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ORIGINAL PAPER

Molecular Phylogenetic Position of *Microjoenia* (Parabasalia: Spirotrichonymphea) from *Reticulitermes* and *Hodotermopsis* Termite Hosts

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Microjoenia **are obligate symbionts of termites. The genus was erected in 1892 for small cells with many flagella that insert near, but not directly from, the cell apex, and an axostyle that can protrude from the cell posterior. Although ultrastructural studies have been carried out on three** ***Microjoenia*** **species to date, no molecular data have been directly attributed to any species.** ***Microjoenia*** **are classified within the parabasalian class Spirotrichonymphea, which is characterized by flagellar bands that emerge near the cell apex and proceed posteriorly in a right-handed helix. In** ***Microjoenia*****, however, the flagellar bands are very short and proceed longitudinally or with a weakly observable helix. In this study, we have amplified and sequenced the 18S ribosomal RNA gene from individually isolated** ***Microjoenia*** **cells from** ***Reticulitermes*** **and** ***Hodotermopsis*** **hosts as part of an ongoing effort to understand the phylogeny of Spirotrichonymphea and their coevolution with termites. In our 18S rRNA gene phylogeny,** ***Microjoenia*** **forms the sister lineage to** ***Spirotrichonympha*****, though many other evolutionary relationships within Spirotrichonymphea remain unresolved.**

Key words: Coevolution; Metamonada; symbiosis; termite.

Introduction

"Lower" termites (all termite families except the apical Termitidae) harbor wood-degrading symbiotic protists belonging to Parabasalia and Oxymonadida (Preaxostyla) in their hindguts (Brune 2014; Chouvenc et al. 2021). This digestive symbiosis began in the common ancestor of termites and their sister lineage, the wood-feeding roach *Cryptocercus*, roughly 175 million years ago, and has led to codiversification of hosts and symbionts (Bourguignon et al. 2015; Bucek et al. 2019; Ohkuma et al. 2009). Today, there are approximately 800 "lower" termite species, each of which harbors a characteristic set of protist symbiont species (Krishna et al. 2013; Yamin 1979). These obligately symbiotic protist species pose special challenges for study because they are very difficult, if not impossible, to maintain in culture.

Most of the termite symbiotic protist genera were described between 1881 and the mid-20th century from light microscopic data (Brugerolle and Lee 2000). Termite symbiotic protists tend to have much larger and more complex cells than those of their non-termite relatives (Čepička et al. 2017; Hampl 2017). However, morphological characteristics such as large cell

size, multiplied flagella, and multiplied nuclei have evolved convergently, according to molecular phylogenies (Gile et al. 2011; Gile and Slamovits 2012; Noda et al. 2012). Although ultrastructural and molecular studies have gradually built a higher-level systematic framework, roughly half of the described genera still lack molecular data, hampering our understanding of their diversity, systematics, and coevolutionary history with their hosts (Čepička et al. 2017; Hampl 2017).

Protist species of lower termites are highly host-specific. In some cases, they have parallel phylogenies to those of their hosts (Harper et al. 2009; James et al. 2013; Noda et al. 2007, 2018), and several parabasalian genera are restricted to a single termite host family. For example, *Pseudotrichonympha* is only found in Rhinotermitidae while *Devescovina* and *Calonympha* are only known from the Kalotermitidae (Yamin 1979). Together, these observations indicate a tight coevolutionary relationship between termite-associated protists and their hosts, in agreement with the vertical inheritance of protists by proctodeal trophallaxis (anal feeding). Host relationships can be useful for clarifying protist symbiont taxonomy. For example, the genus *Spirotrichonympha*, once thought to be widespread across termite families, was shown to be polyphyletic, with each separate clade consisting of symbionts of a single termite family (Brugerolle 2006; Gile et al. 2018; Jasso-Selles et al. 2017; Taerum et al. 2020). The objective of this study was to determine the molecular phylogenetic position of the previously uncharacterized termite symbiont genus *Microjoenia* in order to better understand relationships among termite-specific protist genera and their coevolution with termites.

The genus *Microjoenia* was erected in 1892 for *Microjoenia hexamitoides*, a symbiont of *Reticulitermes lucifugus* (then called *Termes lucifugus*) (Grassi 1892). Its morphology was later described more fully as a small, oval-shaped cell with an axial rod that protrudes from the posterior end, an anteriorly positioned nucleus, and many flagella originating from the apical portion of the cell but not the anterior extremity (Grassi 1917; Grassi and Sandias 1894). *Microjoenia* had been thought to show characteristics of both *Joenia* (Cristamonadida), by bearing many flagella at the cell anterior, and the distantly related *Trichonympha* (Trichonymphida), by having a bald spot, or operculum, at the extreme apex (Grassi 1917; Grassi and Sandias 1894). This combination of characteristics has led to a turbulent taxonomic history. *Microjoenia* has been considered a young form of *Trichonympha* (Leidy 1881), or a young form of *Spirotrichonympha* (Duboscq and Grassé 1928), or a genus of uncertain position

within the Lophomonadida (Brugerolle and Lee 2000). More recently, ultrastructural studies demonstrated that the flagella of *Microjoenia* arise from very short, longitudinal to slightly spiraling bands, confirming its Spirotrichonymphea affinities (Brugerolle 2001). Note that while Spirotrichosomidae (Trichonymphida, Parabasalia) also feature spiraling rows of flagella, they emerge from bilaterally symmetrical rostral structures characteristic of Trichonymphida, and their flagellar bands form a left-handed helix (Carpenter et al. 2010; Radek et al. 2018), thus excluding *Microjoenia*. The simple, tubular axostyle links *Microjoenia* specifically with *Spironympha* (Brugerolle 2005; Brugerolle and Bordereau 2006), another genus that currently lacks molecular data.

