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ORIGINAL ARTICLE

New Species of *Spirotrichonympha* from *Reticulitermes* and the Relationships Among Genera in Spirotrichonymphea (Parabasalia)

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ABSTRACT

Spirotrichonymphea is a class of hypermastigote parabasalids defined by their spiral rows of many flagella. They are obligate hindgut symbionts of lower termites. Despite more than 100 yr of morphological and ultrastructural study, the group remains poorly characterised by molecular data and the phylogenetic positions and taxonomic validity of most genera remain in question. The genus *Spirotrichonympha* has been reported to inhabit several termite genera, including *Reticulitermes*, *Coptotermes*, and *Hodotermopsis*. The type species for this genus, *Spirotrichonympha flagellata*, was described from *Reticulitermes lucifugus* but no molecular data are yet available for this species. In this study, three new *Spirotrichonympha* species are described from three species of *Reticulitermes*. Their molecular phylogenetic position indicates that the genus is not monophyletic, as *Spirotrichonympha* species from *Coptotermes*, *Paraneotermes*, and *Hodotermopsis* branch separately. In contrast, the genus *Holomastigotoides* is monophyletic, as demonstrated using new sequences from *Holomastigotoides* species. The presence of *Holomastigotoides* in *Prorhinotermes* and the distinct phylogenetic positions of *Spirotrichonympha* from *Reticulitermes* and *Coptotermes* are consistent with a previously proposed symbiont fauna replacement in the ancestor of *Reticulitermes*.

SPIROTRICHONYMPHEA is one of six classes in Parabasalia (Cepicka et al. 2010). Its members are found exclusively as symbionts in the hindguts of lower termites where they, like many other termite-dwelling parabasalians, display a great deal of morphological diversity and structural complexity. The group is characterised by having two or more rows of flagella arranged in bands that encircle some or all of the cell with a right-handed helix (Brugerolle and Lee 2000). Because of this shared morphology and similarities in ultrastructure, spirotrichonymphids are considered to be monophyletic (Cepicka et al. 2010). For large and complex parabasalids, morphological characters can be sufficient to make such conclusions, however, in a few cases molecular phylogenetic analyses have shown that morphologically similar body plans are plesiomorphic, as in *Stephanonympha*, or have evolved convergently, as in the nuclear multiplication of *Coronympha* and the calonymphids and the apical tuft of flagella in *Lophomonas*, *Kofoidia*, and the joeniids (Gile and Slamovits 2012; Gile et al. 2011; Tai et al. 2014). Phylogenies of small subunit ribosomal RNA (SSU rRNA) genes support the monophyly of spirotrichonymphids, insofar as they have been sampled (Cepicka et al. 2010; Noda et al. 2012; Ohkuma et al. 2000, 2005). However, of the large and complex parabasalids, commonly referred to as "hypermastigotes", the spirotrichonymphids are also the least studied, and their phylogenetic relationships are mostly untested by molecular data.

Within the Spirotrichonymphea, nine genera in three families are distinguished on the basis of morphological

characters. In the Spirotrichonymphidae, the genus *Spirotrichonympha* has a well-developed axostyle, a centrally located nucleus, and an apical pseudo-rostrum with an internal columella, which is a spiral staircase-like arrangement of basal bodies belonging to the tightly wound flagellar bands (a "true" rostrum is a complex microtubular structure found in Trichonymphea). These characteristics are shared by the related genus *Spironympha* except that in *Spironympha* the spiralling bands of flagella do not reach the cell's posterior (Brugerolle 2001, 2005; Brugerolle and Bordereau 2006). Together, these two genera plus *Microjoenia* make up the Spirotrichonymphidae; though *Microjoenia* lacks a pseudo-rostrum and bears such short flagellar bands that they do not form a spiral, the organisation of its axostyle is similar to *Spironympha* (Brugerolle 2001; Brugerolle and Bordereau 2006). The other two Spirotrichonymphea families are confusingly named Holomastigotidae and Holomastigotoididae. Holomastigotidae includes *Holomastigotes*, *Spiromastigotes*, *Uteronympha*, and *Spirotrichonymphella*. Cells lack a rostrum, the axostyle is reduced or absent (except in *Spirotrichonymphella*), and flagellar rows extend to the posterior (Brugerolle 2006; Brugerolle and Bordereau 2004). Holomastigotoididae includes *Holomastigotoides* and the enigmatic monospecific genus *Rostronympha*. Neither genus bears a rostrum (the name *Rostronympha* refers to a retractile proboscis for attachment to the hindgut wall of *Anacanthotermes ochraceus*), or if a short rostrum is present, it lacks a columella (Brugerolle and Lee 2000; Duboscq and Grassé 1943; Grassi and Foà 1911).

