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ORIGINAL ARTICLE

Morphology and Molecular Phylogeny of *Staurojoenina mulleri* sp. nov. (Trichonymphida, Parabasalia) from the Hindgut of the Kalotermitid *Neotermes jouteli*

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ABSTRACT

Staurojoenina is a large and structurally complex genus of hypermastigont parabasalians found in the hindgut of lower termites. Although several species of *Staurojoenina* have been described worldwide, all *Staurojoenina* observed to date in different species of North American termites have been treated as the same species, *S. assimilis*. Here, we characterize *Staurojoenina* from the North American termite *Neotermes jouteli* using light microscopy, scanning electron microscopy, and phylogenetic analysis of small subunit ribosomal RNA, and compare it with *S. assimilis* from its type host, *Incisitermes minor*. The basic morphological characteristics of the *N. jouteli* symbiont, including its abundant bacterial epibionts, are similar as far as they may be compared with existing data from *S. assimilis*, although not consistently identical. In contrast, we find that they are extremely distantly related at the molecular level, sharing a pairwise similarity of SSU rRNA genes comparable to that seen between different genera or even families of other parabasalians. Based on their evolutionary distance and habitat in different termite genera, we consider the *N. jouteli Staurojoenina* to be distinct from *S. assimilis*, and describe a new species, *Staurojoenina mulleri*, in honor of the pioneering parabasalian researcher, Miklos Muller.

THE genus *Staurojoenina* was erected by Grassi in 1917, who described *S. mirabilis* in *Epicalotermes aethiopicus* Silvestri as part of a large description of numerous new flagellates from several termites (Grassi 1917). The genus was next revisited in 1926 when Kirby undertook a thorough description of a new species, *S. assimilis*, from the North American termite, *Incisitermes* (*Kalotermes*) *minor* (Hagen). Even then, Kirby noted that he faced a dilemma in whether or not to distinguish the new specimen from the single other described species because of the incompleteness of the type description and the variability of cell size and shape; for most measurements, the range of observations overlapped between the two species. Kirby identified one possible distinguishing feature, the distance from the posterior end of the nucleus to the anterior end of the attractophores, but he chose to describe *S. assimilis* as a distinct species based on its generally larger size and on its being found in a distantly related termite (Kirby 1926).

Subsequent new *Staurojoenina* species have been described based on a variety of criteria (Grassé and Hollande 1942, 1945; Hollande 1986), but in some cases, the lack of obvious distinguishing features has led to the decision to expand the range of existing species to other termite hosts (Dolan and Margulis 1997; Maaβ and Radek 2006). In particular, investigation of North American kalotermitids revealed that symbionts in *Neotermes* (*Kalotermes*) *mona* (Banks) and *Neotermes* (*Kalotermes*) *jouteli* (Banks) so closely resembled *S. assimilis* from *I. minor* that the authors could not distinguish them and they were either treated as *S. assimilis* (Dolan et al. 2004) or *Staurojoenina* sp. (Dolan and Margulis 1997). In total, five *Staurojoenina* species have been described and named (Grassé and Hollande 1942; Grassi 1917; Hollande 1986; Kirby 1926), and three species have been described without provision of a name (Dolan and Margulis 1997; Kirby 1926; Maaβ and Radek 2006). With the exception of *S. assimilis*, reported to occur in five different termite host species (Dolan et al. 2004; Kirby 1926; Sutherland 1933), each *Staurojoenina* has a single unique host (Table 1). For an excellent summary of *Staurojoenina* species and their characteristics, see Maaβ and Radek (2006).

Here, we have re-investigated *Staurojoenina* from North American termites, in particular comparing those found in

Table 1. *Staurojoenina* species and their hosts.

Species	Host	Reference
S. mirabilis	*Epicalotermes aethiopicus*	Grassi 1917
S. assimilis	*Incisitermes (Kalotermes) minor*	Kirby 1926
	Bifiditermes condonensis	Kirby 1926
	Bifiditermes improbus (Kalotermes oldfeldi)	Sutherland 1933
	Neotermes jouteli	Dolan et al. 2004
	Neotermes mona	Dolan et al. 2004
S. caullyeri	*Postelectrotermes (Neotermes) praecox*	Grassé and Hollande 1942
S. corbeli	*Bifiditermes rogierae*	Hollande 1986
S. gracilis	*Bifiditermes madagascariensis*	Hollande 1986
S. sp.	*Marginitermes hubbardi*	Kirby 1926
S. sp.	*Neotermes* nr. *jouteli*	Dolan and Margulis 1997
S. sp.	*Neotermes cubanus*	Maaβ and Radek 2006

N. jouteli and *I. minor*, using light and electron microscopy and molecular phylogenetic methods. First, we have barcode identified all termites to confirm their identity (otherwise a common source of misidentified parabasalian symbionts). Next, we have characterized the overall morphology of the *N. jouteli Staurojoenina*, including the surface features of its dense coat of ectosymbiotic bacteria, thus confirming its close physical similarity to *S. assimilis* from *I. minor*. Finally, we sequenced the small subunit ribosomal RNA (SSU rRNA) genes of the *Staurojoenina* from *N. jouteli* and re-characterized the SSU rRNA gene from *S. assimilis* from *I. minor*. Despite their morphological similarity, these two *Staurojoenina* isolates are distantly related at the molecular level, separated by a level of divergence characteristic of other parabasalian families or orders. Based on its molecular distance from *S. assimilis* and its presence in a distantly related host termite, we describe the *N. jouteli* symbiont as a new species, *Staurojoenina mulleri*.

