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ORIGINAL ARTICLE

Molecular Evidence for the Polyphyly of *Macrotrichomonas* (Parabasalia: Cristamonadea) and a Proposal for *Macrotrichomonoides* n. gen.

Gillian H. Gile[a,1], Erick R. James[a], Noriko Okamoto[a], Kevin J. Carpenter[a,2], Rudolf H. Scheffrahn[b] & Patrick J. Keeling[a]

a Department of Botany, Canadian Institute for Advanced Research, University of British Columbia, Vancouver, British Columbia, V6T 1Z4, Canada
b University of Florida Research & Education Center, Davie, Florida 33314, USA

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Correspondence
P.J. Keeling, Department of Botany, University of British Columbia, 3529-6270 University Blvd, Vancouver, British Columbia V6T 1Z4, Canada
Telephone number: +1-604-822-4906; FAX number: +1-604-822-6089;
e-mail: pkeeling@mail.ubc.ca

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ABSTRACT

Macrotrichomonas (Cristamonadea: Parabasalia) is an anaerobic, amitochondriate flagellate symbiont of termite hindguts. It is noteworthy for being large but not structurally complex compared with other large parabasalians, and for retaining a structure similar in appearance to the undulating membrane (UM) of small flagellates closely related to cristamonads, e.g. *Tritrichomonas*. Here, we have characterised the SSU rDNA from two species described as *Macrotrichomonas*: *M. restis* Kirby 1942 from *Neotermes jouteli* and *M. lighti* Connell 1932 from *Paraneotermes simplicicornis*. These species do not form a clade: *M. lighti* branches with previously characterised *Macrotrichomonas* sequences from *Glyptotermes*, while *M. restis* branches with the genus *Metadevescovina*. We examined the *M. restis* UM by light microscopy, scanning electron microscopy, and transmission electron microscopy, and we find common characteristics with the proximal portion of the robust recurrent flagellum of devescovinids. Altogether, we show the genus *Macrotrichomonas* to be polyphyletic and propose transferring *M. restis* to a new genus, *Macrotrichomonoides*. We also hypothesise that the macrotrichomonad body plan represents the ancestral state of cristamonads, from which other major forms evolved.

MACROTRICHOMONAS is a large and distinctive parabasalian flagellate known only from the hindgut of lower termites in the family Kalotermitidae (Yamin 1979). The cells range in size from 26 to over 90 μm, but unlike many other large parabasalian termite flagellates, *Macrotrichomonas* lacks multiple nuclei, massive duplication of flagella, or indeed any replication of the karyomastigont system whatsoever. Instead, it bears a single nucleus associated with a robust and protruding axostyle and three anterior flagella plus a single recurrent flagellum (Grassé and Hollande 1950; Grassi 1917; Hollande and Valentin 1969). The most distinctive feature of *Macrotrichomonas* is its undulating membrane (UM), which superficially resembles those of *Trichomonas* or *Tritrichomonas*. So while the cell has increased in size, it has apparently not increased in morphological complexity, analogous to the large but relatively simple trichomonads *Trichomitopsis* and *Pseudotrypanosoma* (Brugerolle 1999; Grassé and Hollande 1950; Keeling 1998).

The genus was first described in 1917 by Grassi, when he observed *Macrotrichomonas pulchra* in the African termite *Glyptotermes parvulus* (Grassi 1917). Nine additional species have since been described from a variety of kalotermitid hosts: *M. unguis*, *M. ramosa*, and *M. emersoni* from other *Glyptotermes* spp., *M. lighti* from *Paraneotermes simplicicornis*, *M. virgosa* from an unidentified *Procryptotermes* sp., *M. directa* from an unidentified *Kalotermes* sp., *M. procera* from various *Calcaritermes* spp., *M. restis* from *Neotermes jouteli*, and *M. hirsuta* from *Neotermes praecox* (Connell 1932; Grassé and Hollande 1950; Kirby 1942, 1949).

Macrotrichomonas was soon recognised to share significant structural characteristics with *Devescovina*, in particular, the parabasal fibres that wind helically around the stout axostyle, and was united with that genus and others in the Devescovinidae (Kirby 1931). Since then, a similarly coiling parabasal body, thickened recurrent flagellum, and

cresta have been identified in both calonymphids (e.g. *Calonympha*, *Snyderella*) and joeniids (e.g. *Joenia*, *Joenina*), all of which are now included along with devescovinids in the Cristamonadea (Brugerolle and Patterson 2001; Cepicka et al. 2010). Molecular phylogenetics also support the Cristamonadea and the placement of two *Macrotrichomonas* spp., from *Glyptotermes fuscus* and *G. satsumensis*, within it (Cepicka et al. 2010; Noda et al. 2009). In other large cristamonads, such as calonymphids, joeniids, and *Coronympha*, massive increases in cell size are associated with increases in cellular complexity through whole or partial duplication of the karyomastigont system in a process that has taken place multiple times independently (Brugerolle 1986; Brugerolle and Patterson 2001; Cepicka et al. 2010; Gile et al. 2011; Noda et al. 2009). How this increase in complexity took place and the nature of the ancestor of the group are both topics of interest. The nearest recognised sister group to the Cristamonadea is the Tritrichomonadea, a group of small and simple flagellates with a single karyomastigont system including one recurrent flagellum associated with a distinctive "rail" type UM (Brugerolle 1976; Brugerolle and Lee 2000; Cepicka et al. 2010). Thus, the overall structure of *Macrotrichomonas*, particularly the potential presence of an UM, marks this genus among cristamonad flagellates as potentially approximating the ancestral state of the cristamonads as a whole. To date, however, our knowledge of *Macrotrichomonas* remains insufficient for any significant conclusions to be reached: its UM has been investigated in only one species (Hollande and Valentin 1969), and data on its phylogenetic position are limited (Noda et al. 2009).