The distribution of spirotrichonymphids among lower termites might be expected to shed light on their phylogenetic relationships, given that these protists are inherited vertically by the termites' social behaviour of proctodeal trophallaxis (Nalepa 2015). The diversity of termite-symbiotic parabasalids is mainly driven by the evolution of their hosts, and in one case co-speciation has been demonstrated, between *Pseudotrichonympha* (Trichonymphea) and its rhinotermitid hosts (Noda et al. 2007; Tai et al. 2015). Spirotrichonymphids are found in most lower termite families, though not the basal *Mastotermes*, and not the closely related wood-eating roach *Cryptocercus* (Cleveland et al. 1934), but they are best studied in members of the Rhinotermitidae, and in particular the subterranean termites *Heterotermes*, *Coptotermes*, and *Reticulitermes*, where their distribution shows an odd pattern. Termite species belonging to *Heterotermes* and *Coptotermes* consistently harbour protists from three genera: *Holomastigotoides* and *Spirotrichonympha* (Spirotrichonymphea), and *Pseudotrichonympha* (Trichonymphea). *Reticulitermes* species harbour a distinct and more variable community of oxymonads and some combination of *Trichonympha* or *Teranympha* (Trichonymphea) and *Spirotrichonympha*, *Spironympha*, *Holomastigotes*, and *Microjoenia* (Spirotrichonymphea) (Koidzumi 1921; Lewis and Forschler 2006; Yamin 1979). The *Reticulitermes* hindgut protist community is far more similar to that of *Hodotermopsis sjoestedti* (Archotermopsidae) than to those of other Rhinotermitidae. Because of this, it has been proposed that the ancestor of *Reticulitermes* somehow replaced its symbiont fauna with that of *Hodotermopsis* (Kitade 2004).

Only one parabasalid genus is found common in *Reticulitermes, Heterotermes*, and *Coptotermes*: *Spirotrichonympha*. This genus is also found in *Hodotermopsis* (Brugerolle 2005; Kitade et al. 1997). Molecular data are available from *Spirotrichonympha leidyi*, from *Coptotermes formosanus*, and one unknown symbiont clone from *H. sjoestedti* has been provisionally attributed to *Spirotrichonympha cincta* (Brugerolle 2005; Ohkuma et al. 2000), but no sequences are yet available from *Spirotrichonympha* in *Reticulitermes*. To determine whether *Spirotrichonympha* species in *Reticulitermes* are more closely related to their counterparts in *Coptotermes* (suggesting vertical inheritance) or to those in *Hodotermopsis* (suggesting symbiont transfer), we sequenced SSU rRNA genes from spirotrichonymphids inhabiting nine termite species in five genera, including two species and a third distinct, likely species-level haplogroup of *Reticulitermes*. Sequences were obtained from multiple isolated single cells as well as whole gut community DNA. We describe three new species of *Spirotrichonympha* from *Reticulitermes* and find that they branch with the unidentified parabasalid symbiont clones RcF6, Rs7, Ry1, HsS1, and HsS2 (Ohkuma et al. 2000). This position is distinct from both *S. cincta* (clone Hs1) of *H. sjoestedti* and *S. leidyi* of *C. formosanus*.