MATERIALS AND METHODS

Host termite collection and barcoding

Neotermes jouteli was collected on 9 April 2008, from Dagny Johnson Key Largo Hammock State Park, Monroe County, Florida (lat. 25.17608, long. −80. 36945). *Incisitermes minor* was collected on 31 October 2008 at Cibolo Creek, Shafter, Texas (29.82055, −104.30571). Specimens were deposited in the University of Florida termite collection under accessions FL3188 and US1284, respectively. All termites were maintained at room temperature in the laboratory.

Termite identities were determined morphologically and confirmed by barcoding. Genomic DNA was extracted from termite bodies after hindgut removal using the Masterpure Complete DNA and RNA Purification Kit (Epicentre, Madison, WI). For *Incisitermes minor*, a portion of the mitochondrial 16S ribosomal RNA (mt16S rRNA) 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). For *Neotermes jouteli*, a portion of the cytochrome C oxidase subunit II (COII) gene was amplified and sequenced using A-tLeu 5′-ATG GCA GAT TAG TGC AAT GG-3′ and B-tLys 5′-GTT TAA GAG ACC AGT ACT TG-3′ (Wirth et al. 1999). PCR conditions included a 3 min denaturation at 95 °C followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, then an additional 7 min at 72 °C. Both barcode sequences were submitted to GenBank under accessions JX847582 and JX847583.

Symbiont isolation, DNA extraction, PCR, and sequencing

Termites were dissected and hindgut contents were suspended in Trager's medium U (Trager 1934). Individual *Staurojoenina* cells were isolated by micropipette and pooled in samples of 20–50 cells for DNA extraction using the Masterpure Complete DNA and RNA Purification Kit (Epicentre, Madison, WI). 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 in the case of one of the *S. assimilis* reactions, amplified further with the nested SSU primers GGF 5′-CTT CGG TCA TAG ATT AAG CCA TGC-3′ and GGR 5′-CCT TGT TAC GAC TTC TCC TTC CTC-3′ (Gile et al. 2011). 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 both *Staurojoenina* species and assembled into contigs using Sequencher 4.2 (GeneCodes, Ann Arbor, MI) at a stringency of 98%. For *S. assimilis*, two independent PCR reactions were carried out using DNA from isolated cells from two termite individuals. Seven clone sequences from one reaction and one from the other reaction were assembled into a contig 1522 base pairs long, which showed nucleotide disagreements at 25 positions (1.6%), although no single clone differed from the consensus sequence by more than 5 nucleotides. For *S. mulleri*, DNA from isolated cells was used for one PCR and DNA from the whole gut contents was used for an environmental PCR. A contig assembled from one isolated cells clone sequence and three environmental PCR clone sequences showed nucleotide disagreements at 28 of 1573 positions (1.8%), with the four individual clones differing from the consensus by 1, 5, 5, and 12 nucleotides. This level of divergence among clones is within the range so far reported for SSU rRNA clones from termite symbiotic parabasalians (0–50 nucleotide differences, Saldarriaga et al. 2011). For both *Staurojoenina* species, the clone that most closely matched the consen-

sus sequence was used for phylogenetic analyses and submitted to GenBank under accession numbers JX847580 and JX847581.

Phylogenetic analyses

For termite barcoding, all available *Incisitermes* mt16S rRNA and *Neotermes* COII sequences were downloaded from GenBank, aligned with our sequences using MAFFT (Katoh and Toh 2010), and refined by eye using SeaView (Gouy et al. 2010). Sites with gaps in more than 10% of the sequences were removed with trimAl (Capella-Gutiérrez et al. 2009). This procedure resulted in a 365-site alignment for mt16S rRNA (78% of the raw 468-site alignment) and a 534-site alignment for COII (68% of the raw 779-site alignment). Maximum likelihood phylogeny estimation was carried out using RAxML 7.2.5 (Stamatakis 2006) using the GTR-GAMMA-I model of evolution. Support was inferred from 1000 bootstrap replicates.

For the parabasalid SSU phylogenetic analysis, new sequences from *S. assimilis* (from *I. minor*) and *S. mulleri* (from *N. jouteli*) were aligned with previously published sequences spanning the phylogenetic diversity of parabasalians using ClustalX (Thompson et al. 1997). Highly variable regions were removed using GBlocks 0.91b (Castresana 2000) with the minimum block length reduced to 2, the maximum number of contiguous unconserved positions increased to 20, and sites with gaps in up to half of the taxa included for a final alignment of 33 taxa and 1262 positions (76% of the raw 1667-site alignment). Maximum likelihood (ML) and Bayesian phylogenetic analyses were performed with RAxML 7.2.5 (Stamatakis 2006) and MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003), respectively, using the GTR + Γ + I model as specified by Modeltest v3.7 (Posada and Crandall 1998) under the Akaike Information Criterion, implemented in PAUP* 4.0b10 (Swofford 2003). For the ML analysis, support was assessed from 500 bootstrap replicates. For the Bayesian analysis, two independent chains, sampled each 100 generations, were run until they converged (the average standard deviation of partition frequency values between the chains dropped below 0.01) with 25% of the trees discarded as burn-in. Convergence was reached after only 170,000 generations. Consensus trees were computed from the saved trees of both runs, for a total of 2550 trees.