Here, we use light microscopy (LM), scanning and transmission electron microscopy (SEM and TEM), and molecular data from manually isolated cells of two previously described species of *Macrotrichomonas*, *M. restis* Kirby 1946 from *N. jouteli* Banks, 1919 and *M. lighti* Connell 1932 from *P. simplicicornis* Light, 1934. Microscopic observations confirm the *Macrotrichomonas*-type UM in *M. restis*. More surprisingly, molecular data show that the genus *Macrotrichomonas* is polyphyletic: in SSU rRNA trees, *M. lighti* branches with the unidentified *Macrotrichomonas* species characterised previously, but *M. restis* is only distantly related within the Cristomonadea, branching instead with sequences from the genus *Metadevescovina*. We hypothesise that the polyphyly of the "*Macrotrichomonas*" morphology may be due to retention of an ancestral body plan from which other cristamonads evolved.

MATERIALS AND METHODS

Host termite collection and barcoding

Neotermes jouteli was collected on April 9, 2008, from Dagny Johnson Key Largo Hammock State Park, Monroe County, Florida (lat. 25.17608, long. −80.36945). *Paraneotermes simplicicornis* was collected on October 30, 2008, west of Terlingua, Texas (lat. 29.28004, long. −103.71647). Specimens were deposited in the University of Florida termite collection under accessions FL3188 and US1268, respectively. All termites were maintained in falcon tubes with wood from their habitats at room temperature in the laboratory. Termite identities were determined morphologically and, in the case of *N. jouteli*, confirmed by barcoding using the mitochondrial cytochrome C oxidase subunit II gene as described previously (Gile et al. 2013). For *P. simplicicornis*, a portion of the 16S mitochondrial ribosomal RNA gene (which is the large subunit in insect mitochondria, also referred to as LSU) was amplified and sequenced using the primers LR-N-13398 5′-CGC CTG TTT ATC AAA AAC AT-3′ (Simon et al. 1994) and LR-J-13017 5′-TTA CGC TGT TAT CCC TAA-3′ (Kambhampati and Smith 1995) and submitted to GenBank under accession KJ438371. These are the first molecular data available for *P. simplicicornis*.

Symbiont isolation, DNA extraction, PCR, and sequencing

Termites were dissected and hindgut contents were suspended in Trager's medium U (Trager 1934). Individual *Macrotrichomonas* and *Metadevescovina* cells were isolated by micropipette and pooled in samples of 1–100 cells for DNA extraction using the Masterpure Complete DNA and RNA Purification Kit (Epicentre, Madison, WI). DNA was also purified from whole gut contents for environmental PCR. SSU rRNA genes were amplified from purified DNA using the eukaryote specific primers PFI 5′-TGC GCT ACC TGG TTG ATC CTG CC-3′ and FAD4 5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′ and EconoTaq PLUS GREEN (Lucigen, Middleton, WI). PCR conditions included a 3 min denaturation at 95 °C followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min 30 s, then an additional 7 min at 72 °C. Products were purified using the UltraClean 15 gel purification kit (MoBio, Carlsbad, CA), cloned into the pCR2.1 vector using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA), and sequenced on both strands with BigDye Terminator v 3.1 (Applied Biosystems, Carlsbad, CA). Multiple clones were sequenced from each *Metadevescovina* and *Macrotrichomonas* species and assembled into contigs using Sequencher 4.2 (GeneCodes, Ann Arbor, MI) with a stringency of 98%.

For *Macrotrichomonas* from *P. simplicicornis*, cells were isolated in two pools of 25 cells each and DNA from these isolations and a whole gut sample was used for three independent PCR reactions. Five clones, two from each pool of isolated cells and one environmental clone, were sequenced and assembled. The resulting contig was 1,570 nucleotides in length and differed at only six nucleotide positions (0.4%). No single clone differed from the consensus sequence by more than two nucleotides. A clone sequence from one of the pools of isolated cells was identical to the consensus, and it was used for phylogenetic analyses and submitted to GenBank under accession KJ493790.

For *Macrotrichomonas* from *N. jouteli*, DNA was extracted from five separate single cells, and three pools of 2, 10, and 20 cells. Nineteen clones were sequenced

from eight independent PCR reactions and assembled into a contig 1,558 nucleotides in length. A single clone from the whole gut DNA sample PCR also assembled into this contig. At 67 positions of the consensus of the clustered clone sequences, one or more clones differed. Although this represents 4% of the sites in the consensus, it should be noted that no individual clone sequence differed from any other clone by more than 2% of its sites. Most clones differed from the consensus by only 1–6 nucleotides, but three of the four clones sequenced from the 10-cell PCR shared 13 differences from the consensus. One clone from one of the single-cell PCR reactions differed from the consensus by only a single nucleotide, and this was chosen to represent this pool of sequences in phylogenetic analyses and for submission to GenBank under accession KJ493791.

For *Metadevescovina* from *N. jouteli*, DNA from pools of 20 and 100 isolated cells and whole gut contents were used as templates for three PCR reactions. A contig assembled from five clones from the 20 cell PCR, two clones from the 100 cell PCR, and five clones from the environmental PCR were assembled into a contig 1,573 nucleotides in length, with disagreements at a total of 22 positions (1.4%). No single clone differed from the consensus at more than two positions. One clone from the 20-cell PCR that differed from the consensus by a single nucleotide was chosen to represent this group of sequences in phylogenetic analyses and for submission to GenBank (accession KJ493792, henceforth referred to as *Metadevescovina* sp. 1). Two identical clones from the isolated cells could not be assembled into the same contig at 98% stringency, however. This sequence was found to be phylogenetically distinct and is henceforth referred to as *Metadevescovina* sp. 2, and was submitted to GenBank under accession KJ493793. In all, 10 clones from the whole gut DNA PCR were sequenced, of which 5 proved to be *Metadevescovina* sp. 1, 3 were *Staurojoenina mulleri* (Gile et al. 2013), and 1 clustered with the *Macrotrichomonas* sequences.