MATERIALS AND METHODS

Host species collection and identification

The subterranean termite *Reticulitermes hesperus* was collected from a decaying arbutus log on Galiano Island, British Columbia, Canada (48.9236, −123.4415) on July 30, 2015. Individuals belonging to *Reticulitermes* haplotype "O", informally named *R. okanaganensis* (Szalanski et al. 2006), was collected from decaying lumber scraps in Santa Clara county, California, USA (37.2040, −121.9922) on March 17, 2009. *Prorhinotermes simplex* (University of Florida termite collection accession number FL1563), *Reticulitermes virginicus* (UF accession FL2261), and *Coptotermes gestroi* (UF accession FL3578) were collected in Secret Woods County Park, Fort Lauderdale, Florida, USA (26.0857, −80.1800) on September 15, 2002, February 21, 2005, and April 8, 2011, respectively. *Coptotermes testaceus* and *Heterotermes tenuis* were collected above the town of Minca in the Sierra Nevada de Santa Marta, Magdalena, Colombia (11.1256, −74.1197) in May and June 2009. The drywood termite *Paraneotermes simplicicornis* was collected from Tucson, Arizona, USA (32.302, −110.907). All host species were identified morphologically and by DNA barcoding of their mitochondrial 16S rRNA (mt16S) genes using primers LR-N and LR-J (Kambhampati and Smith 1995; Simon et al. 1994). Barcode sequences were obtained in the course of previous studies (James et al. 2013; Saldarriaga et al. 2011; Tai et al. 2014, 2015) and were submitted to GenBank under the following accessions: *Reticulitermes* haplotype O

(*R. okanaganensis*) KJ438377, *R. hesperus* KJ438376, *R. virginicus* JX975354, *P. simplex* JX975355, *C. testaceus* HQ683707, *H. tenuis* HQ683708, and *P. simplicicornis* KJ438371. The mt16S barcode sequence from *C. gestroi* was determined in this study and deposited under accession MF043909. A phylogeny of barcode data is shown in Fig. S1.

Identification, isolation, and molecular characterisation of spirotrichonymphids

Termites were dissected and hindgut contents were suspended in Trager's medium U (Trager 1934). Individual spirotrichonymphid cells were viewed and isolated under a Zeiss Axiovert 2 inverted light microscope with differential interference contrast (DIC) optics. Termite hindgut symbionts were also viewed under a Zeiss Axioplan 2 upright light microscope with DIC optics and photographed with a Canon XL-M1S camera.

At least three individual cells of each spirotrichonymphid morphotype (i.e. *Spirotrichonympha* from *Reticulitermes* species and *Holomastigotoides* from *C. testaceus*, *H. tenuis*, and *P. simplex*) were isolated manually by drawn glass micropipette (see Table S1 for numbers of cells isolated and clones sequenced). DNA was purified from isolated cells and from whole gut contents using the Masterpure Complete DNA and RNA Purification Kit (Epicentre, Madison, WI). SSU rRNA genes were amplified from each purified DNA sample 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′, with further amplification using the nested primers GGF 5′-CTT CGG TCA TAG ATT AAG CCA TGC-3′ and GGR 5′-CCT TGT TAC GAC TTC TCC TTC CTC-3′ for single cells when necessary. For *Holomastigotoides* from *P. simplex*, additional Spirotrichonymphea-specific primers SpiroF 5′-CGG TTG AGC GCC CTA TCA GCT-3′ and SpiroR 5′-CGG GGG TAG TTC GCT CGT TA-3′ were used to amplify the SSU rRNA genes from two pools of 30 isolated cells. PCR 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). At least one clone from each isolated cell PCR and at least eight clones from each whole gut DNA PCR were sequenced on both strands and assembled into contigs using Sequencher 4.2 (GeneCodes, Ann Arbor, MI) with a stringency of 98% (see Table S1 for details). Representative clones were selected for phylogenetic analyses and for submission to GenBank under accessions MF065843–MF065861.

Phylogenetic analyses

New and previously determined SSU rRNA gene sequences from spirotrichonymphids and outgroup tritrichomonads were aligned using MAFFT v7 (Katoh and Standley 2013), and visually inspected and manually trimmed using AliView (Larsson 2014) for a final alignment of 43 taxa and 1,497 sites. Mitochondrial 16S rRNA (mt16S) gene sequences from termites were aligned and trimmed using the same methods, for a final alignment of 39 taxa and 390 sites. Alignments are available upon request from the authors. Maximum likelihood (ML) and Bayesian phylogenetic analyses were performed using RAxML v8 (Stamatakis 2014) and MrBayes 3 (Ronquist and Huelsenbeck 2003) respectively, under the GTR model with four evolutionary rate categories approximated by a gamma distribution. For the ML analyses, support was assessed from 1,000 bootstrap replicates. For the Bayesian analyses, two independent chains, sampled every 100 generations, were run until they converged (the average standard deviation of partition frequency values between the chains dropped below 0.01) with the first 25% of the trees discarded as burn-in. For the parabasalid SSU rRNA gene tree, convergence was reached after 230,000 generations and consensus trees were computed from the saved trees of both runs, for a total of 3,452 trees. For the termite mt16S gene tree, convergence was reached after 570,000 generations and consensus trees were computed from the saved trees of both runs, for a total of 8,552 trees.