There are eight described species of *Microjoenia*, nearly all from *Reticulitermes* host species in Europe or North America (Brown 1930; Brugerolle 2001, 2005; Cutler 1920; Duboscq and Grassé 1928; Grassé 1952; Grassi 1892) (Table 1). The type species, *Microjoenia hexamitoides*, was described from *Reticulitermes lucifugus* in Sicily (Grassi 1892). One species inhabiting *Reticulitermes hesperus* was described as a distinct genus, *Torquenympha*, but was later synonymized with *Microjoenia* (Brown 1930; Yamin 1979). The non-*Reticulitermes* hosts include *Porotermes grandis* (Stolotermitidae, possibly a synonym of *P. adamsoni*) (Brugerolle 2001; Hill 1933), *Archotermopsis wroughtoni* (Archotermopsidae) (Cutler 1920), and *Hodotermopsis sjostedti* (Archotermopsidae) (Brugerolle 2005). Because the symbiont community of *H. sjostedti* bears close resemblance to those of *Reticulitermes* spp., likely due to horizontal symbiont transfer, the presence of *Microjoenia* in both host genera is not surprising (Kitade 2004). In this study, we have used a combination of single cell PCR and fluorescence *in situ* hybridization (FISH) to determine the molecular identity of *Microjoenia* species from *Reticulitermes flavipes*, *Reticulitermes tibialis*, and *Hodotermopsis sjostedti.*

Results and Discussion

Identification of *Microjoenia*

In the hindguts of *Hodotermopsis sjostedti*, *Reticulitermes tibialis*, *Reticulitermes flavipes*, and *Reticulitermes lucifugus,* we observed small cells (< 20 μm in length) with the morphological characteristics of *Microjoenia* (Fig. 1). These cells were sub-spherical to pyriform, bore many flagella near the apical end of the cell but not on the cell apex itself, and often had a posteriorly

protruding axostyle. These morphological features are similar to those of *Spironympha*, except that *Spironympha* cells are larger and the flagella arise from bands that spiral further around the apical portion of the cell (Koidzumi 1921). In *Microjoenia*, the flagella arise from similar bands, but the bands are short and do not complete a spiral turn around the cell (Brugerolle 2005; Brugerolle and Bordereau 2006). We also used protargol staining to visualize the subcellular features of *Microjoenia* from *H. sjostedti*. A darkly stained nucleus, darkly stained parabasal bodies arranged in a crown, and a faintly stained, tube-like axostyle are recognized, which are typical morphological features of this genus (Brugerolle and Lee 2000; Grassé 1952) (Fig. 1C-D).

In order to determine the molecular phylogenetic position of *Microjoenia*, we used a single-cell PCR approach. We isolated 7 single cells and 6 pools of 2-6 cells each from *H. sjostedti*, *R. flavipes*, and *R. tibialis* (we were unsuccessful with single cells from *R. lucifugus*) and amplified and sequenced their 18S rRNA genes (Table 2). These cells ranged in length from 6-17 μm, but we targeted the smallest cells with *Microjoenia* morphology in order to avoid isolating small *Spironympha* cells, which can look superficially similar to *Microjoenia* in live material. It is likely that other *Microjoenia* cells in our samples exceeded 17 μm in length, given that previous reports from *H. sjostedti* and *Reticulitermes* spp. indicated cell lengths of 20 μm (Brugerolle 2005; Brugerolle and Bordereau 2006; Grassé 1952). All new sequences from *Microjoenia* cells clustered together in a moderately supported clade sister to *Spirotrichonympha* (Fig. 2). Within this clade were several supported subclades and several previously published sequences from termite guts.

Microjoenia* from *H. sjostedti

The *H. sjostedti* symbiont sequences formed two clades, one that included the previously sequenced clone HsS1 (AB032236) while the other included HsS2 (AB032237). Both HsS1 and HsS2 were obtained from the small cell fraction after differential centrifugation of *H. sjostedti* hindgut contents and were considered to derive from either *Microjoenia* or *Holomastigotes* (Ohkuma et al. 2000). A later ultrastructural study described *Microjoenia minima* from *H. sjostedti* (Brugerolle 2005) and speculated that its 18S rRNA gene sequence was likely to match either the HsS1 or HsS2 clones (Brugerolle 2005). Our new single cell data indicate that both HsS1 and HsS2 derive from *Microjoenia* cells, and their distinct positions in our phylogeny,

separated by Asian *Reticulitermes* symbionts, suggest that these represent two distinct species. We were unable to detect morphological differences that might distinguish these two species. Likewise, the ultrastructural methods employed in the description of *M. minima* would have been unable to distinguish between two species, and therefore the description of *M. minima* was likely based on data from cells of both phylotypes (Brugerolle 2005).

To confirm the molecular identity of *Microjoenia*, we also carried out FISH using a probe designed from clone M1 from a previously generated library of amplified 18S rRNA gene sequences obtained by whole gut RT-PCR of *H. sjostedti* (Fig. 3). The M1 sequence belongs to the same subclade of *Microjoenia* as HsS2, and the probe matches both HsS1 and HsS2 sequences. In agreement with our single cell PCR data, the 6FAM-tagged *Microjoenia*-specific probe hybridized to small cells with the morphological characteristics of *Microjoenia*, while the Texas Red-tagged general eukaryotic probe hybridized to a range of cell types (Fig. 3).