Phylogenetic analyses

New *Macrotrichomonas* and *Metadevescovina* sequences from *N. jouteli* and *P. simplicicornis* were aligned with previously published sequences spanning the phylogenetic diversity of parabasalians using MAFFT (Katoh et al. 2002). Highly variable regions were removed using BMGE (Criscuolo and Gribaldo 2010), resulting in a final alignment of 60 taxa and 1,395 positions (83% of the raw 1,673-site alignment). The alignment is available upon request from the authors. Maximum likelihood (ML) and Bayesian phylogenetic analyses were performed with RAxML 7.2.5 (Stamatakis 2006) and PhyloBayes v3.2 (Lartillot and Philippe 2004) respectively, using the GTR + Γ model (GTR + Γ + CAT in phylobayes). For the ML analysis, support was assessed from 1,000 bootstrap replicates. For the Bayesian analysis, two independent chains, sampled each 10 cycles, were run for 106 h, at which point the chains had converged (maxdiff = 0.023) and more than 11,200 trees had accumulated for each. The first 1,260 trees from each chain were discarded as burn-in, and the remaining 20,000 trees were used to compute the majority rule consensus tree.

Confidence in alternate topologies was assessed using approximately unbiased (AU) tests (Shimodaira 2002). Optimised ML trees for two topological constraints, one in which *Macrotrichomonas* was constrained as monophyletic and one in which *Macrotrichomonas* and *Metadevescovina* together formed a clade were computed in RAxML and added to a set of 1,000 trees from the ML bootstrap analysis for testing. AU tests were performed using the program CONSEL 1.19 (Shimodaira and Hasegawa 2001) from site-likelihoods computed in RAxML.

Light and electron microscopy

Hindgut contents suspended in Trager medium U were viewed on an Axioplan 2 compound microscope (Zeiss, Oberkochen, Germany) using differential interference contrast and photographed with a MicroImager II (QImaging, Surrey, BC, Canada). Cell isolations were performed by hand using hand-drawn glass micropipettes and an Axiovert 200 (Zeiss) inverted microscope.

Samples were processed for scanning electron microscopy as described previously (Gile et al. 2013). For TEM, hindgut contents of *N. jouteli* were fixed in 1% glutaraldehyde (final concentration) in 0.1 M phosphate buffer (PB) pH 7 for 1 h, rinsed twice with PB, then post fixed with 1% OsO4 for 1 h at room temperature. Following fixation, samples were dehydrated in an ethanol series (15%, 30%, 50%, 75%, 80%, 90%, 95%, and 100% twice), then embedded in SPI-Pon 812 resin in BEEM capsules (SPI, West Chester, PA). Serial ultrathin sections (50 nm thickness) were collected on Formvar-coated slot grids. Ultrathin sections were post-stained with 2% uranyl acetate for 15 min and Reynold's lead citrate for 5 min (Reynolds 1963), then observed under a Hitachi H7600 electron microscope (Hitachi, Tokyo, Japan), and post-processed using Photoshop CS5 software (Adobe, San Jose, CA).

RESULTS AND DISCUSSION

Phylogenetic characterisation of *Macrotrichomonas* from *N. jouteli* and *P. simplicicornis*

Molecular barcoding of termites morphologically identified as *N. jouteli* confirmed their identity, as described previously (Gile et al. 2013; GenBank accession JX847582). Termites morphologically identified as *P. simplicicornis* were also barcoded (GenBank accession KJ438371), but there is not a publicly available collection of reference sequences from this species with which to compare the barcode. Nevertheless, the sequence branched where expected compared to other kalotermitids (Thompson et al. 2000), the termite morphology matched that of *P. simplicicornis*, and its hindgut contents also matched the unusual and distinctive complement of species previously observed in this host (Yamin 1979). We are therefore confident in the identification.

Previous studies of these two termites based on morphology concluded that each host is home to a single species of *Macrotrichomonas: M. restis* in *N. jouteli* (Kirby 1942), and *M. lighti* in *P. simplicicornis* (Connell 1932). To determine the phylogenetic position of these two symbiont species, we characterised the SSU rRNA from manually isolated single cells or small pools of cells all matching the previously described morphology of *Macrotrichomonas* (including five independently isolated single *M. restis* cells, see Materials and methods for details). Although we are confident in the assignment of each SSU sequence to its respective manually isolated protist morphotype, future studies employing fluorescence in situ hybridisation techniques would help remove any doubt. In each termite, all sequences from isolated cells shared a high level of sequence identity (greater than 98%—see Methods). Therefore, we conclude that a single species of *Macrotrichomonas* likely exists in each termite host.

In phylogenetic analyses, both *Macrotrichomonas* sequences branch within the Cristamonadea, as expected. The *M. lighti* sequence branches (without strong support) with two previously characterised *Macrotrichomonas* species isolated from *Glyptotermes* spp. (Fig. 1). In contrast, the sequence from *M. restis* branches with *Metadevescovina* (Fig. 1), most closely to a sequence from an unidentified symbiont of *N. jouteli*.

Neotermes jouteli is reported to harbour two species of *Metadevescovina*, *Me. nudula* and *Me. turbula* (Kirby 1945; Yamin 1979), and we likewise observed cells with *Metadevescovina* morphology. Because the placement of *M. restis* with *Metadevescovina* was unexpected, we explored the possibility that the *M. restis* sequences might be derived from contaminating *Metadevescovina* cells. We amplified *Metadevescovina* SSU from two pools of isolated cells (a total of 120 cells) and from a whole-hindgut sample and sequenced a total of 12 clones from the three independent PCRs. These clones assembled into two distinct contigs, neither of which matched the *M. restis* sequence.

We also sought to assess confidence in the deduced polyphyly of *Macrotrichomonas* using AU tests (Shimodaira 2002; Shimodaira and Hasegawa 2001). The monophyly of *Macrotrichomonas* was rejected ($p = 0.0002$), but the possibility that all *Macrotrichomonas* sequences (i.e. *M. restis*, *M. lighti*, and two unidentified species from *Glyptotermes*) and *Metadevescovina* together form a clade could not be ruled out ($p = 0.835$). Overall, we conclude that *M. restis* is not closely related to *M. lighti* and other cells with *Macrotrichomonas* morphology that have been characterised at the molecular level to date; instead, it is more closely related to *Metadevescovina*.

