not only that of their rhinotermitid termite hosts, but also that of their bacterial endosymbionts, which account for more than two-thirds of the total bacterial population in the termite's gut. Each of the termite species they investigated was found to harbor only one *Pseudotrichonympha* species, as defined by >98% small subunit (SSU) ribosomal RNA (rRNA) gene sequence identity among clones, and the phylogeny of *Pseudotrichonympha* species was fully congruent with the phylogeny of the host termites. Of the 15 *Pseudotrichonympha* ''species'' investigated by Noda et al. (2007), only three have been formally named.

Here we use light microscopy (LM), molecular methods, and the first use of scanning electron microscopy (SEM) to investigate the *Pseudotrichonympha* symbionts of two species of rhinotermitid termites collected in Colombia. One of the organisms that we investigate is the type species of the genus, *P. hertwigi* from *C. testaceus*, while the other is *Pseudotrichonympha paulistana* described from *Heterotermes tenuis* (De Mello 1954b).

MATERIALS AND METHODS

Host termite collections. Termites were collected in May and June of 2009 in the framework of a faunistic study of the isopterans of the Caribbean Basin. Rhinotermitid termites of the genera *Heterotermes* and *Coptotermes* were collected above the town of Minca in the Sierra Nevada de Santa Marta, Magdalena, Colombia (11.1256°N, 74.11972°W). Collected specimens were

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subsequently identified by morphology and DNA barcoding (mitochondrial SSU [mt SSU] rRNA gene).

Light and SEM. Whole termite guts were dissected, submerged in Trager medium U (Trager 1934), and macerated on glass slides. Light microscopy was performed on living cells using a Zeiss Axioplan 2 compound microscope (Zeiss, Oberkochen, Germany) with Plan Apochromat objectives and differential interference contrast (DIC) optics. Image capture was carried out using a Q-imaging Micro Imager II digital camera or using a Canon XL-M1S (Canon, Tokyo, Japan) for HD video, from which stills were captured and live video archived.

For SEM, whole gut contents were diluted approximately 1:1 with medium U, and prefixed with OsO_4 vapor for 30 min. The prefixed material was transferred to small containers with a floor that consisted of a 10-μm polycarbonate membrane filter (Corning Separations Division, Acton, MA) and fixed for 30 min with a mixture of 8% (v/v) glutaraldehyde and 4% (w/v) OsO_4, giving a final concentration of 2.5% glutaraldehyde and 1% OsO_4. Samples were postfixed with a few drops of 4% OsO_4 for 30 min and washed 3 times in medium U to remove the fixative. Samples were dehydrated using a graded ethanol series and critical point dried in a Tousimis Sandri 795 CPD (Rockville, MD). The dried filters carrying the cells were mounted onto aluminum stubs and sputter-coated with 5-nm thick gold (Cressington High Resolution Sputter Coater, Cressington Scientific Instruments Ltd., Watford, UK). The SEM used to view the samples and take the photographs was a Hitachi S4700 (Hitachi, Tokyo, Japan).

Molecular methods. *Pseudotrichonympha* cells were isolated by micropipette using a Zeiss Axiovert 2 microscope and isolated cells were photographed using a QImaging MicroImager II camera (data not shown). DNA was isolated using the Epicentre Masterpure Complete DNA and RNA purification kit (Madison, WI). Nearly full-length SSU rRNA gene sequences were amplified using eukaryote-specific primers 5′-TGC GCT ACC TGG TTG ATC CTG CC-3′ and 5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′ as described previously (Harper et al. 2009). Polymerase chain reaction (PCR) products were separated by agarose gel electrophoresis, cloned using the StrataClone PCR Cloning Kit (Stratagene, Mississauga, ON), and sequenced on both strands using BigDye Terminator v 3.1. For symbionts from *C. testaceus*, four independent SSU rRNA gene PCR products from isolations of two cells and four cells from one termite individual and eight cells and 15 cells from another termite were cloned, and one clone from each reaction was sequenced on both strands. A contig assembled from these sequences contained eight single nucleotide disagreements and two ambiguous positions where the two clones from one termite shared a different nucleotide from the two clones of the other termite. The greatest number of disagreements from the consensus observed in a single clone was three, from the two-cell PCR product, the other clones showed just one or two differences. The SSU rRNA gene clone from the four-cell isolation, which differed from the consensus at only one position, was chosen to represent the species for phylogenetic analyses and for submission to GenBank. For symbionts of *H. tenuis*, DNA was extracted from one pool of 20 cells as well as from the entire gut contents of another *H. tenuis* individual. Two clones of SSU rRNA gene PCR products from the isolated cells and one environmental clone from the whole gut contents were sequenced. One clone from the isolated cells was found to be very similar to the environmental clone (five mismatches between them), while the second clone from the isolated cells was found to be very similar to the existing unidentified *H. tenuis* whole gut sequence in GenBank (AB262491: three mismatches between them, Fig. 1). The distance between these two pairs was greater, at 66 nucleotide differences. The clone from the isolated cells that was most dissimilar to the existing GenBank sequence (isolate clone 1, Fig. 1) was submitted to GenBank as *P. paulistana* from *H. tenuis*.

In order to confirm the identity of the host termites, partial mt SSU rRNA gene sequences were amplified using the primers 5′-TTA CGC TGT TAT CCC TAA-3′ and 5′-CGC CTG TTT ATC AAA AAC AT-3′ (Kambhampati and Smith 1995; Simon et al. 1994) from genomic DNA extracted using the Epicentre Masterpure Complete DNA and RNA purification kit. Polymerase chain reaction products were sequenced directly on both strands. New sequences determined in this study were deposited in GenBank under accessions HQ683705–HQ683708.