Microjoenia* from *Reticulitermes

Sequences from North American and European *Reticulitermes* symbionts formed a clade to the exclusion of those from *H. sjostedti* and the Asian *Reticulitermes* spp. This European/North American clade included, as its deepest branch, sequences from *Reticulitermes hesperus* and *Reticulitermes okanaganensis* that were amplified from *Spirotrichonympha* cell isolates but branched separately (Gile et al. 2018). It is now clear that these were contaminating sequences from *Microjoenia* cells. In the case of *R. hesperus*, they might represent either *Microjoenia ratcliffei* or *Microjoenia (=Torquenympha) octoplus*, which were both described from this host. Also within this clade, *R. flavipes* symbionts branched in three places, though only two were supported clades. Sequences from the cell PES6 (collected in New Jersey, USA) formed a supported clade with the previously published U17508, which was amplified from the gut contents of *R. flavipes* collected in Woods Hole, Massachusetts, USA (Gunderson et al. 1995). Sequences from PES9 and PES24 formed an additional supported clade while sequences from the cells FPS10 and FPS21 branched separately. Although our *R. flavipes* sequences suggest that there should be two or three *Microjoenia* species present, only one has been described so far: *M. fallax* from *R. flavipes* (formerly known as *R. santonensis*) collected in France (Grassé 1952). This symbiont was initially considered a young form of the *Spirotrichonympha* cells in this host (Duboscq and Grassé 1928). Finally, sequences from *R. tibialis* symbiont cells RDAM and

RDCM clustered together near the *R. flavipes* symbiont cells FPS10 and FPS21, but neither their monophyly nor their sister relationship with the FPS10 and FPS21 cells were supported. No species of *Microjoenia* from *R. tibialis* have been named or formally described to date.

Evolution of Spirotrichonymphea

The sequences reported here from *Microjoenia* cells demonstrate that *Microjoenia* is a valid genus, not a life cycle stage of *Spirotrichonympha*. In particular, the symbionts of *R. hesperus* demonstrate this, because sequences from isolated cells of both *Spirotrichonympha* and *Microjoenia* have been determined from this host. Furthermore, *H. sjostedti* harbors *Microjoenia* but no true *Spirotrichonympha*, which appears to be restricted to *Reticulitermes* hosts (Gile et al. 2018). *Spirotrichonympha cincta*, reported to be the only *Spirotrichonympha* in *H. sjostedti*, branches separately (AB032226, Fig. 2), and should be placed in a new genus (Brugerolle 2005). The sequences reported here from *Microjoenia* cells also support the inclusion of this genus within Spirotrichonymphea, in agreement with ultrastructural characteristics, as opposed to the earlier suggested affinity to the "lophomonads" (Cristamonadida, formerly Lophomonadida) (Brugerolle 2001; Brugerolle and Bordereau 2006; Brugerolle and Lee 2000).

Currently a family-level taxonomy is lacking for Spirotrichonymphea. Because of the lack of morphotype-linked molecular data and the difficulty of establishing monophyletic families on the basis of ultrastructural data, recent classification schemes have lumped all Spirotrichonymphea genera into a single family, Holomastigotoididae (Čepička et al. 2010, 2017). Gradually, molecular data have been improving the situation. The placement of true *Spirotrichonympha* (i.e., from *Reticulitermes* hosts) was determined (Gile et al. 2018), and *Spirotrichonympha* species from *Coptotermes* and *Heterotermes* hosts were split out into the reinstated *Cononympha* (Jasso-Selles et al. 2017, 2020), while *Spirotrichonympha* from *Paraneotermes* was split into the new genus *Cuppa* (Taerum et al. 2020). Additionally, the 18S rRNA gene sequences of *Holomastigotes* were determined (Taerum et al. 2019) and a new genus *Fraterculus* was established (Taerum et al. 2020). In total, there are now 7 Spirotrichonymphea genera with directly attributed molecular data (*Cononympha*, *Cuppa*, *Fraterculus*, *Holomastigotes*, *Holomastigotoides*, *Microjoenia*, and *Spirotrichonympha*), and 6 described genera for which molecular data are still lacking (*Micromastigotes*, *Rostronympha*, *Spiromastigotes*, *Spironympha*, *Spirotrichonymphella*, and *Uteronympha*). Unfortunately, 18S

rRNA gene sequences seem to be insufficient for determining inter-generic relationships in Spirotrichonymphea. Here, we recovered a sister relationship between *Microjoenia* and *Spirotrichonympha*, and the *Paraneotermes* symbionts *Cuppa* and *Fraterculus* also have a supported sister relationship (Fig. 2), but other relationships remain unresolved. Future studies should characterize additional Spirotrichonymphea genera with additional phylogenetic markers.