Morphology of *Macrotrichomonas* with particular reference to the UM

Undulating membranes are found in multiple parabasalian groups, and have different, potentially nonhomologous morphologies. Brugerolle identified three types of UM in the Parabasalia: the lamelliform type, the rail type, and the devescovinid type (Brugerolle 1976, 1986). The devescovinid type is fully developed in only two genera, *Macrotrichomonas* and *Gigantomonas*, and it resembles the thickened recurrent flagellum and its associated structures in other devescovinids. The lamelliform and rail type UMs are each subtended by a costa, a microfibrillar structure with periodic striations, while the devescovinid type lacks a costa. However, the lamelliform and rail type UMs may not be homologous: the rail type UM's costa has longitudinal striations identical to those of the parabasal fibres (A-type pattern) while the lamelliform UM's costa has a lattice structure and is striated in cross section (B-type pattern), though note that *Trichomitus* has an A-type costa and a lamelliform UM (Brugerolle 1976; Brugerolle and Lee 2000). (Note that *Trichomitus* is an exception to this rule, having an A-type costa and a lamelliform UM, Brugerolle and Lee 2000.) Meanwhile, though the devescovinid UM lacks a costa, it shares some similarities with the rail type: in both UMs the recurrent flagellum is thickened by paraxonemal fibres, and the cresta, which subtends the recurrent flagellum in the devescovinid UM, may be homologous to the microfibrils between the costa and cell margin in the rail type UM (Brugerolle 1976; Brugerolle and Patterson 2001). For a diagram of the three parabasalid UM types, see Brugerolle (1976). This is a point of some interest because the sister group to cristamonads as a whole in most molecular analyses is the tritrichomonads, whose UMs are of the rail type (Cepicka et al. 2010; Noda et al. 2009, 2012). This suggests that the UM may be ancestral to cristamonads, and by extension that the UM in *Macrotrichomonas* might be of some evolutionary significance. But little is currently known about the *Macrotrichomonas* UM at the ultrastructural level, with only one species examined to date (Hollande and Valentin 1969). To improve our understanding of the *Macrotrichomonas* UM and to characterise the phylogenetically distinct UM of *M. restis*, we studied *M. lighti* and *M. restis* by LM and *M. restis* by SEM and TEM.

In LM, both *M. lighti* (Fig. 2A–C) and *M. restis* (Fig. 3A–C) resemble previous descriptions, including their overall size and shape, the single anterior nucleus, the parabasal fibres helically coiled about the posteriorly protruding axostyle, and the UM (Connell 1932; Kirby 1942). In SEM, the recurrent flagellum of *M. restis* is sometimes separated from the cell, but in most well preserved cells (e.g. Fig. 4A, C) it is clearly and specifically associated with the UM, which is in line with its motion observed under LM (not shown). In cells where the flagellum is dissociated, the UM retains its structure (Fig. 4B). At high magnification, the surface characteristics of the UM are distinct from the rest of the cell, but only on one side of the protruding UM (Fig. 4B, C), i.e. where the UM meets the cell appears as a sharp transition zone (Fig. 4C). In cells where the flagellum has been dislodged from the UM (Fig. 4B), the point of contact appears as an invagination at the most distal point of the UM. In the single SEM of *M. lighti*, the UM was not well preserved but could be seen as an indentation in the surface (Fig. 2D).