Molecular phylogenetic analyses. The identities of *C. testaceus* and *H. tenuis* were determined by BLAST similarity and confirmed by phylogenetic analysis of their mt SSU rRNA gene sequences, because this gene has been the most commonly used barcode for lower termites (e.g. see Scheffrahn et al. 2004; Szalanski et al. 2004). All available *Coptotermes* and *Heterotermes* mt SSU rRNA gene sequences and select *Reticulitermes* sequences with high BLAST similarity were downloaded from GenBank, aligned with our new sequences using MAFFT (Katoh et al. 2002), and refined by eye. To reduce the number of taxa included, sequences were removed from the alignment if they were both identical to and from the same collection location as other *C. testaceus* or *H. tenuis* sequences. Identical sequences from other species were removed whether from the same location or not. Finally, a few annotation changes were made: AY558901 was changed from *Coptotermes crassus* to *C. testaceus* for clarity as these species are synonymous (Scheffrahn et al. 2011); AY558902 was changed from *Coptotermes* sp. to *Coptotermes sjoestedti* (Scheffrahn et al. 2004); AY380297, erroneously attributed to *Heterotermes convexinotatus* in GenBank, was corrected to *Heterotermes cardini* (Szalanski et al. 2004); and AY558898, erroneously attributed to *Coptotermes vastator* but in fact a contaminating sequence from *C. testaceus*, was removed from the analysis. The final, manually edited mt SSU barcode alignment includes 61 taxa and 371 sites.

Phylogenetic analyses of termite barcodes were carried out using maximum likelihood, distance, and Bayesian methods. The HKY+G+I substitution model was chosen as the best fit by Modelgenerator (Keane et al. 2006) under the uncorrected Akaike information criterion 2 and the Bayesian information criterion. Maximum likelihood phylogeny estimation was carried out using PHYML v. 3.0 (Guindon and Gascuel 2003) with the rate parameter alpha, nucleotide frequencies, transition to transversion ratio, and proportion of invariable sites specified by Modelgenerator, and using four γ-distributed rate categories, on 500 bootstrap replicates. The distance-based analysis used SEQBOOT to generate 500 bootstrapped data sets, PUZZLEBOOT (shell script by A. Roger and M. Holder, http://www.tree-puzzle.de) to call TREE-PUZZLE (Strimmer and von Haeseler 1996) to compute distance matrices for each data set using parameters estimated by Modelgenerator, NEIGHBOR to construct phylogenies using the neighbor-joining algorithm, and CONSENSE to compute the 50% majority rule consensus tree. SEQBOOT, NEIGHBOR, and CONSENSE are part of PHYLIP v. 3.69 (Felsenstein 2005). The Bayesian analysis used MRBAYES v. 3.1 (Ronquist and Huelsenbeck 2003). Two independent chains were run for five million generations each, saving each 100th tree. The first 25% of saved trees was discarded as burn-in, and the remaining 75,000 trees were used to compute a 50% majority rule consensus tree. The runs converged (i.e. the average standard deviation of partition frequency values between the chains dropped below 0.01) after 1,180,000 generations. Both phylogenies and BLAST analyses gave consistent termite species identifications.

Pseudotrichonympha SSU rRNA gene sequences were added to an existing alignment of parabasalian diversity (Carpenter, Horák, and Keeling 2010), and re-aligned using SINA Webaligner (http://www.arb-silva.de/aligner), which aligns the query sequences to a