Light and electron microscopy

Hindgut contents suspended in Trager medium U were viewed on a Zeiss Axioplan 2 (Zeiss, Oberkochen, Germany) compound microscope using differential interference contrast and photographed with a QImaging MicroImager II (QImaging, Surrey, BC, Canada). Cell isolations were performed on a Zeiss Axiovert 200 (Zeiss, Oberkochen, Germany) inverted microscope. For scanning electron microscopy, a few drops of hindgut contents in Trager medium U were pipetted onto a Millipore Isopore membrane filter (pore size of 3 or 5 μm) held in a plastic Millipore Swinnex cartridge affixed to a 10 ml syringe. Five milliliters of 2.5% glutaraldehyde in Trager medium U buffer were then poured into the syringe, and material was allowed to fix for 30 min. The syringe barrel was then inserted and used to force the glutaraldehyde out of the cartridge. Immediately after, the cartridge was unscrewed from the syringe, and 10 ml of buffer was pulled into the syringe. Then the cartridge was screwed back on, and the buffer was pushed through to rinse. This was repeated once or twice before a few milliliters of 1% OsO_4 was pulled into the syringe (with the Swinnex cartridge off) and then placed into contact with the material, which was allowed to fix for 30 min. Following fixation, filters were rinsed with buffer, dehydrated in an ethanol series (50%, 70%, 90%, 2 × 100%), and critical point dried in a Tousimis Autosamdri 815B CO2 critical point dryer. Dried filters were affixed to aluminum SEM stubs with double-sided carbon sticky tape, and coated with 5 nm of Au or Au/Pd in a Cressington 208 HR sputter coater. Samples were examined and photographed in a Hitachi 4700 FESEM (Hitachi, Tokyo, Japan) at 3–5 kV.

RESULTS

Staurojoenina mulleri, sp. nov.

Taxonomic synopsis

Staurojoenina mulleri Gile and Keeling, **sp. nov.** (Fig. 2–11)

Type host: *Neotermes jouteli* (Banks)

Type locality: lat. 25. 17608, long. −80. 36945. Dagny Johnson Key Largo Hammock State Park, Monroe County, FL, USA.

Host collection: University of Florida termite collection, accession number FL3188. Collector R. H. Scheffrahn. Collected 9 April 2008.

Diagnosis: Large (115–190 μm long and 50–75 μm wide) mononucleate flagellate with four prominent anterior flagellated zones comprising hundreds of flagella and separated by prominent ectoplasmic lobes. Flagella emerge from thick parabasal plates. Found in the hindgut of the neotropical kalotermitid *Neotermes jouteli* and distinguished by SSU rRNA sequence.

Hapantotype: SEM stub deposited at the Beaty Biodiversity Museum, University of British Columbia, Vancouver, Canada under accession number MI-PR109.

Gene sequence: SSU rRNA accession number JX847580.

Etymology: This species is named in honor of Prof. Miklos Muller, a pioneer of parabasalian research, whose work transformed our understanding of their biochemistry and evolution, and who has been a longtime mentor and supporter of parabasalian researchers. While Muller is often spelled with a ü, Prof. Muller never uses "Mueller" and prefers "Muller", and so the specific epithet is mulleri rather than muelleri.

Barcode identification of *I. minor* and *N. jouteli*

There has been some confusion between host species in the description of their parabasalian symbionts, and

between *N. jouteli* and other species of *Neotermes* in particular (Gile et al. 2011). Moreover, the *Staurojoenina* isolates from *Neotermes* have sometimes been considered conspecific with *S. assimilis*, of which the type-host is *I. minor*. Therefore, we identified *N. jouteli* and *I. minor* using established DNA barcoding markers for lower termites, mitochondrial 16S rRNA (mt16S rRNA) and cytochrome oxidase C subunit II (COII). COII was used for *Neotermes* because it is the only marker that has been sequenced from *N. jouteli* and enough other species of *Neotermes* to ensure a correct identification, and mt16S rRNA was sequenced from *I. minor* for the same reason (neither marker could identify both species due to current barcode library sampling). Barcode sequences showed the two species to be identified correctly (Fig. 1). Our *I. minor* isolate shared 96% identity with other *I. minor* barcodes, and only 79% identity to the next nearest species, *I. snyderi* (Fig. 1A). Our *N. jouteli* isolate shared 99% identity with other identified *N. jouteli* isolates (Fig. 1B), and only 81% identity with the next nearest species, *N. castaneus* (which has been misidentified as *N. jouteli* previously, Gile et al. 2011).

Morphology of *S. mulleri*

The gut of *N. jouteli* was observed to contain the expected range of flagellates, including *Macrotrichomonas*, *Metadevescovina*, and *Oxymonas* (Yamin 1979), and a single morphologically distinguishable type of *Staurojoenina*, here described as *S. mulleri*, which fits all the criteria of the genus *Staurojoenina* (Grassi 1917; Kirby 1926). *Staurojoenina mulleri* is a large cell (length: 115–190 μm, mean = 144 μm, width: 50–75 μm, mean = 66 μm, $n = 18$) with four apical flagellated zones, each extending from near the anterior end to approximately half the length of the body (Fig. 2–4, 8, 9). The flagellated zones are subtended by thick parabasal plates that meet at the anterior end (Fig. 4, 5). In cells well preserved for SEM, the flagella can be seen to emerge from deep clefts separated by thick cytoplasmic lobes (Fig. 8, 9). The single large and distinctive nucleus is located centrally and posterior to the flagellated zones and attractophores (Fig. 4, 5). The distance from the posterior end of the nucleus to the anterior end of the attractophores was 61–68 μm (mean: 66 μm, $n = 7$), which is a similar range to those observed previously for other *Staurojoenina* species (Maaβ and Radek 2006). The posterior of the cell is typically oval in shape, though it can be greatly distended by wood particles (Fig. 3, 4, 8, 9). The size range of *S. assimilis* is recorded as 105–190 μm, mean 142 μm (Kirby 1926), which is nearly identical to the size range we observed for *S. mulleri*, whereas the nucleus-attractophore distance in *S. assimilis* is recorded as 57–65 μm, mean 60 μm, slightly smaller than the distances we observed in *S. mulleri*.