RESULTS AND DISCUSSION

Molecular characterisation of new *Spirotrichonympha* species

Parabasalid cells with morphological characteristics of the genus *Spirotrichonympha*, namely bands of flagella that spiral around the cell, a pseudo-rostrum with internal columella, a centrally positioned nucleus, and plentiful ingested wood particles (Fig. 1), were observed inhabiting the hindguts of *Reticulitermes* species. We isolated multiple *Spirotrichonympha* cells from each termite and sequenced their amplified SSU rRNA genes (Table S1). The nine clones we sequenced from *R. virginicus* all clustered together under a 98% identity threshold, and two clones (Rv1, Rv2) were selected to represent this organism in phylogenetic analyses and for submission to GenBank. The eight *Spirotrichonympha* clones we sequenced from *R. hesperus*, however, did not all cluster together. All but one formed a contig of highly similar sequences (Rh1, Rh2) that were found to branch with *Spirotrichonympha* from *R. virginicus* but one (Rh3) branched separately, sharing only 95.1% and 95.2% of sites with the main contig, represented by Rh1 and Rh2 (Fig. 2). The same pattern was observed for *Spirotrichonympha* from *Reticulitermes* haplotype O (hereafter referred to as *R. okanaganensis* Szalanski et al. 2006), with the single anomalous clone (Ro3) forming a weakly supported clade with the anomalous clone from *R. hesperus*, and sharing 95.8% and 95.6% identity with Ro1 and Ro2 (Fig. 2).

These deeper-branching sequences do not appear to be chimeric, being similar to the main *Spirotrichonympha* clade throughout their length, and having polymorphisms evenly distributed among the variable regions of the