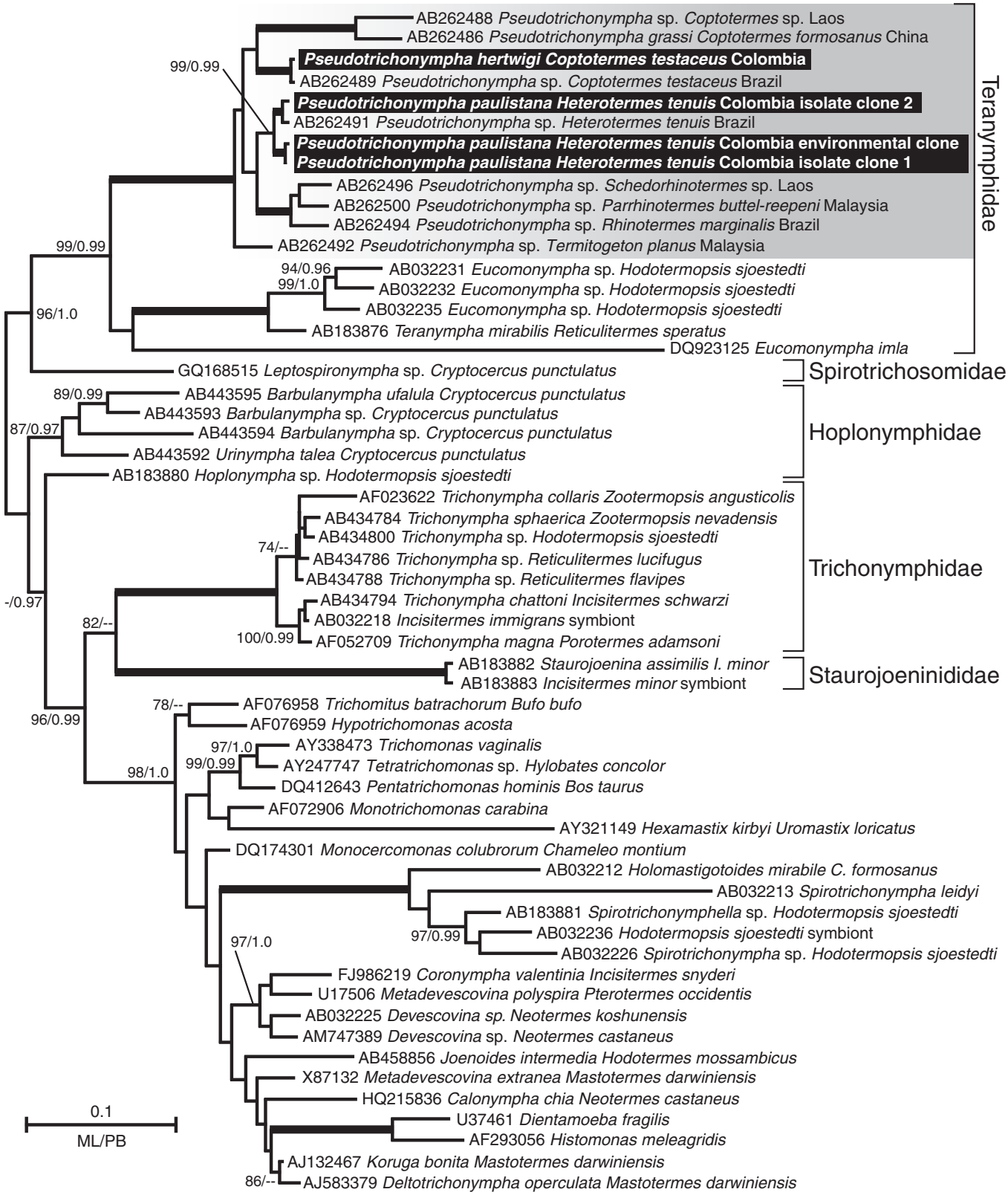

Fig. **1.** Maximum likelihood (ML) phylogeny of parabasalian flagellates based on nuclear small subunit (SSU) rRNA gene sequences, with an emphasis on the Trichonymphida, and showing the phylogenetic relationships of *Pseudotrichonympha paulistana* and *Pseudotrichonympha hertwigi*, which are indicated by white text in a black box. Families within the Trichonymphida are bracketed and named on the right, and the *Pseudotrichonympha* clade is indicated by gray shading. Support for nodes from 500 ML bootstrap replicates and Bayesian posterior probabilities are indicated (in that order) where ML bootstrap values are $>70\%$ and the posterior probability is >0.95. Nodes with thick lines indicate complete support from both methods (100/1.0).

''backbone'' curated SSU rRNA alignment. Ambiguous sites were manually removed using SeaView 4 (Gouy, Guindon, and Gascuel 2010), leaving 1,279 sites in the final alignment. Maximum likelihood phylogeny was inferred using RAxML 7.2.5 (Stamatakis 2006) using the GTR-GAMMA model of evolution, as specified by Mr AIC (Nylander 2004). Support was inferred from 500 bootstrap replicates. Bayesian posterior probabilities were computed with Phylobayes 3.2 (Lartillot, Lepage, and Blanquart 2009), using the Dirichlet process mixture of components, weights, and profiles combined with exchange rates as defined by the GTR model (-gtr -cat option). Two independent chains were run for 250,000 cycles each, saving every 10th tree, at which point the maxdiff value (largest discrepancy between the frequencies of bipartitions between the two chains) was 0.09. The first 25% of saved trees was discarded as burn-in, and the remaining 37,500 trees from both chains were used to compute a 50% majority rule consensus tree.

RESULTS

Termite identification by DNA barcoding. The two Colombian termites were determined to be *H. tenuis* (Hagen) and *C. testaceus* (L.) based on sequence similarity with existing mt SSU rRNA barcodes (Fig. 2). Our *C. testaceus* barcode shares 99% sequence similarity, based on BLAST alignments, with existing *C. testaceus* barcodes from the Caribbean, and is equally distant from known *C. testaceus* barcodes as they are from one another. Our *H. tenuis* sample is more distantly related to existing *H. tenuis* barcodes from the Caribbean than they are to one another. However, at 96–97% similarity, it is considerably closer to the Caribbean *H. tenuis* sequences than to any other species; the next closest species is *Heterotermes longiceps* at only 91% similarity (Fig. 2). There are no other data to suggest that the Colombian isolate represents a cryptic sister species to *H. tenuis*, and Constantino (2002) has shown that *H. tenuis* is found throughout northern South America, so unless such data emerge we are conservatively identifying our Colombian sample as *H. tenuis* and interpreting the sequence divergence as a result of geographical variation.

Morphology and phylogeny of *Pseudotrichonympha hertwigi*. *Pseudotrichonympha* from *C. testaceus* was described by Hartmann (1910) and emended by Grassi and Foà (1911) before the termite was formally described, and when the *Pseudotrichonympha* SSU rRNA was isolated from *C. testaceus* by Noda et al. (2007), the circuitous connection between this termite and the one studied by Hartmann, Grassi, and Foà was not yet made. During the course of this study it became evident that this is the type species of *Pseudotrichonympha*, and the data that we provide using LM, SEM, and molecular methods therefore serve to characterize it much more thoroughly than it was previously, in particular by providing an unambiguous molecular marker.