The most distinctive characteristic observed in SEM is a dense, uniform coat of epibiotic bacteria (Fig. 8–11), which is also visible in light microscopy (Fig. 6, 7). The bacteria are rod-shaped with tapered ends (Fig. 10, 11). The bacteria are semi-organized in longitudinal rows (head to tail), so they form visually pleasing patterns reminiscent of 'currents' on the surface of the cell (Fig. 8–11). These are not entirely random, as in every cell we observed that the currents meet at a specific sub-dorsal focal point (Fig. 8, 9 at the posterior of the cell to the left, and Fig. 10 for close-up): here, all the bacterial currents converge and a small cluster of bacteria can be seen protruding from the surface on their ends, as though pushed up (Fig. 10). We hypothesize that the bacteria are actively dividing on the surface and creating these currents along their division plane so that at the convergent point bacteria are physically pushed off the surface. These observations suggest the bacteria may therefore represent a homogeneous 'colony'.

The epibiotic bacteria we observed in LM and SEM are a conserved feature in other *Staurojoenina* descriptions. Kirby noted them and drew surface striations in *S. assimilis* (Kirby 1926). Hollande reported their presence on both *S. corbeli* and *S. mirabilis* (Hollande 1986). They were also noted in TEM on *S. assimilis* from *Neotermes mona* and given the name Candidatus *Cuticobacterium kirbyi* (Dolan et al. 2004; Wier et al. 2004) and on *Staurojoenina* sp. from *Neotermes cubanus*, named Candidatus *Vestibaculum illigatum* (Stingl et al. 2004).

In addition to the dominant rod-shaped bacteria, we consistently observed a second bacterial type in lower numbers and with a looser physical association. These bacteria were much longer and appeared morphologically similar to spirochetes (Fig. 8, 9 and Fig. 10, 11 for detail). They were scattered on the surface and loosely connected, but were always observed and we therefore conclude that they are likely in a specific association with *S. mulleri*.

Phylogenetic analysis of *S. mulleri*

The SSU rRNA gene from *S. mulleri* was sequenced from a pool of 40 manually isolated single cells and from a pool of hindgut contents. Nearly identical sequences were found in all cases (98% identity across all clones). The phylogenetic position of *S. mulleri* SSU rRNA was inferred using maximum likelihood and Bayesian methods, which consistently placed *S. mulleri* with strong support as sister to the only other *Staurojoenina* sequence characterized to date, *S. assimilis* (Ohkuma et al. 2005) (Fig. 12). Together, *S. mulleri* and *S. assimilis* branch as sister to *Trichonympha*, albeit with poor support, both falling within the Trichonymphida, as seen in previous analyses (Carpenter et al. 2009; Cepicka et al. 2010; Ohkuma et al. 2005). The *S. assimilis* sequence was acquired from a termite identified as *I. minor* (Ohkuma et al. 2005), but there is no molecular record of its host termite identification. Because the *N. jouteli* symbiont has been referred to as *S. assimilis* in the past (Dolan et al. 2004), and because the type host of *S. assimilis* is *I. minor*, it is particularly important to confirm the identity of the *I. minor* symbiont. Therefore, we re-sequenced the *S. assimilis* SSU rRNA from a pool of 20 *Staurojoenina* cells manually isolated from termites confirmed by barcoding to be *I. minor*. Sequenced clones were found to share 98% identity with the existing *S. assimilis* sequence (Fig. 12), comparable to the variation we observed between clones of *S. mulleri* rRNA sequences.