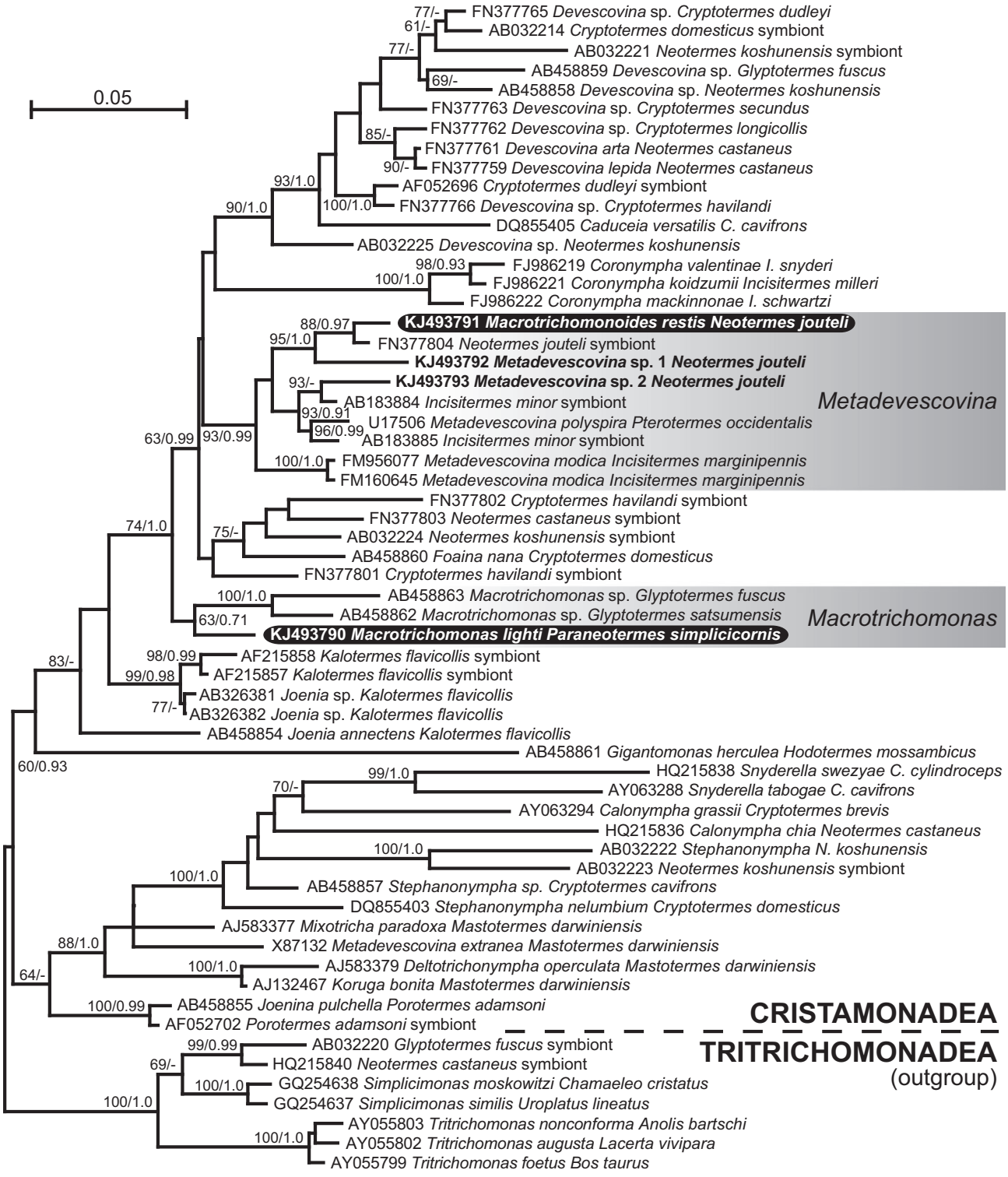

Figure 1 Maximum likelihood (ML) phylogeny of SSU rRNA sequences of Cristamonadea rooted with sequences from Tritrichomonadea. The genera *Macrotrichomonas* and *Metadevescovina* (not including *Me. extranea* from *Mastotermes darwiniensis*) are shaded in grey. New *Macrotrichomonas* and *Macrotrichomonoides* sequences from this study are indicated by white type on a black background; new *Metadevescovina* sequences are indicated by bold type. Numerical values at nodes indicate % bootstrap support (out of 1,000 replicates, where greater than 60%) and posterior probabilities from the Bayesian analysis (where in agreement with the ML topology and greater than 0.90).

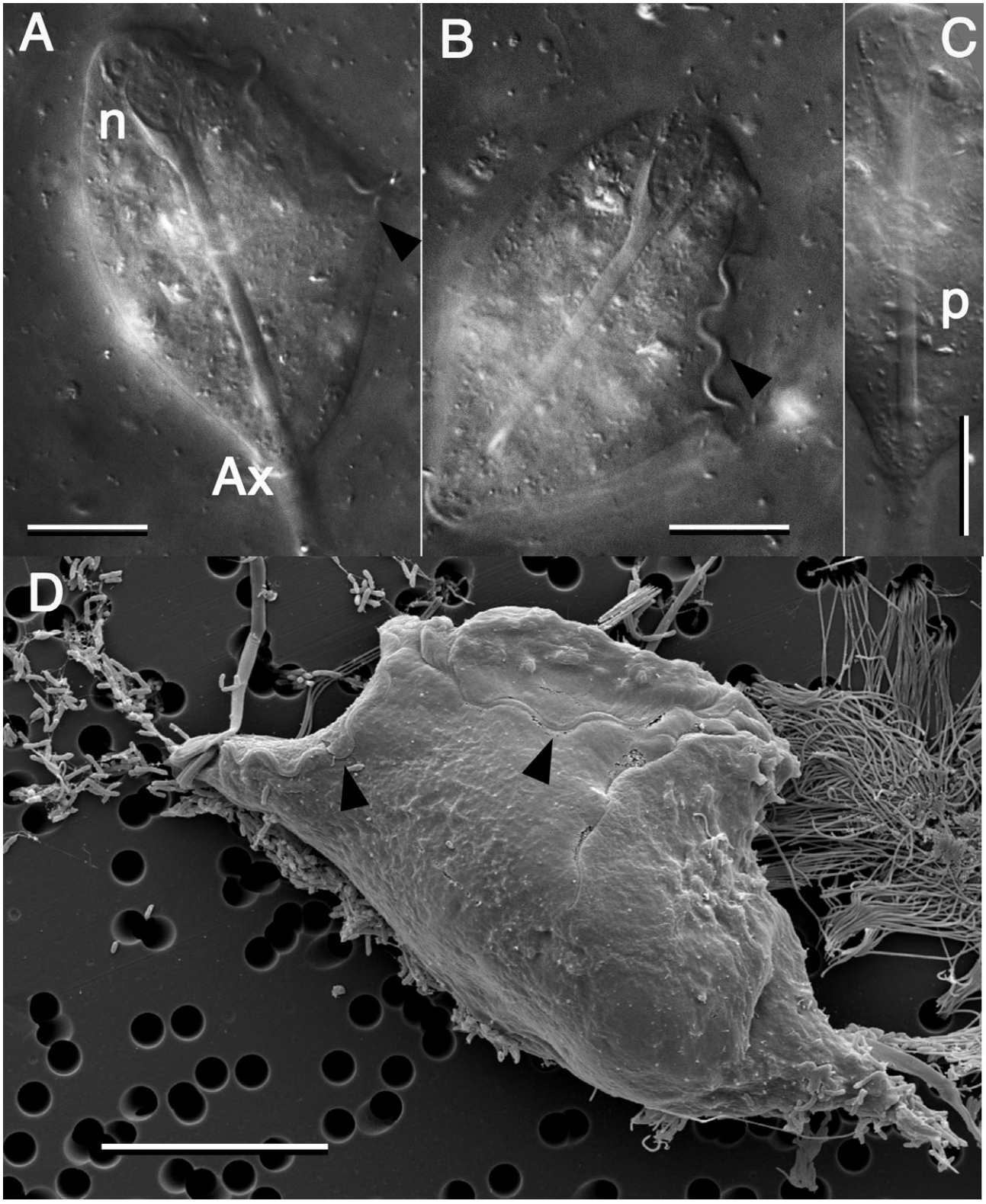

Figure 2 General morphology of *Macrotrichomonas lighti* from *Paraneotermes simplicicornis*. **A, B**. Differential interference contrast light micrographs showing overall shape and size, as in the original description. The single anterior nucleus (n) is cupped in the anterior part of the axostyle (Ax), which protrudes. **C**. The parabasal fibres (p) are coiled around the axostyle. **D**. Scanning electron micrograph showing a furrow where the band-shaped recurrent flagellum has come detached (arrowhead). Scale bars: 20 μm.