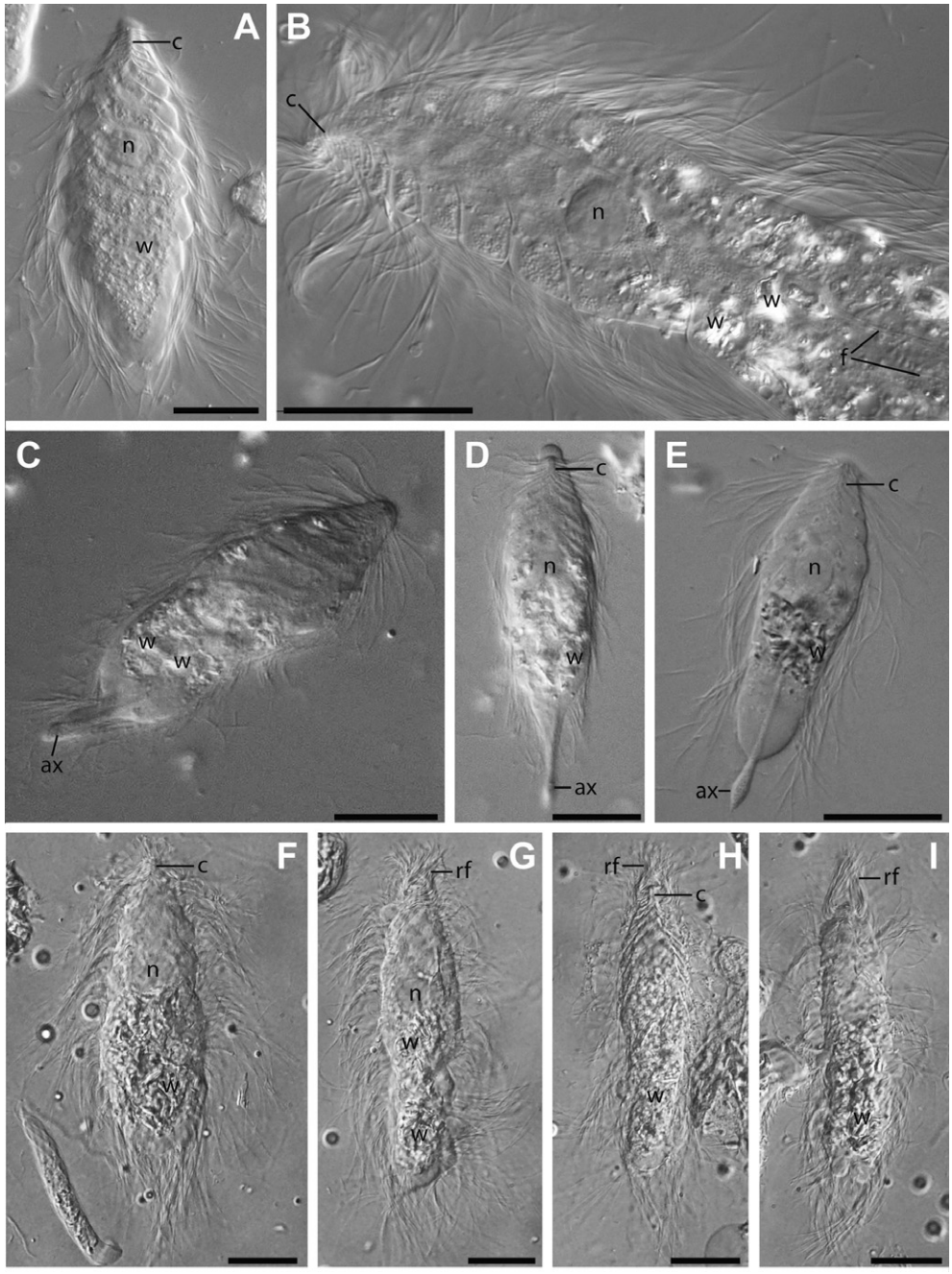

Figure 1 Light micrographs of *Spirotrichonympha* species from *Reticulitermes*. For all species, note the apical pseudo-rostrum with axial columella (c), centrally positioned nucleus (n), and ingested wood particles (w). (**A, B**) Differential interference contrast (DIC) light micrographs of *S. okanaganensis*, n. sp. (A) Whole cell view. Columella is in the plane of focus; centrally positioned nucleus is just out of the plane of focus. (B) Detail of flattened cell. Axostylar filaments (f) can be seen posterior to the nucleus amidst ingested wood particles. (**C**–**E**) DIC light micrographs of whole cells of *Spirotrichonympha virginica* with its characteristic protruding axostyle (ax). (**F**–**I**) Brightfield light micrographs of whole cells of *Spirotrichonympha hespera* with its characteristic recurved apical flagella (rf). A *Dinenympha* sp. can be seen in the lower left corner of (F). All scale bars = 20 μm.

alignment. This raises the question of whether these sequences represent distinct *Spirotrichonympha* species in *R. hesperus* and *R. okanaganensis*, or whether they represent distinct SSU rRNA gene loci from the same species but on different evolutionary trajectories (as occurs in *Plasmodium*, Rooney 2004). Supporting the second possibility, the two distinct sequence types were obtained from one isolated *Spirotrichonympha* cell in *R. okanaganensis* (Fig. 2). Distinct variants of the SSU rRNA gene have been found within individual parabasalid cells previously (e.g.