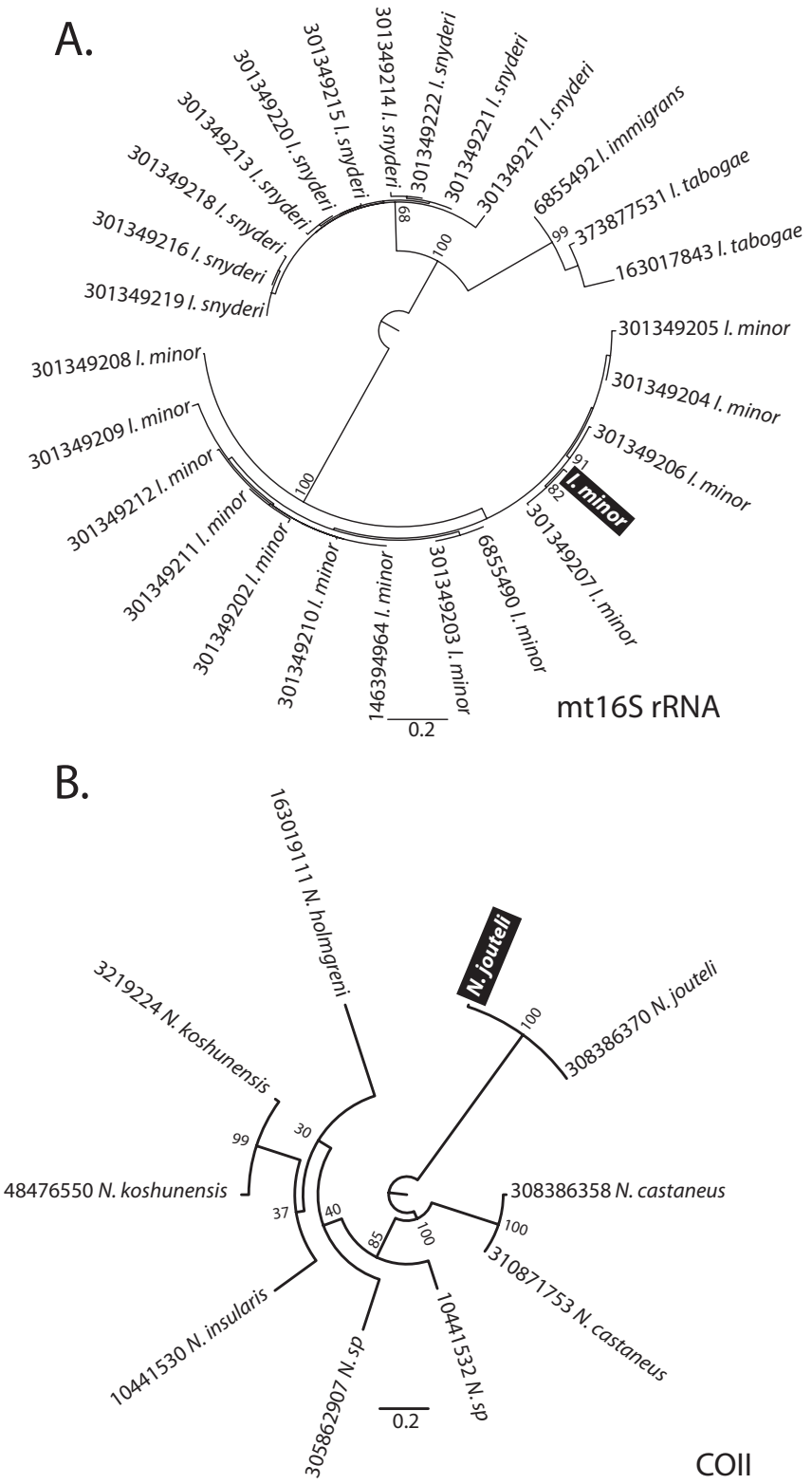

Fig. 1. Barcoding termites to confirm the identification of *Incisitermes minor* and *Neotermes jouteli*. (**A**) Maximum likelihood tree of mt16S rRNA barcodes from the genus *Incisitermes*, showing the Texas isolate to share a near-identical barcode with other *I. minor* isolates. The nearest sister species (*I. snyderi*) shares 79% identity. (**B**) Maximum likelihood tree of COII barcodes from the genus *Neotermes*, showing the Florida isolate to share a near-identical barcode with the only other *N. jouteli* sequence. The nearest sister species (*N. castaneus*) shares 81% identity.