Pseudotrichonympha hertwigi cells are very large, 253.2 ± 26.6 μm in length and 96.6 ± 14.2 μm in width ($n = 6$), plastic, and completely covered in flagella that fall into three zones: short anterior flagella, long postrostral flagella, and uniform intermediate-sized flagella covering the main cell body, in agreement with the original description of the ''male'' form of *T. hertwigi* (Hartmann 1910, Fig. 3–7). Some early line drawings of *Pseudotrichonympha*, although not those of Hartmann (1910) or De Mello (1954a, b), misinterpreted these flagellar zones by depicting the most anterior zone as having very long flagella (e.g. Cleveland et al. 1934, Fig. 432 in plate 59, and Brugerolle and Lee 2000, Fig. 49B). From LM one can see the origin of this misunderstanding, because the short anterior flagella followed by very long flagella can appear to be long flagella lying flat on the surface of the cell (e.g. Fig. 4, 9). The cell size ranges given by Hartmann (1910), however, appear to contain an error. The cell length range is stated as 760–330 μm, but the stated magnification of the line drawings indicates cells of 208, 261, and 278 μm in length, which fall within our observed range (Hartmann 1910). We suspect therefore that 760–330 μm is a typographical error that was meant to read 160–330 μm.

The rostral cap of *P. hertwigi* was consistently observed in both LM and SEM to be pointed in shape (Fig. 3–7), as was the zone around the rostral tube (Fig. 4, 5). Diagrams in Hartmann (1910) are inconsistent on this point, some showing a pointed rostral cap as we observe (e.g. Fig. 15, 17, and 21 in Hartmann 1910), and others showing it rounded with a bulbous tip (e.g. Fig. 18 in Hartmann 1910). The rostral tube tends to be short in proportion to the rest of the body, at about 5% of the total body length (Fig. 4, 6). Hartmann's (1910) diagrams also show a short rostral tube, indeed even shorter by proportion to the length of the cell.

The SSU rRNA gene sequence was characterized from four batches of manually isolated *P. hertwigi* cells. Phylogenetic reconstruction shows that it forms a clade with a previously unidentified *Pseudotrichonympha* sequence from a *C. testaceus* from Brazil, where Hartmann collected his termites, distinct from all other *Pseudotrichonympha* sequences (Fig. 1).

Phylogeny and morphology of *Pseudotrichonympha paulistana*. Barcoding demonstrates that the *Heterotermes* we collected in Colombia is most closely related to *H. tenuis*, though we note that its distance from existing *H. tenuis* specimens is greater than we observed for *C. testaceus*. The symbiont fauna of *H. tenuis* in Brazil was investigated 50 years ago, and the *Pseudotrichonympha* symbiont observed there was named *P. paulistana* (De Mello 1954b). No SSU rRNA sequences exist for the *P. paulistana* described by De Mello (1954b), but Noda et al. (2007) did obtain SSU rRNA data from a *Pseudotrichonympha* sp. in a Brazilian *H. tenuis*. From our manually isolated *Pseudotrichonympha* cells from Colombian *H. tenuis* we have identified a sequence that is very closely related to the sequence determined by Noda and colleagues (Fig. 1). Interestingly, however, a second related cluster of sequences was also identified from isolated cells and whole guts of from Colombian *H. tenuis* (Fig. 1). It is unfortunately not possible to link these genotypes to morphological variation based on the present data. Because all sequences from *H. tenuis* form a distinct and strongly supported cluster in the tree, we conservatively conclude that these sequences are all derived from a single species, *P. paulistana*, and consider this intraspecific variation. However, this does not exclude the possibility that the distinct genotypes represent two *Pseudotrichonympha* species in the same termite (see ''Discussion'').

The overall structure of *P. paulistana* is similar to other *Pseudotrichonympha* species: cells are large, at 147.2 ± 18.0 μm in length and 51.9 ± 9.0 μm in width ($n = 13$), and tapered at the posterior end (Fig. 8, 9, 10). Cells are entirely covered in flagella except for a very small nonflagellated section at the posterior end (not shown), and the rostral cap (Fig. 12). The rostral cap is a hemispherical structure in live cells that can appear flattened in dehydrated SEM preparations (Fig. 8, 12). Under the outer cap there is an inner one similar to structures described in other species (Fig. 9, 10).

A rostral tube is located immediately under the inner cap (Fig. 9, 10) and runs for about 10% of the total length of the cell. Flagella emerge from around the rostral tube in very high numbers, and SEM reveals that the flagella emerging from the anterior section of this region are much shorter than elsewhere on the surface of the cell; only 2–3 μm long at a maximum (Fig. 8, 12). The flagella emerging immediately posterior to the rostral tube are by contrast ~ 12 μm long, and indeed are longer than the flagella emerging from the main portion of the cell (Fig. 8, 12). These