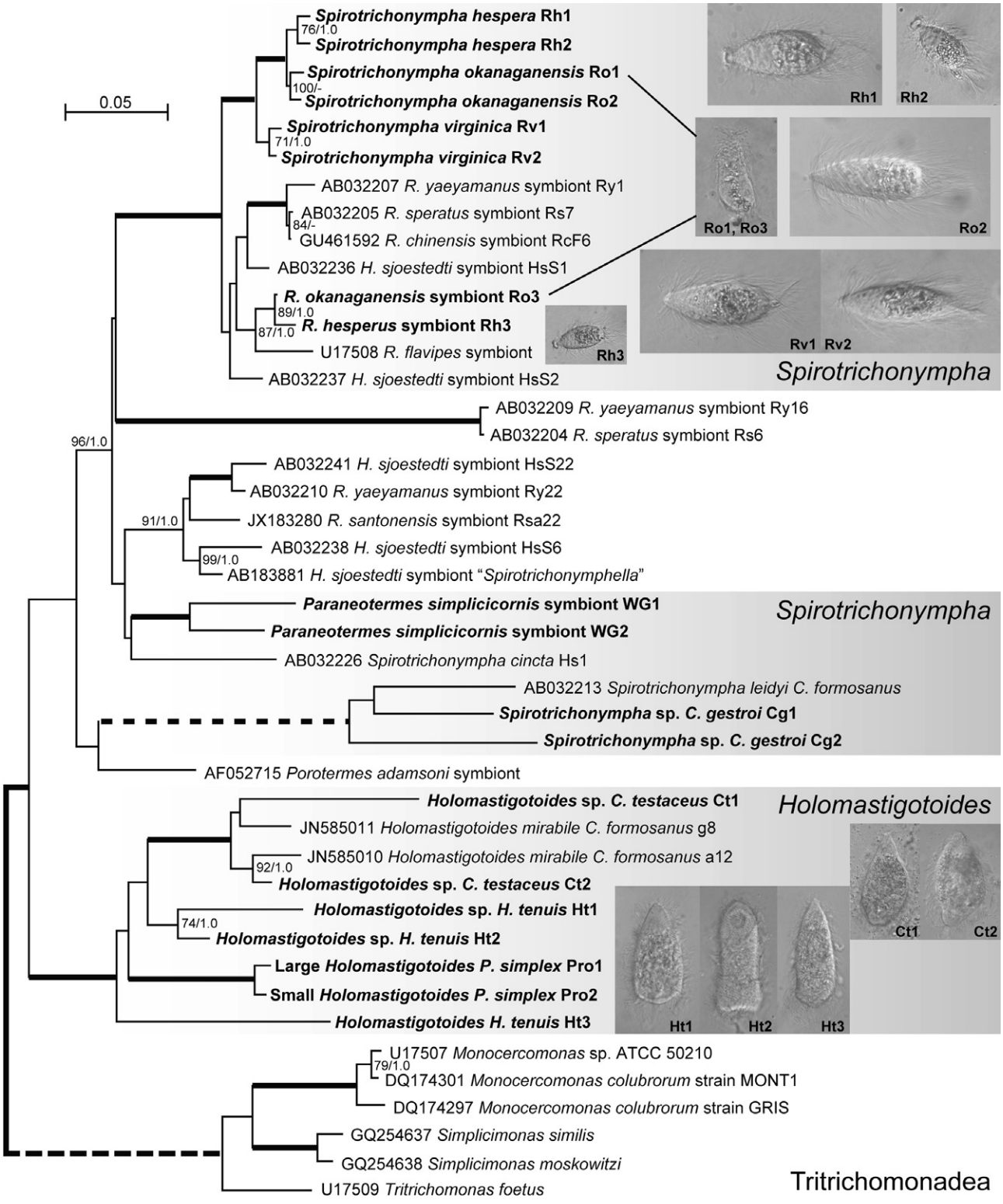

Figure 2 Maximum likelihood phylogenetic analysis of SSU rRNA gene sequences from Spirotrichonymphea and outgroup Tritrichomonadea. Isolated *Spirotrichonympha* and *Holomastigotoides* cells are pictured and labelled with their corresponding clone name(s). New sequences obtained in this study are indicated by bold text. Support at nodes is shown where greater than 70% out of 1,000 bootstrap replicates and greater than 0.95 Bayesian posterior probability. Fully supported nodes (100% BS/1.0 BPP) are indicated by thick branches. Dashed branches are shown at half their actual length.

Macrotrichomonoides, Kofoidia, and *Trichonympha* Tai et al. 2013, 2014; Gile et al. 2015). However, we cannot rule out the possibility that Ro3, obtained from the same cell as Ro1 might represent a contaminating sequence. We therefore consider the clade of clones 1 and 2 from each termite to be representative of the *Spirotrichonympha* species described here, and the clade of clones 3 from *R. okanaganensis* and *R. hesperus* to be of undefined origin.