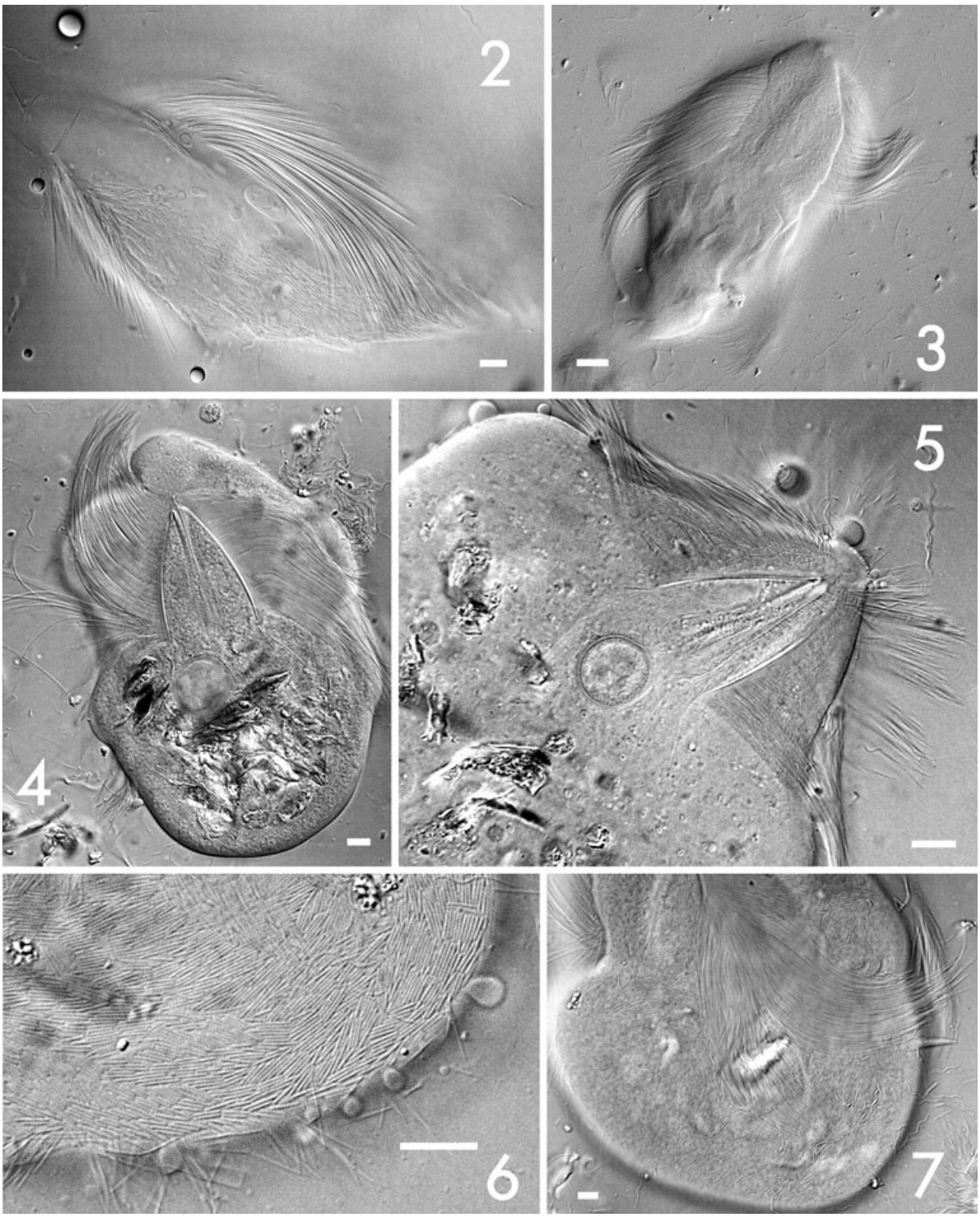

Fig. 2–7. Differential interference contrast (DIC) light micrographs of living *Staurojoenina mulleri*. **Fig. 2, 3:** overall body plan showing the basic shape and cell size, and the multiple symmetrical emergence of flagellar zones. **Fig. 4, 5:** internal detail, including the single central nucleus and the V-shaped parabasal plates with associated flagellar zones extending from the apex to the center of the cell just anterior of the nucleus. The large and refractile bodies in the cell posterior are ingested fragments of wood. **Fig. 6, 7:** surface morphology by DIC shows regular striations that are rod-shaped bacteria and less frequent spiral bodies that are spirochete bacteria (see also Figs 8–11).

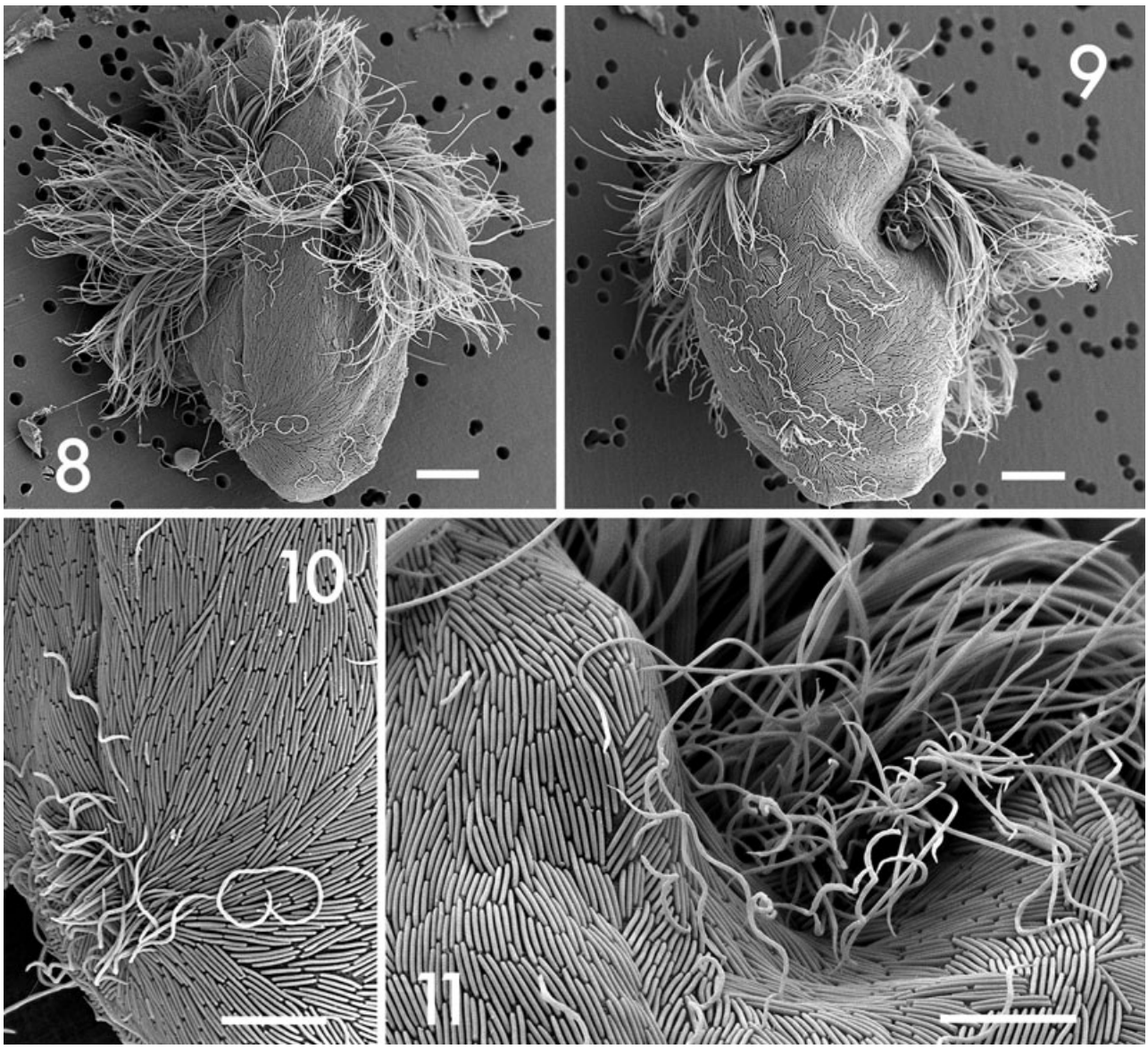

Fig. 8–11. Scanning electron micrographs of *S. mulleri*. **Fig. 8, 9:** two aspects of *S. mulleri* showing the overall shape and size of the cell and the multiple flagellar zones separated by large cytoplasmic lobes. All non-flagellated surfaces are completely covered in a monolayer of rod-shaped bacteria with identical morphology. The bacteria are oriented to form a pattern with a convergence point at a slightly sub-posterior location in all cells observed. At this point (see **Fig. 10** for detail), the converging bacteria appear to be physically forced off the cell surface and take on a vertical orientation. In addition to the monolayer of rod-shaped bacteria, there is a consistent presence of larger spirochete bacteria (see **Fig. 11** for detail).