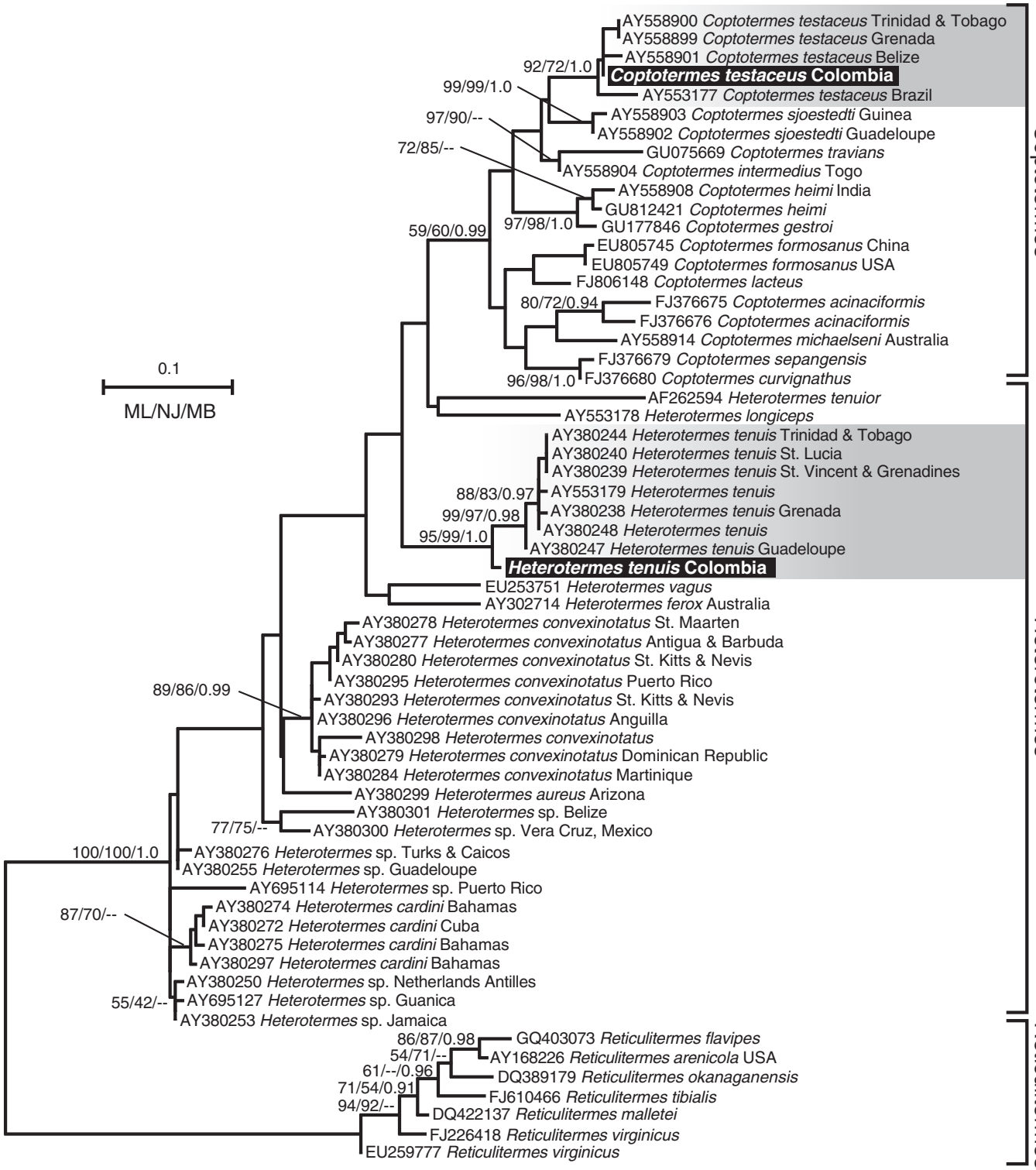

Fig. **2.** Maximum likelihood (ML) phylogeny of partial mitochondrial small subunit (mt SSU) ribosomal RNA gene sequences from termites. Sequences obtained in this study are indicated by white text in a black box. Major subgroups are bracketed and named on the right, and clades of primary interest are indicated by gray shading. All available nonidentical *Coptotermes* and *Heterotermes* sequences are included, plus all available *Coptotermes testaceus* and *Heterotermes tenuis* sequences, except where identical and from the same location (see ''Materials and Methods'' for details and exceptions). Select *Reticulitermes* sequences were chosen as an outgroup because *Reticulitermes* has been shown to branch sister to a clade of *Coptotermes* and *Heterotermes* (Inward et al. 2007; Lo et al. 2004). Support for nodes from 500 ML and 500 neighbor-joining bootstrap replicates and Bayesian posterior probabilities are indicated (in that order) where ML and distance bootstrap values are $>50\%$ and the posterior probability is >0.95.