The three new *Spirotrichonympha* species, including the anomalous clones from isolated symbionts of *R. okanaganensis* and *R. hesperus*, branched with strong support with a clade of unidentified environmental sequences from other species of *Reticulitermes* and with *H. sjoestedti*. This clade is strongly supported (100% bootstrap, 1.0 BPP) and recovered in all analyses (Fig. 2). Within this clade, sequences from the three new species form a discrete and strongly supported subgroup, with the *R. hesperus* and *R. okanaganensis* symbionts showing a sister relationship. This is consistent with the phylogeny of their hosts, as *R. okanaganensis* is considered to be a recently diverged sister haplogroup of *R. hesperus* (Szalanski et al. 2006), though our termite mt16S gene phylogeny did not resolve this sister relationship (but did show they are distinct, Fig. S1).

We also characterised environmental sequences from *P. simplicicornis* and *C. gestroi* that branch within the Spirotrichonymphidae. The two distinct sequences from *P. simplicicornis* likely correspond to each of the two *Spirotrichonympha* species that have been described from that termite, *S. polygyra* and *S. bispira* (Cleveland 1938; Cupp 1930). No other spirotrichonymphids have been described from that termite (Yamin 1979), and we did not observe any other spirotrichonymphids (data not shown). These sequences branch together with strong support (100% BS, 1.0 BPP), but their position is unresolved. The two distinct environmental sequences from *C. gestroi* branch with *Spirotrichonympha leidyi* from *C. formosanus* with strong support (100% BS, 1.0 BPP, Fig. 2), but no parabasalid species are yet described from *C. gestroi*.

Polyphyly of *Spirotrichonympha*

It has been noted previously that the genus *Spirotrichonympha* is likely not monophyletic, on the basis of *S. leidyi* branching separately from a clone attributed to *Spirotrichonympha* (Hs1) from *H. sjoestedti* (Ohkuma et al. 2005). The new species of *Spirotrichonympha* described here form yet another clade, indicating that *Spirotrichonympha* from *Reticulitermes* are phylogenetically distinct from their counterparts in both *Coptotermes* and *H. sjoestedti* (Fig. 2).

However, two unidentified *H. sjoestedti* clones (HsS1 and HsS2) also branch with the *Spirotrichonympha* from *Reticulitermes*, so it is possible that these derive from *Spirotrichonympha* species. Although the symbiont clone Hs1 AB032226 from *H. sjoestedti* was attributed to the genus *Spirotrichonympha* by fluorescent in situ hybridisation (Ohkuma et al. 2000), the spindle-shaped fixed cell displaying fluorescent signal lacks morphological detail, and its size falls within the range of other spirotrichonymphids in *H. sjoestedti*, including *Holomastigotes elongatum*, *Spironympha obtusa,* and *Spironympha oblonga* (Brugerolle 2005, 2006; Ohkuma et al. 2000). Overall, the phylogenetic position of *Spirotrichonympha* from *H. sjoestedti* may be uncertain, but *Spirotrichonympha* from *Reticulitermes* is clearly distinct from *Spirotrichonympha* from *Coptotermes*.

The monophyly of *Spirotrichonympha* is doubtful, because the symbiont clones Ry16, Rs6, HsS22, Ry22, Rsa22, and HsS6 that branch between the *Reticulitermes-Spirotrichonympha* and the *Coptotermes-Spirotrichonympha* likely belong to other spirotrichonymphid genera. For example, the *H. sjoestedti* symbiont sequence AB183881 was obtained by isolating a pool of 30 cells with *Holomastigotes* morphology (Brugerolle 2006; Ohkuma et al. 2005). Ohkuma et al. attributed this sequence to the similar genus *Spirotrichonymphella*, but independent morphological and ultrastructural studies (Brugerolle 2005; Kitade et al. 1997) do not report this genus from *H. sjoestedti*, rather they consider that morphotype to be *Holomastigotes elongatum*. The genus *Spirotrichonympha* was established to accommodate a symbiont of *Reticulitermes lucifugus* that was originally misidentified as *Pyrsonympha* (Grassi and Foà 1911). Although no molecular data are yet available from the type species, it is reasonable to expect that it would branch with *Spirotrichonympha* from *Reticulitermes* rather than with those from *Coptotermes*. Accordingly, if *Spirotrichonympha* is confirmed to be polyphyletic, species from this clade should retain the name *Spirotrichonympha* and new genera should be erected for distinct lineages of *Spirotrichonympha* morphotypes found in non-*Reticulitermes* hosts.