Another interesting question for future studies would be whether the *Microjoenia* species described from *P. grandis* and *A. wroughtoni* are truly *Microjoenia*. In this study, we did not acquire a sequence from the type species, *M. hexamitoides*, from its type host, *R. lucifugus*, but we did acquire *Microjoenia* single cell sequences from three related host species which all formed a clade (Fig. 2), and the *Microjoenia* cells we observed in *R. lucifugus* had very similar morphology to the sequenced cells (Fig. 1F). Therefore, it is quite likely that the type species (*M. hexamitoides*) from the type host (*R. lucifugus*) would branch with the other *Reticulitermes* symbionts, as was the case for *Holomastigotes elongatum* from its type host (also *R. lucifugus*) (Taerum et al. 2019). While *H. sjostedti* is not closely related to *Reticulitermes*, it shares many of the same symbiont genera, likely due to horizontal symbiont transfer (Kitade 2004). If the *Microjoenia* symbionts of *P. grandis* and *A. wroughtoni* were found to branch with true *Microjoenia*, they would be the first symbionts from hosts other than *Reticulitermes* and *H. sjostedti* to branch in the *Holomastigotes/Spirotrichonympha/Microjoenia* portion of the Spirotrichonymphea phylogeny (Fig. 2). Perhaps they will instead branch separately, in which case they should be transferred to a new genus (or genera), as was the case for *Spirotrichonympha* symbionts from non-*Reticulitermes* hosts (Gile et al. 2018; Jasso-Selles et al. 2017; Taerum et al. 2020).

Methods

Termite collections: *Hodotermopsis sjostedti* was collected in Tam Dao (Vietnam), *R. flavipes* in New Jersey (USA), *R. lucifugus* in Corsica (France), and *R. tibialis* in Arizona (USA). The identities of these same termite collections were confirmed previously by molecular barcoding using the mitochondrial 16S rRNA gene (Taerum et al. 2019) and voucher specimens for all but *R. lucifugus* were deposited in the Hasbrouck Insect Collection at Arizona State University (ASUHIC) under accessions ASUHIC0095054-ASUHIC0095056 (Taerum et al. 2019). Additionally, *H. sjostedti* was collected in Kagoshima (Japan) for protargol staining and FISH (this study).

Protist observation and sequencing: Hindguts of termite workers were dissected from live termites and macerated in Ringer's solution (8.5 g NaCl, 0.20 g KCl, 0.20 g $CaCl_2$, 0.10 g $NaHCO_3$ per liter, HiMedia Laboratories). Live *Microjoenia* cells were viewed using an AxioImager upright compound microscope and photographed using an AxioCam 503 monochrome camera (Zeiss). Protargol-stained cells were prepared as previously described (Honigberg and Davenport 1954; Kitade et al. 1997) and imaged on an Olympus BX-63 compound microscope.

Individual *Microjoenia* cells were observed on a Zeiss AxioVert inverted compound microscope, photographed using an Axiocam 105 color camera (Zeiss), and isolated using hand-drawn glass capillaries. Each isolated *Microjoenia* cell was washed twice in fresh Ringer's solution and ejected into a 0.5 ml tube for DNA extraction using the MasterPure DNA Purification Kit (Epicentre, Madison, Wisconsin, USA) following the manufacturer's protocol except purified DNA was resuspended in 5 µl of TE buffer. The 18S rRNA gene was amplified from purified DNA from isolated single cells using a nested PCR approach, using outer primers SpiroF1/R1 and inner primers GGF/GGR as previously described (Jasso-Selles et al. 2017; Taerum et al. 2019). PCR products were ligated into the pCR 4-TOPO vector using the TOPO TA Cloning Kit and cloned with the One Shot TOP10 chemically competent *E. coli* (Invitrogen, Carlsbad, California, USA), following the manufacturer's protocols. Inserts from positive transformant colonies were amplified using standard sequencing primers M13F and M13R, purified using the GeneJet PCR purification kit (ThermoFisher, Waltham, MA, USA), and sequenced on both strands using an Applied Biosystems 3730 capillary sequencer (Applied Biosystems, Waltham, Massachussetts, USA). Two representative sequences from each isolated cell were selected for detailed phylogenetic analyses and were submitted to GenBank under accession numbers MZ663673-MZ663698.

Fluorescence in-situ hybridization: Previously, we obtained a large clone library of 18S rRNA gene sequences amplified by RT-PCR from the hindgut of *H. sjostedti* (unpublished data). Several of these were expected to belong to Spirotrichonymphea. In order to identify the genus *Microjoenia,* a probe specific for the sequence M1 was designed (HsM1-FAM: GACCCCACCCGTAGATGT), and we carried out fluorescence in-situ hybridization (FISH) as described previously (Noda et al. 2003, 2018). Briefly, termite gut contents were fixed in 4% paraformaldehyde. Fixed cells were then spotted onto a silane-coated glass slide (Matsunami Glass, Osaka, Japan), dehydrated in ethanol, and incubated in hybridization solution (0.9 M NaCl and 0.1 M Tris-HCl) containing fluorescently labeled probes at 48 ºC for 2 h. Specimens were then washed for 20 min in a washing buffer (0.2 M NaCl and 0.1 M Tris-HCl) at 48 ºC, mounted using a Fluoro-Keeper antifade reagent (Nacalai Tesque, Kyoto, Japan), and observed under an Olympus BX-63 epifluorescence microscope. The *Microjoenia*-specific probe was tagged with 6-carboxyfluorescein (6-FAM), while the general eukaryotic probe Euk1190: GGRCATCACRGACCTGTTAT was tagged with Texas Red (Amann et al. 1995; Ohkuma et al. 2000).

Phylogenetic analyses: Parabasalian 18S rRNA gene clone sequences from isolated single cells were trimmed of vector and sequences from both strands of each clone were assembled using Geneious R9 (Kearse et al. 2012). New 18S rRNA gene sequences were aligned with representatives of all previously published Spirotrichonymphea species and environmental clades using MAFFT v. 7.205 (Katoh and Standley 2013) with iterative refinement using the G-INS-i option. Ambiguously aligned sites were removed by eye in AliView (Larsson 2014). Preliminary analyses including all new and published Spirotrichonymphea sequences. Final analyses used a reduced alignment with all *Cononympha* spp. and *Holomastigotoides bigfooti* sequences removed, after noting that these sequences failed a chi-square test of base composition carried out in IQ-TREE v. 1.6.12 web server (Nguyen et al. 2015, Trifinopoulos et al. 2016). The reduced alignment had a final size of 77 taxa and 1,420 sites.