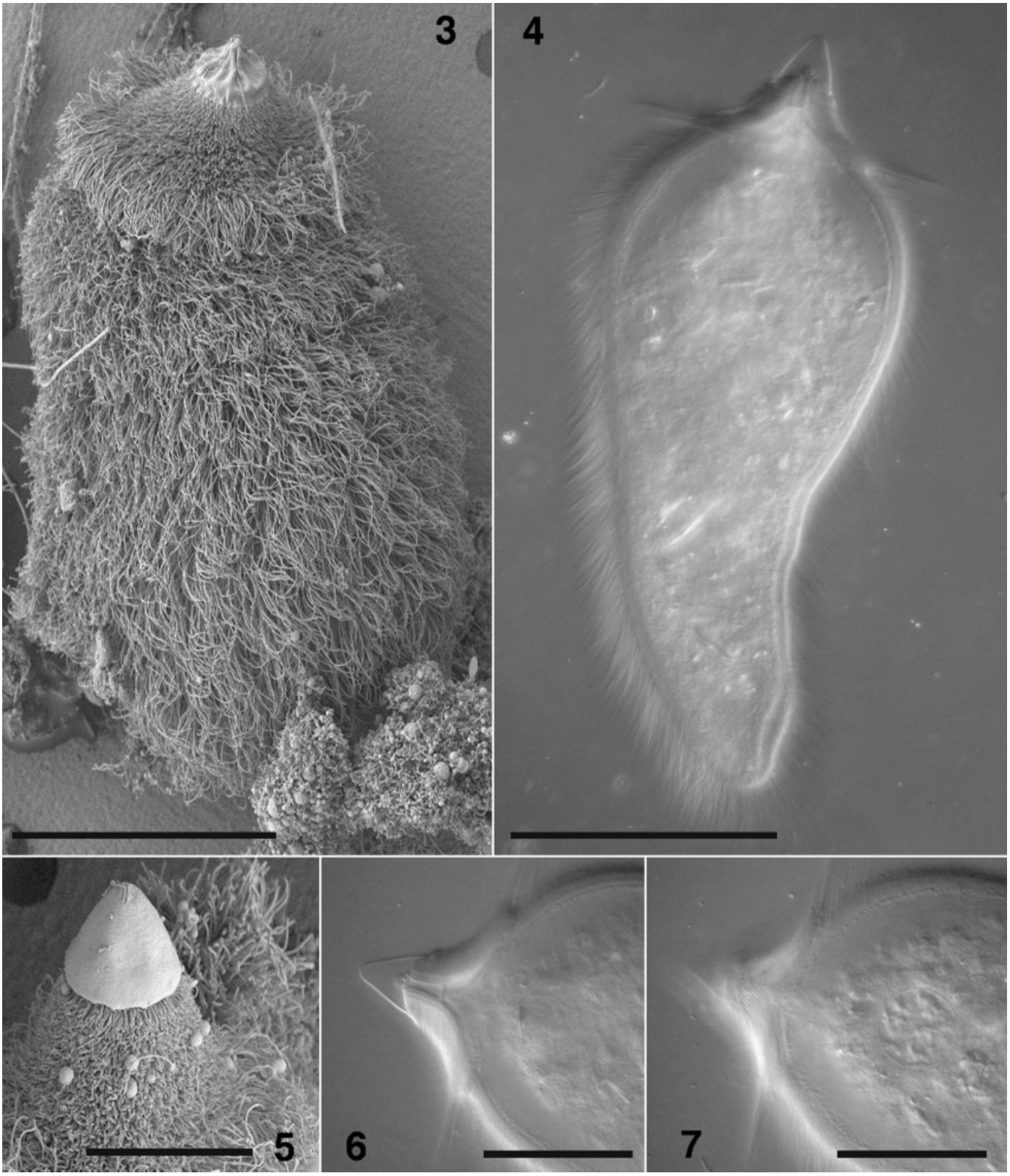

Fig. **3–7.** Morphology of *Pseudotrichonympha hertwigi* from *Coptotermes testaceus* collected in Colombia. Whole cells are tapered at both extremities, with a more pronounced taper at the posterior end. The rostral tube accounts for approximately 5% of the body length (6, 7, which are two focal planes from the same cell), and the rostral cap is distinctively pointed in both light microscopy (LM) (4, 6, 7) and scanning electron microscopy (SEM) (5). Some deflated specimens were observed in SEM as well (3), and those tended to be pinched rather than flattened. In many views, the anterior-most flagellated zone with short flagella was raised to be continuous with the angle of the rostral cap (4–7). Scale bars correspond to 50 μm (3, 4), 20 μm (5), and 25 μm (6, 7).