With the *S. assimilis* sequence from the type host confirmed, we can conclude that the *N. jouteli* symbiont is evolutionarily distinct from *S. assimilis*. Despite being morphologically similar, the two symbionts share only 82% sequence identity, a level of divergence inconsistent with a single species in other parabasalians. Indeed, in the tree in Fig. 12, lower levels of divergence are observed between pairs of genera (e.g. *Trichomonas* and *Trichomitopsis*), and even between species in different classes (e.g. *Devescovina* and *Trichomitus*). SSU rRNA genes in Trichonymphida tend to evolve quickly, but even still the level of divergence is far beyond any two isolates of a species, reinforcing our conclusion that *S. assimilis* and *S. mulleri* are distinct species.

DISCUSSION

Each new species of *Staurojoenina* has been described using different criteria than those described before. When Kirby described the second species, he noted that the

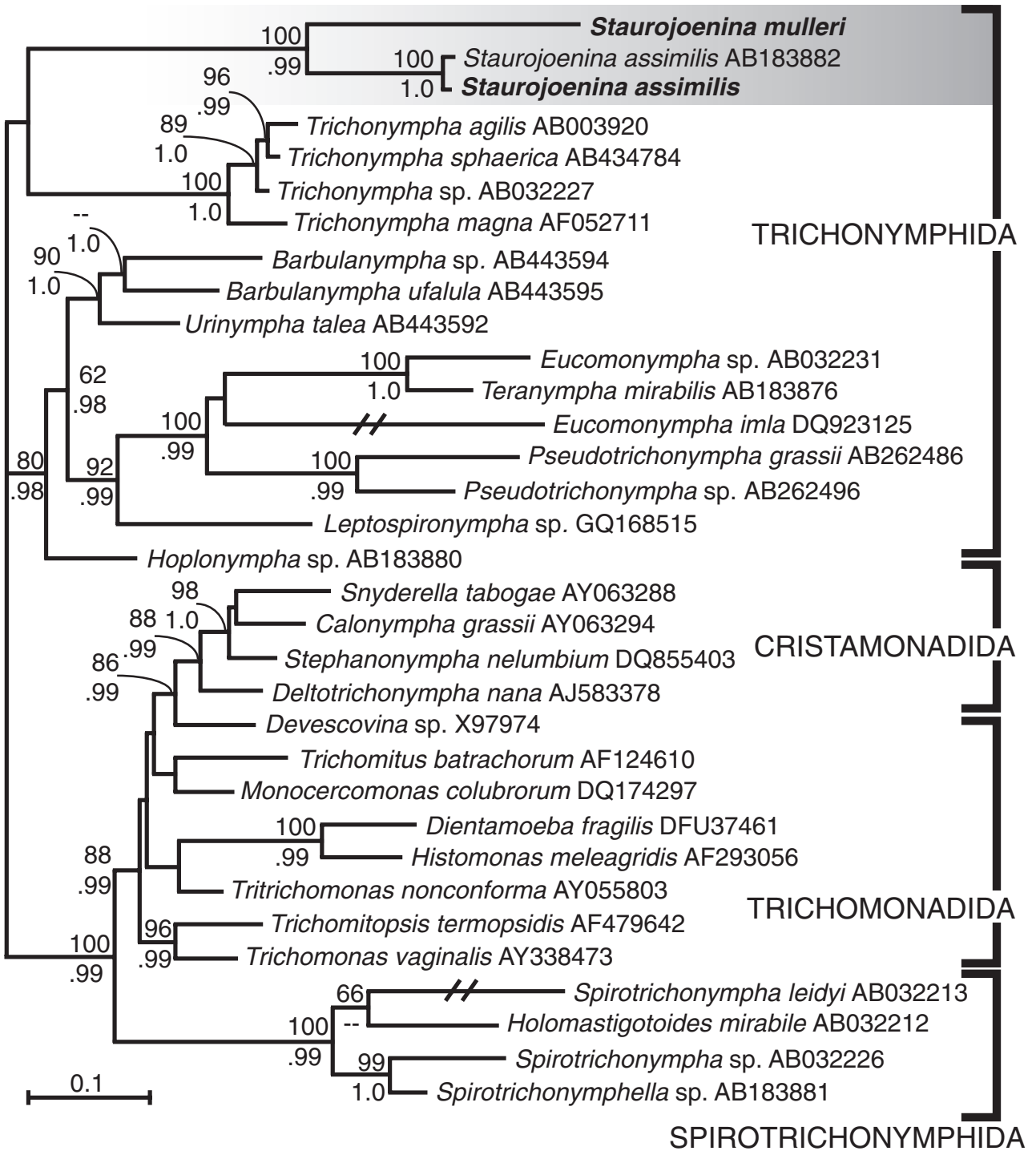

Fig. 12. Maximum likelihood (ML) phylogenetic analysis of small subunit rRNA sequences from representative parabasalians. New sequences acquired in this study are indicated in bold type and the genus *Staurojoenina* is indicated by a shaded box. Numbers at nodes indicate ML bootstrap support greater than 60% (above) and Bayesian posterior probabilities above 0.95 (below). Hatch marks indicate where the branch lengths of *Spirotrichonympha leidyi* and *Eucomonympha imla* have been reduced by one-half for clarity. Major subgroups of parabasalians are bracketed and named to the right.