Maximum Likelihood (ML) and Bayesian phylogenetic analyses were performed using IQ-TREE v. 1.6.12 (Nguyen et al. 2015) and MrBayes v. 3.2.6 (Ronquist et al. 2012) respectively. ML analyses used the TIM3e+R3 model as specified by ModelFinder implemented in IQ-TREE (Kalyaanamoorthy et al. 2017), which was considered the best fit for the data under the Bayesian Information Criterion (the best fit model under both the Akaike and Corrected Akaike Information Criteria was GTR+F+R3). Support for nodes was assessed from 1,000 ultrafast bootstrap replicates (Hoang et al. 2018). Bayesian analyses were carried out under the GTR model with four evolutionary rate categories approximated by a gamma distribution. Two independent chains, sampled once every 100 generations, were run until they converged (the average standard deviation of partition frequency values between the chains dropped below 0.01). Convergence was reached after 240,000 generations. The first 25% of trees were then discarded as burn-in and majority rule consensus trees were computed from the remaining 3602 trees from both runs. Support at nodes is given by posterior probabilities.

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Tables

Figure Legends

Figure 1. Light micrographs of *Microjoenia*. **A**. *Microjoenia* sp. from *Reticulitermes tibialis*. Note the diagnostic distribution of flagella near the cell apex but not on the apical pole. The posteriorly protruding axostyle is barely visible beyond the plane of focus of this specimen. Differential interference contrast (DIC) optics. **B-D**. *Microjoenia* sp. from *Hodotermopsis sjostedti*, displaying anteriorly positioned flagella and bare apical pole. **B**. DIC optics. **C-D**. Protargol-stained specimens, brightfield optics. The parabasal bodies (dictyosomes with associated fibers) are visible as short, darkly staining granules surrounding the cell apex. Nucleus

(n) and axostyle (ax) are indicated. **E**. *Microjoenia* sp. from *Reticulitermes flavipes*, DIC. **F**. *Microjoenia* sp. from *Reticulitermes lucifugus*, DIC. Scale bars = 10 μm.

Figure 2. Maximum likelihood phylogeny of Spirotrichonymphea 18S rRNA gene sequences with outgroup Tritrichomonadida. *Cononympha* sequences are not included due to anomalous base frequencies. New *Microjoenia* sequences determined in this study are indicated by bold type. Support for nodes is given when equal to or greater than 90% ultrafast bootstrap support and greater than 0.95 Bayesian posterior probability. Filled circles at nodes indicate full support, i.e., 100/1.0. Images of isolated cells are inset at right and labeled with their cell code that corresponds to the clone names in the tree. Scale bars = 10 μm.

Figure 3. Fluorescence detection of *Microjoenia* sp. from *Hodotermopsis sjostedti*. The HsM1-specific probe, labeled with 6FAM (green) and the general eukaryotic probe, labeled with Texas Red (red), were used simultaneously. **A-C**. Images taken from a slide region including *Pyrsonympha* cells (elongated, top left and bottom middle), *Trichomonoides trypanoides* (arrows), and *M. minima* (arrowheads). **A**. Green wavelength filter, 6FAM label is only applied to one cell. **B**. Red wavelength filter, TexasRed label is applied to all protist cells. **C**. Phase contrast micrograph. The cell that was labeled in A bears multiple flagella near its apex and a protruding axostyle at its posterior, confirming the identification of *Microjoenia*. **D-F**. Images taken from another slide region, with filters and phase contrast imaging as for A-C. **D**. Green wavelength filter, 6FAM label is only applied to two small cells. **E**. Red wavelength filter, TexasRed label is applied to all protist cells. **F**. Phase contrast micrograph. Cells that were labeled in D are very small (<10 μm). Scale bars = 20 μm.

Michael Melkonian
Editor-in-Chief, *Protist*

20 September 2021

Dear Dr. Melkonian,

Please find included with this letter a revised manuscript entitled “Molecular phylogenetic position of *Microjoenia* (Parabasalia: Spirotrichonymphea) from *Reticulitermes* and *Hodotermopsis* termite hosts”. We have incorporated the reviewers’ suggestions and we hope

that it is sufficiently improved for publication in your journal. Specific reponses to reviewer comments are included below.

All of the work presented in this manuscript is original, has not been published elsewhere, and is not currently under consideration for publication elsewhere. All authors have seen the final version of this manuscript and approved its submission. The manuscript has been formatted according to instructions for *Protist*.

We look forward to further correspondence regarding our manuscript.