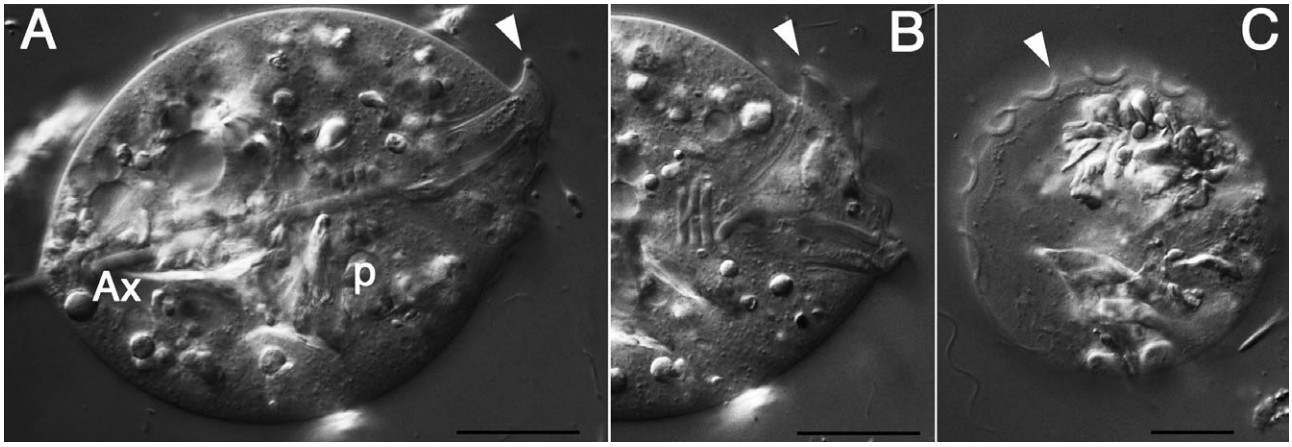

Figure 3 General morphology of *Macrotrichomonoides restis* from *Neotermes jouteli*. **A**–**C**. Differential interference contrast light micrographs on different focal planes of the same cell showing general size and shape, the axostyle (Ax) and parabasal fibres (p), and the undulating membrane (arrowhead). Scale bars: 20 μm.

Transmission electron microscopy of *M. restis* supports the observations made from SEM, and clarifies the cytoskeletal structures of the UM. First, TEM confirms the physical association of the UM with the recurrent flagellum, which is also more robust than the other flagella. The recurrent flagellum is expanded in diameter and filled with a dense granular material associated with the 9 + 2 microtubules of the axoneme (Fig. 5B, C). Second, the surface characteristics of the two sides of the UM are also distinct in TEM (see for example Fig. 5B, which is a section though an entire fold of the UM). The cytoplasmic portion of the UM that extends from the cell is dominated by a broad multilayered electron-dense band of unknown composition that matches the description of the cresta in devescovinids (Fig. 5A–D, cf. Brugerolle and Lee 2000; fig. 7a, b). The cresta swells or forms flat plates at the point where the UM contacts the recurrent flagellum (Fig. 5B, C), and also at its opposite end where it contacts the parabasal fibres (Fig. 5A, D). Outside the folds of the UM, the cresta appears as a light band of varying width with a dense-staining core that extends for its entire length, but which sometimes appears fragmented (Fig. 5A). No TEM was possible on *M. lighti* due to the death of the host animal.

Reconstructing the evolution of UMs and the ancestral state of Cristamonadea

Synthesising the phylogenetic relationships of *Macrotrichomonas* with other cristamonads with the comparative morphology of the *M. restis* and tritrichomonad UMs and the cytoskeleton of devescovinids suggests a simple model for cristamonad evolution. The rail type UM characteristic of *Tritrichomonas* is not identical to the *M. restis* UM, but it is more similar than either is to the lamelliform UM found in *Trichomonas*. On the basis of the polyphyly of *Macrotrichomonas* in our analysis, the sister relationship between cristamonads and tritrichomonads (Fig. 1, Cepicka et al. 2010; Noda et al. 2009, 2012), and the similarities previously noted between lamelliform and devescovinid type UMs (Brugerolle 1976; Brugerolle and Patterson 2001), we hypothesise that the cristamonad and tritrichomonad UMs are homologous, though distantly related, and as a corollary, the *Macrotrichomonas* morphology is plesiomorphic.

The morphology and phylogenetic position of *Gigantomonas herculea* is also consistent with this view. The UMs of *Macrotrichomonas* and *Gigantomonas* are so similar that for a time the two genera were considered synonymous (Connell 1932; Grassi 1917; Kirby 1938, 1942). The lack of a parabasal fibre coiled around the axostyle and the genuine amoeboid stage of *Gigantomonas* led Kirby (1938, 1942) to maintain separate genera, though the near-identity of their UMs led him to later propose a new subfamily for them, the Gigantomonadinae (Kirby 1946). Molecular phylogenetic analyses have since shown that *Macrotrichomonas* and *Gigantomonas* are not sisters (Cepicka et al. 2010; Noda et al. 2009). Here, we further show that cells with *Macrotrichomonas* morphology are themselves not closely related. This suggests that the UM-possessing cristamonads (*Gigantomonas* and at least two clades of "*Macrotrichomonas*") retain this morphology in parallel, and by extension that the characteristics shared by these organisms may represent the ancestral state of cristamonads as a whole (i.e. that this ancestor included a UM associated with the recurrent flagellum).