morphological plasticity of the cells from a single termite was so high compared with the variability between *Staurojoenina* cells from different termites as to make clear criteria for species identification difficult, although he suggested that the nucleus-attractophore distance might be a stable enough character to be useful (Kirby 1926). In sub-

sequent treatments, the presence of "hamuli" (little hooks) on the parabasal plates (Grassé and Hollande 1945), or the presence of dictyosomes free in the cytoplasm vs. associated with parabasal fibers (Hollande 1986) has been used to distinguish new species, but none of these characters has been consistently reported in all species descriptions (Maaβ and Radek 2006). The present study agrees that readily accessible morphological characters are not sufficient to distinguish among *Staurojoenina* species, even though these cells are exceptionally large and structurally complex. This is due to an apparently low rate of morphological change between species together with a high degree of morphological plasticity within each species (or indeed within a single cell because they are highly active, motile cells).

In North American termites, no new species has been proposed since 1926, when Kirby decided to erect *S. assimilis* due to its presence in a distinct host rather than attempting a morphology-based circumscription. Here, our molecular data indicate that the "*S. assimilis*" from two different termite species are in fact quite distantly related. While *N. jouteli* and *I. minor* are relatively distant hosts, we would expect the same to hold for *Staurojoenina* from congeneric hosts (e.g. *N. jouteli*, *N. mona,* and *N. cubanus,* Dolan et al. 2004; Maaβ and Radek 2006). The assumption that symbionts co-speciate with their hosts has not been thoroughly tested across parabasalian diversity, but there is evidence of co-speciation between *Pseudotrichonympha* species and their hosts (Noda et al. 2007) and between parabasalians and their bacterial symbionts (Carpenter et al. 2009; Desai et al. 2010; Ikeda-Ohtsubo and Brune 2009; Noda et al. 2007). If a high degree of host specificity is borne out by testing many symbiont/host pairs with molecular data, this will be a useful criterion for *Staurojoenina* species circumscription.

A potential difficulty in circumscribing *Staurojoenina* species according to their host identities, however, is the possibility of horizontal transfer of symbionts. Although to date no single hypermastigont species has been confirmed by molecular data to live in two different hosts, the reported cyst-forming ability of *Staurojoenina* (Dolan et al. 2004), experimental transfaunation experiments (Light and Sanford 1928), and the overall patchy distribution of *Staurojoenina* within a variety of termites suggest that horizontal transfer is possible. The host termites investigated here are both geographically and climatically allopatric, making horizontal transfer unlikely: *Incisitermes minor* is a southwestern nearctic species (Banks and Snyder 1920) ranging over the arid lands of coastal northern California, southern California, southern Arizona, and into western Texas, while *Neotermes jouteli* is a moist tropical species ranging from Tampico, Mexico (Banks and Snyder 1920) south to the Yucatan peninsula and east to the Turks and Caicos Islands (Scheffrahn et al. 2000). Whether *Staurojoenina* from sympatric hosts will show sufficient molecular divergence to justify establishing new species remains to be seen. Accordingly, we propose that a combination of molecular data and host identity (with morphological information where informative) is currently the most useful option for distinguishing *Staurojoenina* species.

Our emphasis on molecular data for species identification, although somewhat of a departure from traditional taxonomy and still not universally accepted, is not without precedent. Not only are molecular data more readily comparable from one study to the next, they can uncover cryptic diversity in protists where morphology is particularly stable (e.g. Cepicka et al. 2005; Yubuki et al. 2010). Furthermore, such data have revealed that two morphologically defined genera are actually life cycle stages of the same organism (Harper et al. 2009), and, conversely, confirmed the distinct identities of two genera suspected of being life stages of a single organism (Moriya et al. 2003; Stingl and Brune 2003). In the present study, two *Staurojoenina* species share a remarkably low sequence identity of 82%, despite being indistinguishable morphologically. While this level of divergence may be justification for erecting a new genus in other groups, the current morphology-based demarcation between *Staurojoenina* and morphologically comparable genera (e.g. *Idionympha* and *Hoplonympha*) is clear and effective, and erecting a new, morphologically indistinguishable genus on the basis of sequence divergence would not be appropriate. Moreover, the rate of SSU rRNA evolution is known to have increased in the Trichonymphida (as well as other parabasalian lineages such as Spirotrichonymphida and the *Histomonas* and *Dientamoeba* clade, see Fig. 12). As a result, SSU rRNA sequence identity of 82% between *Staurojoenina* species from distantly related hosts may be somewhat high, but perhaps not surprisingly so. *Trichonympha* itself is a particularly extreme example if symbionts from termites and *Cryptocercus* are compared: previously published SSU rRNA sequences from the two host lineages (Carpenter et al. 2009; Ohkuma et al. 2009) can share as little as 48% sequence identity. While such rapid evolution suggests that alternative markers may be more appropriate for reconstructing higher level relationships (and a handful of protein-coding genes have been characterized for that purpose, Gerbod et al. 2004; Noda et al. 2012; Ohkuma et al. 2007, 2009), it can be beneficial for species delimitations. In some parabasalid lineages, SSU rRNA evolution is too slow for distinguishing species, and characterization of more rapidly evolving regions such as the internal transcribed spacer region (ITS) is necessary (Hampl et al. 2007). Similarly, when our sampling approaches the true diversity of a genus, even rapidly evolving genera such as *Staurojoenina* and *Trichonympha,* SSU rRNA may have insufficient information to distinguish the most closely related species. Accordingly, we consider the symbiont from *N. jouteli* to be a new species of *Staurojoenina*, *S. mulleri*.

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