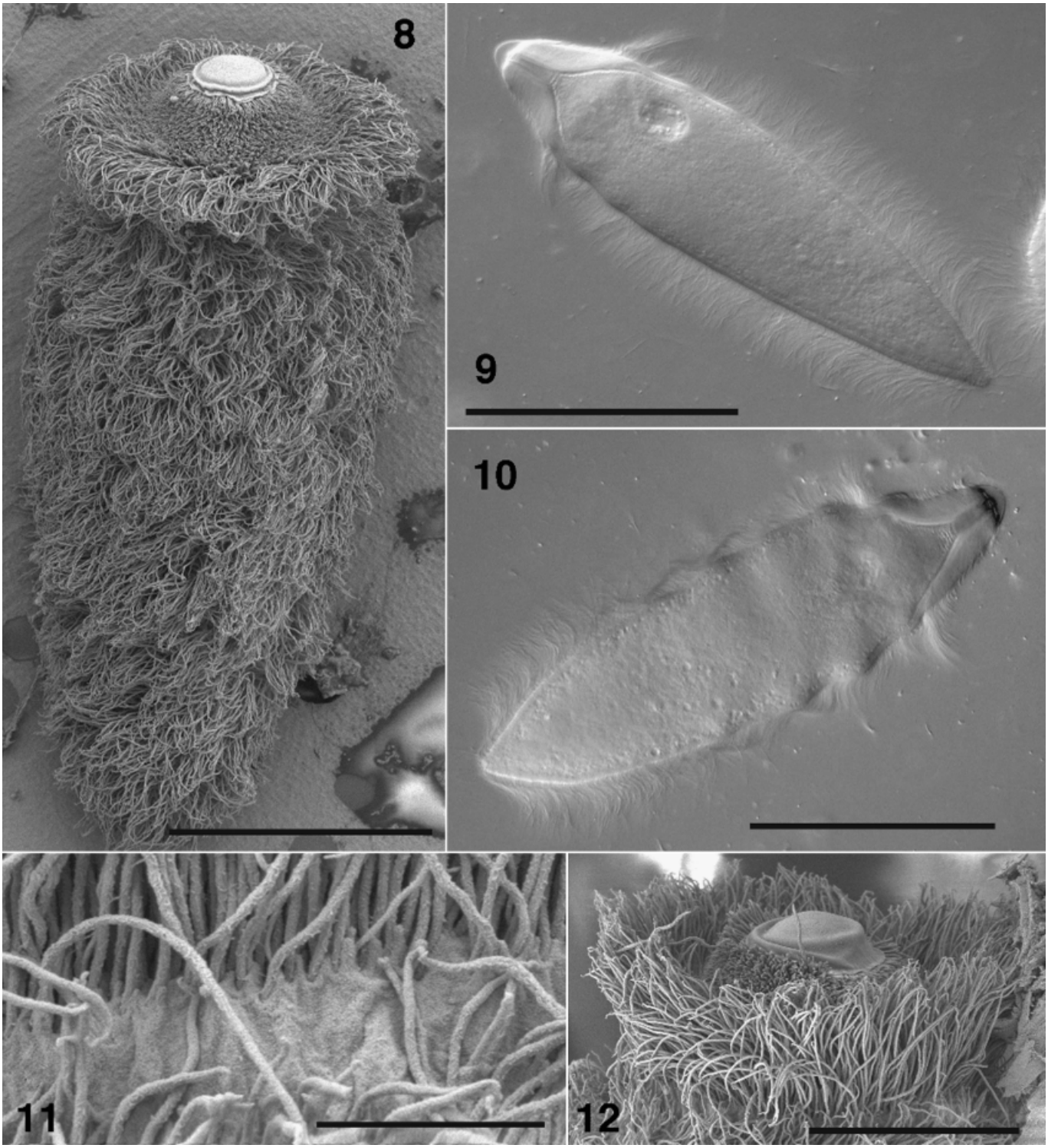

Fig. **8–12.** Morphology of *Pseudotrichonympha paulistana* from *Heterotermes tenuis* collected in Colombia. The morphology of *P. paulistana* closely resembles that of *Pseudotrichonympha hertwigi*, including a similar overall body shape (8–10) and flagellar zones (8, 12). The rostral tube is comparatively longer, at approximately 10% of the length of the cell body, and the single prominent nucleus is positioned anteriorly (9, 10). The rostral cap is rounded in light microscopy (9, 10) and appears deflated in scanning electron microscopy (8, 12). From anterior to posterior, the pattern of flagellation begins with a zone of very short flagella followed by a zone of very long flagella, then intermediate sized flagella covering the majority of the body (8, 12), with a nonflagellated zone between the long and intermediate zones (11). Coordinated beating of flagella across zones was observed, associated with rippling of the cell surface (10). Scale bars correspond to 50 μm (8–10), 5 μm (11), and 25 μm (12).

flagella tend to point anteriorly, and their action seems to produce the thrust when live cells are moving.

Light microscopy shows that there is no pronounced furrow between the mid- and posterior zones of the organism, as is the case in closely related genera like *Eucomonympha* (Carpenter and Keeling 2007). There is, however, a distinct nonflagellated zone between the long flagella of the rostral tube and the more uniform flagella covering the main portion of the cell (Fig. 11).

The remainder of the cell is made up of a large zone completely covered in flagella of intermediate length ($\sim$ 8.5–9 μm), which can beat synchronously and create waves (Fig. 10). There is no significant nonflagellated region at the extreme posterior, but in some views a very small nonflagellated zone was observed in a few cells (not shown). The single prominent nucleus is generally found toward the anterior end, centrally within the cytoplasm of this zone.

DISCUSSION

The molecular characterization of *P. hertwigi*, the type species of *Pseudotrichonympha,* is relatively straightforward. A single sequence type with low variation was found to be closely related to a sequence characterized from the same termite sampled in Brazil (Noda et al. 2007). The gene sequence of the Colombian *C. testaceus* host termite is also very closely related to the Brazilian one and to sequences from specimens collected in several Caribbean countries.

In contrast, the variability of both host and symbiont sequences in *H. tenuis* are much greater. Termite sequences from the Caribbean are on average only 96% similar to the Colombian sample, but the Colombian sample remains far more closely related to other *H. tenuis* specimens than to any other known termite. In the absence of any evidence to the contrary, we therefore suggest this represents intraspecific variation. With respect to the symbionts, we cannot rule out the possibility that the two clone types that we found in isolated *H. tenuis Pseudotrichonympha* cells represent two different species of *Pseudotrichonympha* endosymbionts, but without additional characterization (in particular direct demonstration that this variation is not between copies of the gene within single cells and preferably that it correlates with morphological variation) it would seem more conservative to consider the approximately 4% divergence between them to be intraspecies variation as well. Observed intraspecific sequence divergence can vary greatly among gut-symbiotic parabasalids: in *Snyderella yamini* and *Coronympha koidzumii* all clones characterized (5 and 18, respectively) were identical (Gile et al. in press; Harper et al. 2009), while in *Kofoidia* over 50 differences were observed among seven clones from a two-cell isolation (unpubl. data). Furthermore, hypermastigote parabasalids of the Teranymphidae have long branches in SSU rRNA phylogenetic analyses (e.g. Fig. 1; Carpenter and Keeling 2007; Ohkuma et al. 2005), an observation consistent with higher rates of evolution in this gene in this lineage.

We have observed a few morphological variations between the *P. paulistana* cells in Colombian *H. tenuis* specimens and the descriptions of De Mello (1954b) from Brazilian *H. tenuis* specimens, though it is impossible to tell whether these differences are due to the use of different methods: we observed live cells and used SEM, whereas he used fixed and stained cells (Bouin/hematoxylin, Heidenhain azan, protargol) for LM. De Mello (1954b) worked from three specimens of *H. tenuis* collected in Sao Paulo State, Brazil. From his descriptions and drawings based on fixed and stained specimens, the Brazilian form appears to be longer and more slender than the Colombian one: it is 165.3 ± 35.9 μm in length and 38 ± 11.1 μm in width ($n = 70$, De Mello 1954b), while the Colombian form is 147.2 ± 18.0 μm in length and 51.9 ± 9.0 μm in width ($n = 13$). De Mello also described features such as ''deux barbelles sidérophiles élégamment recourbées'' (two elegantly curved iron-staining barbs) attached to the rostral cap of the cells and a series of spheres (i.e. ''lentilles sphériques'') inside the rostral tube. We did not observe these features in unstained cells using DIC optics, but this is likely due to the use of stains (De Mello 1954b). It would be interesting to compare Brazilian and Colombian *P. paulistana* populations using the same methodology to see whether any of these observations represent real differences.