In this model, the other major morphological types of cristamonad (e.g. calonymphids, coronymphids, joeniids) evolved from *Macrotrichomonas*-like ancestors, at least partially independently. This is not inconsistent with their morphology, since many cristamonads have increased their cell size and complexity by massively replicating either the whole karyomastigont system or parts of it. The remaining relatively simple cells (e.g. *Devescovina*, *Metadevescovina*) have also retained much of the ancestral morphology, but the UM has become partly or completely

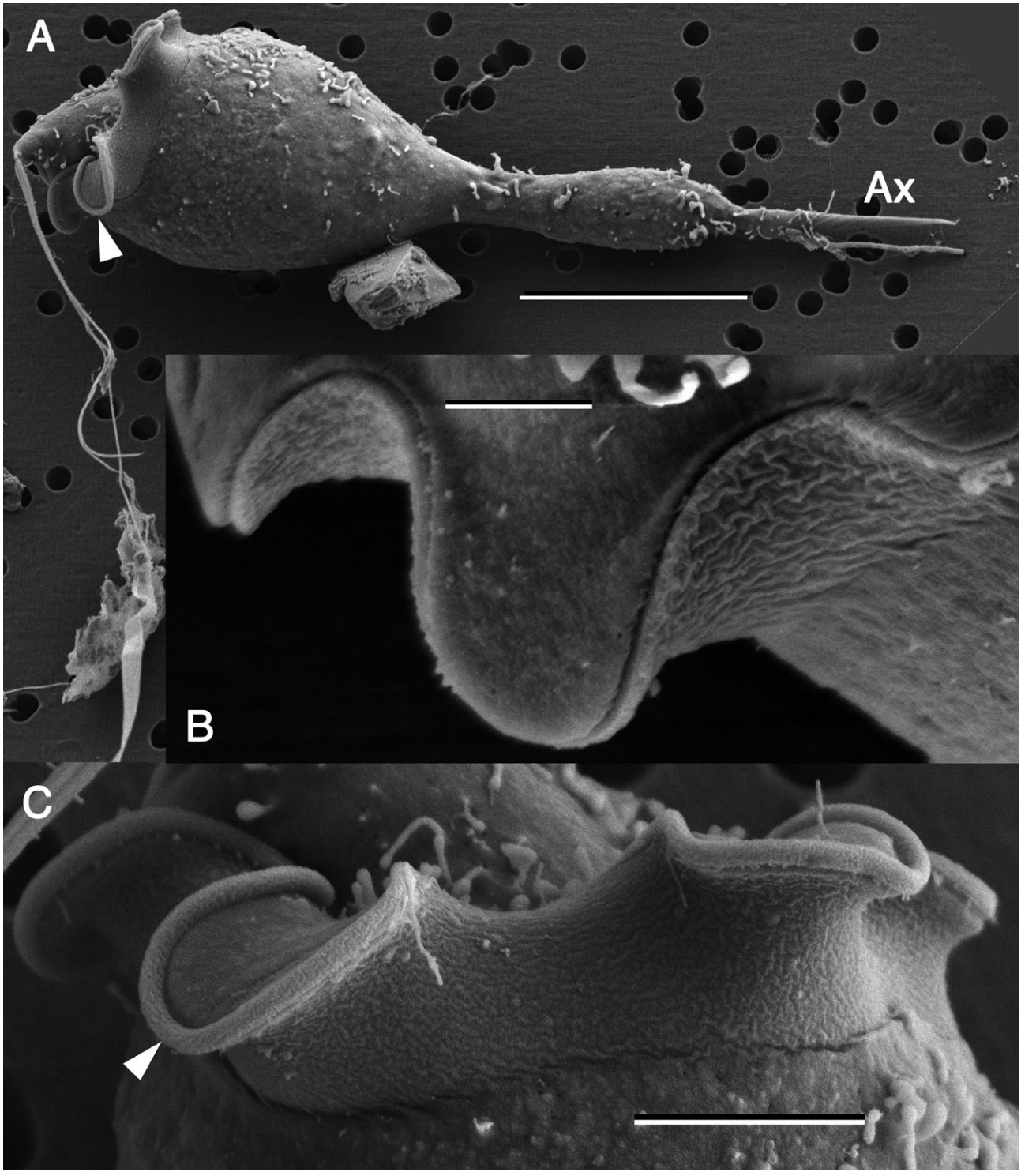

Figure 4 Surface morphology of *Macrotrichomonoides restis* from *Neotermes jouteli*. **A.** A whole cell. Three anterior flagella emerge from the anterior cell apex and the axostyle protrudes from the posterior cell apex. The undulating membrane (UM) and associated recurrent flagellum are indicated by an arrowhead. **B**, **C**. Detailed surface structure of the UM showing the smooth surface on the anterior side and the rough texture on the posterior side. (C) and (A) are images from the same cell, whereas in the cell shown in (B) the recurrent flagellum has been dislodged. Scale bars: (A) 20 μm; (B) 5 μm; (C) 2 μm.