Yours sincerely,
Gillian Gile

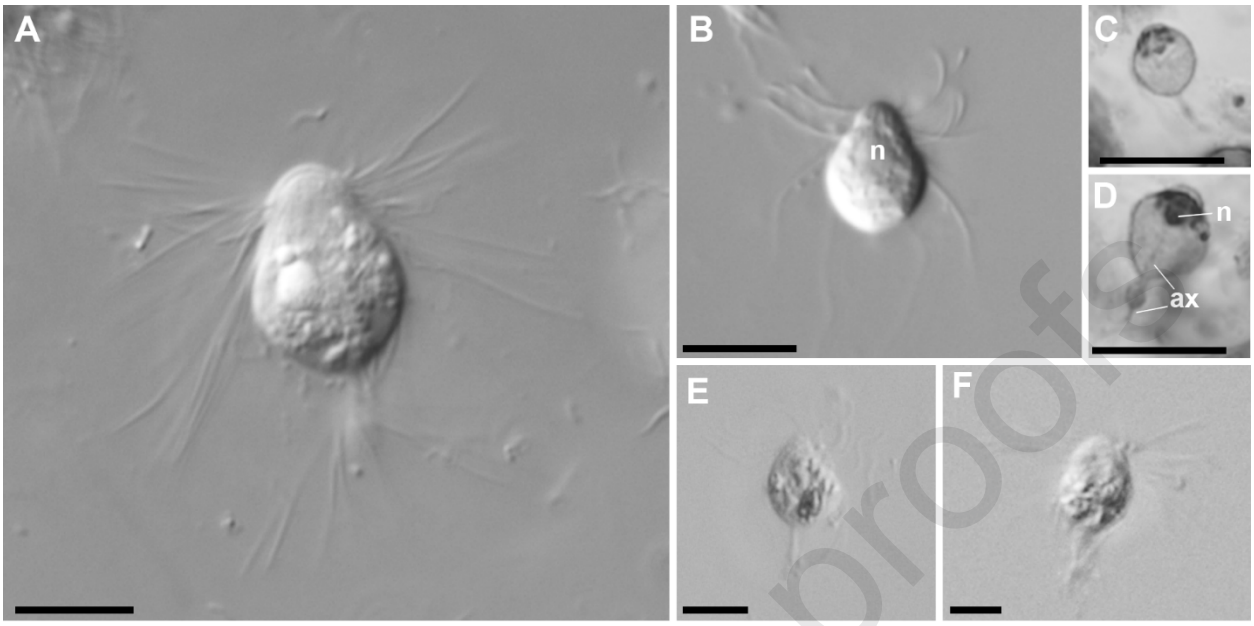
On behalf of my co-authors:

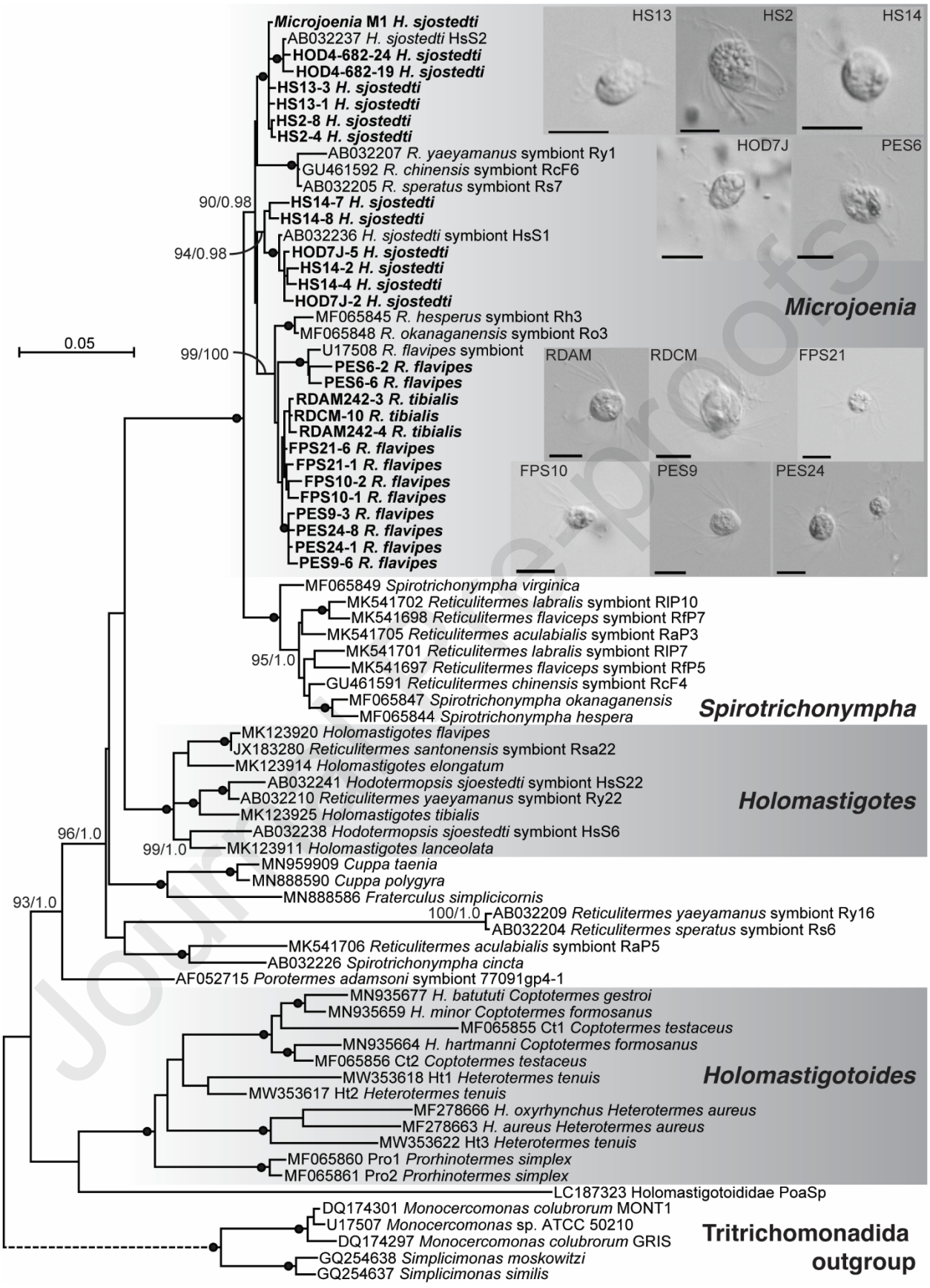
Dr. S. J. Taerum
Mr. D.E. Jasso-Selles
Dr. Sillam-Dussès
Dr. Moriya Ohkuma
Dr. Osamu Kitade
Dr. Satoko Noda