Since the genus *Pseudotrichonympha* was first described in 1910 a number of different species have been described from a variety of rhinotermitids and from the kalotermitid genus *Rugitermes* (Table 1). They may also exist in serritermitids like *Serritermes* or *Glossotermes*, but the *Pseudotrichonympha*-like symbionts of those genera have not been formally described

Table **1.** Host distribution of described species of *Pseudotrichonympha*.

Species	Host	Reference
P. bachmani	An unidentified termite from San Juan, Puerto Rico[a]	Calkins (1936)
P. belari	*Heterotermes indicola*	De Mello (1927)
P. cardiformis	*Coptotermes heimi*	Karandikar and Vittal (1954)
	Heterotermes malabaricus	Karandikar and Vittal (1954)
P. grassei	*Psammotermes hybostoma*	Bobyleva (1969)
P. grassii	*Coptotermes formosanus*	Koidzumi (1917)
	Coptotermes heimi	Saleem (1952)
	Heterotermes indicola	Saleem (1952)
P. hertwigi (type)	''*Coptotermes hartmanni*'' (*C. testaceus*)	Hartmann (1910)
	Coptotermes sjoestedti	Cleveland (1926)
	Prorhinotermes flavus	Sutherland (1933)
	Coptotermes acinaciformis	Sutherland (1933), Mannesmann (1969)
	Heterotermes longiceps	Dini and Cesar (1960)
P. hertwigi var. major	*Coptotermes lacteus*	Grassi (1917)
P. hertwigi var. minor	*Coptotermes sjoestedti*	Grassi (1917)
P. hertwigi var. simplex	*Coptotermes* sp. from Daman, India	De Mello (1937)
P. indica	*Heterotermes indicola*	Chakravarty and Banerjee (1956)
	Coptotermes heimi	Das (1976)
P. introflexibilis	*Schedorhinotermes putorius*	Dogiel (1922)
P. magnipapillosa	*Schedorhinotermes putorius*	Grassi (1917)
P. parvipapillosa	*Schedorhinotermes intermedius*	Grassi (1917)
P. paulistana	*Heterotermes tenuis*	De Mello (1954b)
P. pisciformis	*Coptotermes heimi*	Karandikar and Vittal (1954)
	Heterotermes malabaricus	Karandikar and Vittal (1954)
P. pristina	*Archotermopsis wroughtoni*	Cutler (1921)
P. ramani	*Heterotermes* sp. from India	De Mello (1937)
P. sertaneja	*Rugitermes* sp.	De Mello (1954a)
P. sphaerophora	*Rhinotermes nasutus*	Dunkerley (1923)
P. subapicalis	*Coptotermes heimi*	Karandikar and Vittal (1954)
	Heterotermes malabaricus	Karandikar and Vittal (1954)

[a]According to Scheffrahn et al. (2003) and Szalanski et al. (2004), only three rhinotermitids occur in Puerto Rico: *Heterotermes convexinotatus*, *Prorhinotermes simplex* (possibly introduced), and *Coptotermes gestroi* (recently introduced).

(Cancello and DeSouza 2004; Costa-Leonardo and Kitayama 1991). Table 1 makes it apparent that in some cases *Pseudotrichonympha* species have been described from more than one species of host, and that in others one species of termite seems to harbor more than one species of *Pseudotrichonympha*. However, some of the publications that record these relationships (e.g. Sutherland 1933 recording *P. hertwigi* in *Prorhinotermes flavus*) are very sketchily described and simply state that the symbiont was found in a particular termite without providing any images. It is obviously very difficult to evaluate this kind of record without more data. In other cases, for example Cleveland 1926 recording *P. hertwigi* in *C. sjoestedti*, beautiful images are provided that suggest to us that the species being drawn is different from what we consider *P. hertwigi*. In that particular case the *Pseudotrichonympha* endosymbiont has a very slender shape, quite different from what is observed in *C. testaceus*. All in all, we suspect that the true diversity of *Pseudotrichonympha*, like that of most protists, has been greatly underestimated; the issue will certainly have to be investigated in the future.

Another aspect of the distribution of *Pseudotrichonympha* in host species is also of interest. The genus is found in many rhinotermitids (Table 1), and in two genera, *Parrhinotermes* and *Termitogeton*, *Pseudotrichonympha* has been reported to be the only parabasalian symbiont (Kitade and Matsumoto 1998). However, it has never been described from *Reticulitermes*, a well-studied rhinotermitid closely related to *Coptotermes* and *Heterotermes* (Inward, Vogler, and Eggleton 2007; Lo et al. 2004). In contrast to its close relatives, *Reticulitermes* contains an entirely different set of protist endosymbionts, resembling that of the termopsid termite *Hodotermopsis* (Kitade and Matsumoto 1998). It has been proposed that an ancestor of *Reticulitermes* may have replaced its gut fauna through a process of horizontal transfer involving a relative or ancestor of *Hodotermopsis* (Kitade and Matsumoto 1998; Noda et al. 2007). A more thorough investigation of *Reticulitermes* symbiont diversity may shed some light on this hypothesis: if, for instance, such a replacement of hindgut flora took place within the diversification of the termite genus, then some *Reticulitermes* species might still retain *Pseudotrichonympha*, as well as other symbionts more typical of closely related rhinotermitids.

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