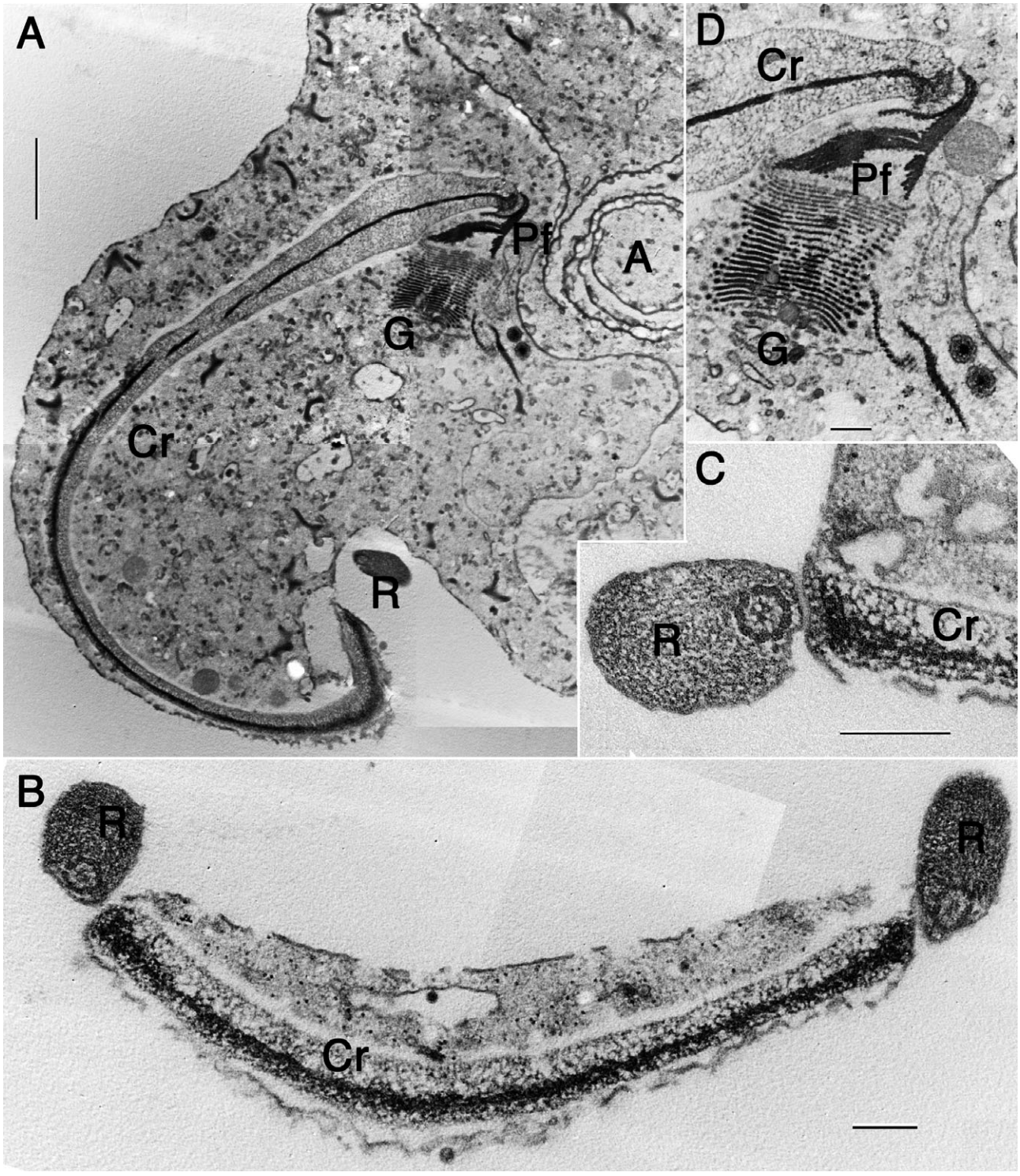

Figure 5 Ultrastructure of the undulating membrane of *Macrotrichomonoides restis* from *Neotermes jouteli*. **A.** Transverse section of the cell showing axostyle (A), cresta (Cr) and the recurrent flagellum (R). Cresta shows amorphous contents with a dark-staining core. **B**, **C.** 9 + 2 axoneme of the recurrent flagellum (R) maintains a close association with the cresta (Cr) through its entire length. **D**. Cresta (Cr) is associated with the parabasal fibre (Pf) adjacent to Golgi apparatus. Scale bars. (A) 2 μm; (B–D) 500 nm.

dissociated from the cell. Thus the UM has been lost multiple times among cristamonads. The alternative interpretation would be that a devescovinid-like ancestor gave rise to *Macrotrichomonas*-like morphology multiple times by independently extending the cresta-recurrent flagellum association down the length of the cell body.

Taxonomic considerations

If the genus *Macrotrichomonas* is polyphyletic, then one or perhaps both of *M. lighti* and *M. restis* should be reassigned to a new genus. This depends on which, if either, is specifically related to the type species for the genus *Macrotrichomonas*: *M. pulchra*, from type host *G. parvulus* (Grassi 1917). This species has not been examined at the molecular level, so it is impossible to conclusively state which of the two *Macrotrichomonas* clades in current molecular phylogenies corresponds to the one including the type species. However, Grassi and others have described *M. pulchra* as existing in many species of *Glyptotermes*, and the *M. lighti* sequence branches with two *Macrotrichomonas* sp. sequences that were derived from different species of *Glyptotermes* (*G. fuscus* and *G. satsumensis*, Noda et al. 2009). It seems very likely therefore that the type species, which is also described from *Glyptotermes*, will fall within this clade. In this case, *M. lighti* would represent a true *Macrotrichomonas* species, but *M. restis* should be transferred to a new genus, as we propose below.

TAXONOMIC SUMMARY

Class Cristamonadea
Order Cristamonadida
Family Lophomonadidae

Macrotrichomonoides restis n. gen., n. comb. Gile & Keeling, 2014

Diagnosis. Parabasalian flagellate with three anterior flagella free of the cell body, and a single, robust recurrent flagellum. Recurrent flagellum is adhered to an UM that is subtended by a well-developed multilayered cresta for most or all of the length of the cell body. Single anterior nucleus. Prominent axostyle that partially surrounds the nucleus and extends to the cell posterior, sometimes protruding. Large parabasal fibre coiled helically around the axostyle at the anterior half of the cell. Molecular phylogenetic position of SSU rRNA gene within the Cristamonadida, most closely related to *Metadevescovina* sequences.
Etymology. Genus name refers to its morphological similarity to *Macrotrichomonas*.
Zoobank ID. urn:lsid:zoobank.org:pub:F1D56D96-9807-497B-BB80-B24A20A77CCC
Gene sequence. SSU rRNA gene, accession number KJ493791.
Host. *Neotermes jouteli* Banks (Isoptera, Kalotermitidae, barcode JX847582).
Locality. Lat. 25.17608, long. −80.36945. Dagny Johnson Key Largo Hammock State Park, Monroe County, FL.
Host collection. University of Florida termite collection, accession number FL3188. Collector R. H. Scheffrahn. Collected April 9, 2008.
Archive material. SEM stub deposited at the Beaty Biodiversity Museum, University of British Columbia, Vancouver, Canada under accession number MI-PR109.

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[1]Present address: Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, Nova Scotia, B3H 4R2, Canada
[2]Present address:Biological Sciences Department, California State Polytechnic University Pomona, Pomona, California, 91768, USA