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:




Microjoenia M1 H. sjostedti
AB032237 H. sjostedti HsS2
HOD4-682-24 H. sjostedti
HOD4-682-19 H. sjostedti
HS13-3 H. sjostedti
HS13-1 H. sjostedti
HS2-8 H. sjostedti
HS2-4 H. sjostedti
AB032207 R. yaeyamanus symbiont Ry1
GU461592 R. chinensis symbiont RcF6
AB032205 R. speratus symbiont Rs7
90/0.98
HS14-7 H. sjostedti
HS14-8 H. sjostedti
AB032236 H. sjostedti symbiont HsS1
94/0.98
HOD7J-5 H. sjostedti
HS14-2 H. sjostedti
HS14-4 H. sjostedti
HOD7J-2 H. sjostedti
MF065845 R. hesperus symbiont Rh3
MF065848 R. okanaganensis symbiont Ro3
0.05
99/100
U17508 R. flavipes symbiont
PES6-2 R. flavipes
PES6-6 R. flavipes
RDAM242-3 R. tibialis
RDCM-10 R. tibialis
RDAM242-4 R. tibialis
FPS21-6 R. flavipes
FPS21-1 R. flavipes
FPS10-2 R. flavipes
FPS10-1 R. flavipes
PES9-3 R. flavipes
PES24-8 R. flavipes
PES24-1 R. flavipes
PES9-6 R. flavipes
HS13
HS2
HS14
HOD7J
PES6
RDAM
RDCM
FPS21
FPS10
PES9
PES24
Microjoenia
MF065849 Spirotrichonympha virginica
MK541702 Reticulitermes labralis symbiont RlP10
MK541698 Reticulitermes flaviceps symbiont RfP7
MK541705 Reticulitermes aculabialis symbiont RaP3
95/1.0
MK541701 Reticulitermes labralis symbiont RlP7
MK541697 Reticulitermes flaviceps symbiont RfP5
GU461591 Reticulitermes chinensis symbiont RcF4
MF065847 Spirotrichonympha okanaganensis
MF065844 Spirotrichonympha hespera
Spirotrichonympha
MK123920 Holomastigotes flavipes
JX183280 Reticulitermes santonensis symbiont Rsa22
MK123914 Holomastigotes elongatum
AB032241 Hodotermopsis sjoestedti symbiont HsS22
AB032210 Reticulitermes yaeyamanus symbiont Ry22
MK123925 Holomastigotes tibialis
96/1.0
AB032238 Hodotermopsis sjoestedti symbiont HsS6
99/1.0
MK123911 Holomastigotes lanceolata
Holomastigotes
MN959909 Cuppa taenia
MN888590 Cuppa polygyra
93/1.0
MN888586 Fraterculus simplicicornis
100/1.0
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AB032204 Reticulitermes speratus symbiont Rs6
MK541706 Reticulitermes aculabialis symbiont RaP5
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MN935677 H. batututi Coptotermes gestroi
MN935659 H. minor Coptotermes formosanus
MF065855 Ct1 Coptotermes testaceus
MN935664 H. hartmanni Coptotermes formosanus
MF065856 Ct2 Coptotermes testaceus
MW353618 Ht1 Heterotermes tenuis
MW353617 Ht2 Heterotermes tenuis
MF278666 H. oxyrhynchus Heterotermes aureus
MF278663 H. aureus Heterotermes aureus
MW353622 Ht3 Heterotermes tenuis
MF065860 Pro1 Prorhinotermes simplex
MF065861 Pro2 Prorhinotermes simplex
Holomastigotoides
LC187323 Holomastigotoididae PoaSp
DQ174301 Monocercomonas colubrorum MONT1
U17507 Monocercomonas sp. ATCC 50210
DQ174297 Monocercomonas colubrorum GRIS
GQ254638 Simplicimonas moskowitzi
GQ254637 Simplicimonas similis
Tritrichomonadida outgroup

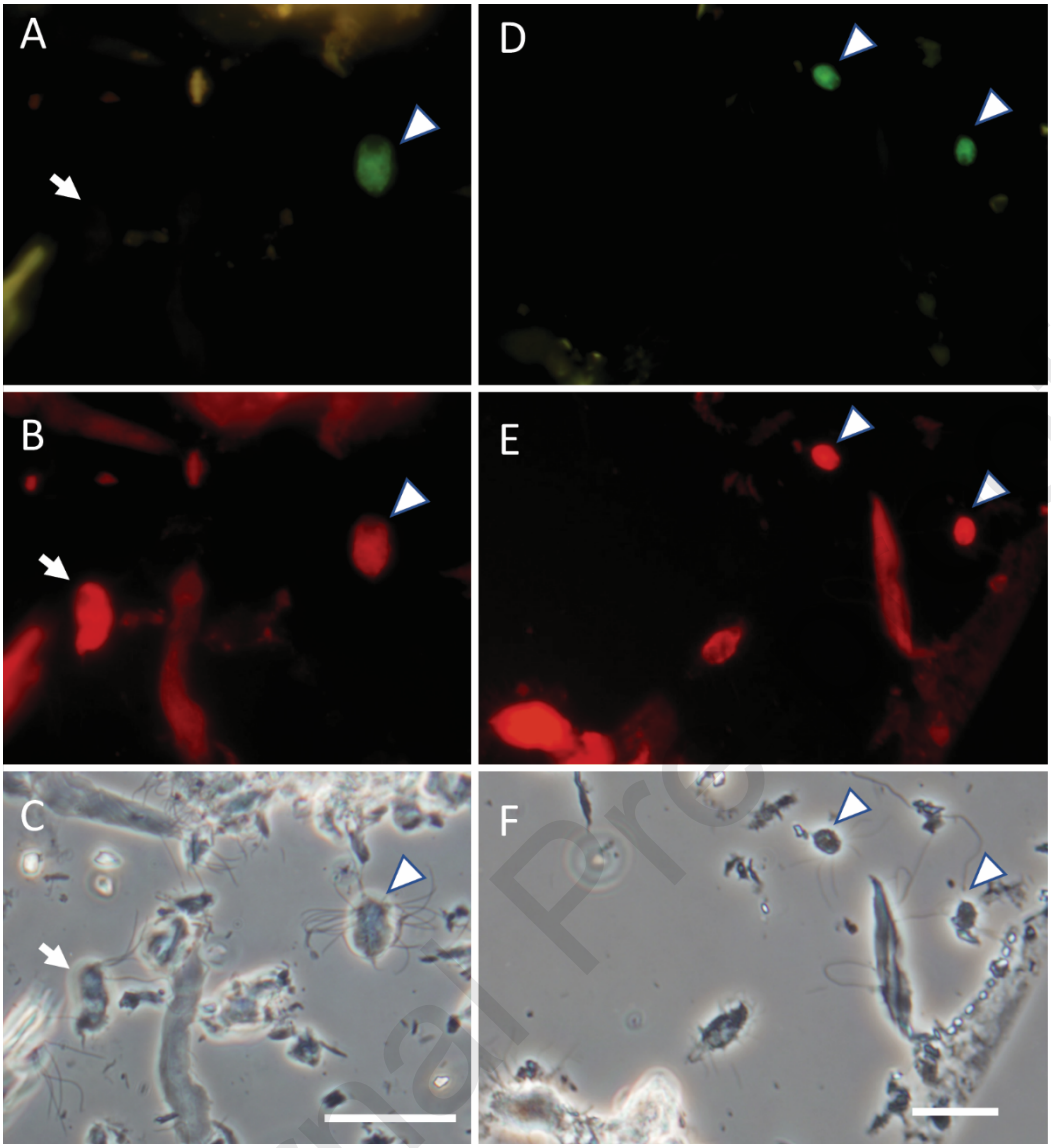

Table 1. Species of *Microjoenia*.

Species	Host	Reference
M. fallax	*Reticulitermes flavipes (= R. santonensis)*	Grassé 1952, Brugerolle & Bordereau 2006 emended
M. hexamitoides	*Reticulitermes lucifugus*	Grassi 1892
M. octoplus (=Torquenympha octoplus)	*Reticulitermes hesperus*	Brown 1930
M. ratcliffei	*Reticulitermes hesperus*	Brown 1930
M. pyriformis	*Reticulitermes hageni*	Brown 1930
M. anterodepressa	*Porotermes grandis*	Brugerolle 2001
M. minuta	*Hodotermopsis sjostedti*	Brugerolle 2005
M. axostylis	*Archotermopsis wroughtoni*	Cutler 1920

Table 2. Host collections, cell isolations, and sequenced clones.

Cell code	Host species	Host locality	Number of cells	Number of clones sequenced
RDAM-242	*Reticulitermes tibialis*	Wolf Creek, Prescott, Arizona, USA (34.4548, -112.4848)	4	3
RDCM	*Reticulitermes tibialis*	Wolf Creek, Prescott, Arizona, USA (34.4548, -112.4848)	4	1
FPS-21	*Reticulitermes flavipes*	Eagle Rock Reservation, New Jersey, USA (40.8114, -74.2385)	1	5
FPS-10	*Reticulitermes flavipes*	Eagle Rock Reservation, New Jersey, USA (40.8114, -74.2385)	1	5
PES-9	*Reticulitermes flavipes*	Eagle Rock Reservation, New Jersey, USA (40.8114, -74.2385)	6	5
PES-24	*Reticulitermes flavipes*	Eagle Rock Reservation, New Jersey, USA (40.8114, -74.2385)	3	7
PES-6	*Reticulitermes flavipes*	Eagle Rock Reservation, New Jersey, USA (40.8114, -74.2385)	1	5
HOD4-682	*Hodotermopsis sjostedti*	Tam Dao National Park, Vietnam (21.4564, 105.61)	2	7
HOD7-J	*Hodotermopsis sjostedti*	Tam Dao National Park, Vietnam (21.4564, 105.61)	2	8
HS2	*Hodotermopsis sjostedti*	Tam Dao National Park, Vietnam (21.4564, 105.61)	1	8
HS13	*Hodotermopsis sjostedti*	Tam Dao National Park, Vietnam (21.4564, 105.61)	1	4
HS14	*Hodotermopsis sjostedti*	Tam Dao National Park, Vietnam (21.4564, 105.61)	1	7
M1 (clone)	*Hodotermopsis sjostedti*	Kagoshima, Japan	whole gut	N/A