Only three *Spirotrichonympha* species have been reported from two termite species in the family Kalotermitidae, *S. bispira* and *S. polygyra* from *P. simplicicornis* and *S. minor* from *Kalotermes sinaicus* (Radek 1997), now *Longicaputermes sinaicus* (Ghesini et al. 2014). No other spirotrichonymphids are reported from any termite in this family (Yamin 1979). The presence of *Spirotrichonympha* in kalotermitids thus seems anomalous and hints at symbiont transfer. However, the phylogenetic position of our spirotrichonymphid clones from *P. simplicicornis* is not resolved and therefore provides no clue as to their origin, though they are clearly excluded from both the *Coptotermes* and the *Reticulitermes Spirotrichonympha* clades (Fig. 2). Curiously, *P. simplicicornis* also harbours the trichonymphid *Hoplonympha natator*, which is otherwise only known from *H. sjoestedti*. Clearly more data are needed to understand the evolution and distribution of termite symbiotic parabasalids, and particularly the Spirotrichonymphea, for which seven of the nine accepted genera still lack molecular data and the majority of available sequences have not been attributed to genera.

Diversity and phylogeny of *Holomastigotoides*

The genus *Holomastigotoides* is characterised by spiral bands of flagella that encircle the cell nearly to its

posterior, the lack of a rostrum or a short pseudo-rostrum with no columella, and are xylophagous (Fig. 3) (Brugerolle and Lee 2000). We isolated single cells with these morphological characteristics from *C. testaceus*, *H. tenuis*, and *P. simplex* and sequenced multiple clones of the amplified SSU rRNA genes from these cells and from whole-gut DNA extracts (see Table S1 for details). From *C. testaceus*, we found two distinct *Holomastigotoides* sequences with 89.8% identity. From *P. simplex*, we isolated two distinct size morphs of *Holomastigotoides* and found that their SSU rRNA gene sequences were 98.5% identical. For *H. tenuis*, the isolated cell and whole gut PCR clones yielded three distinct sequence types, with pairwise identities of 93.5%, 89.2%, and 87.6%.

Each of the termite species, we examined has prior record of *Holomastigotoides* inhabitants in the literature. *C. testaceus* is now known to be the type host for *Holomastigotoides*, but only through a circuitous route of study. *Holomastigotoides* was initially described as the female form of *Trichonympha hertwigi*, from an undetermined *Coptotermes* species in Brazil (Hartmann 1910). In the following year, a new species was erected for the host, *Coptotermes hartmanni*, and the genus *Holomastigotoides* was erected for the protist morphotype (Grassi and Foà 1911; Holmgren 1911). However, the species *C. hartmanni* is invalid due to lack of a formal description. Termite surveys have concluded that only one species of *Coptotermes* is native to the New World, *C. testaceus*, and that must therefore be the type host species for *Holomastigotoides hertwigi* (Saldarriaga et al. 2011; Scheffrahn et al. 2015). Our molecular data suggest the presence of two species of *Holomastigotoides* in *C. testaceus*, with Ct1 and Ct2 sharing only 89.8% sequence identity (Fig. 2), but we are unable to determine which one corresponds to *H. hertwigi*. Early light microscopy investigations of *C. formosanus* similarly reported just one *Holomastigotoides* species, *H. mirabile*, while more recent molecular data suggest two *Holomastigotoides* species (Koidzumi 1921; Xie et al. 2011).

Three species of *Holomastigotoides* have been described from light microscope investigations of *P. simplex*: *H. mitotica*, *H. diversa*, and *H. tusitala* (Cleveland 1949; Jennings 1942). *Holomastigotoides mitotica* lacks a formal description, but *H. diversa* and *H. tusitala* were both named from large *Holomastigotoides* cells on the basis of transverse (*H. tusitala*) or longitudinal (*H. diversa*) cell division, while the smaller cells were left unnamed (Cleveland 1949). We likewise observed small and large *Holomastigotoides* cells, but the sequences obtained from isolated cells of each class were highly similar, at least 98.5% for all pairwise comparisons, strongly suggesting that they belong to the same species. We also designed and used Spirotrichonymphea-specific primers on pools of 30 small cells and 30 large cells, and found no evidence of distinct sequence types based on cell size, despite sequencing a total of 26 clones. The level of sequence variation between large and small *Holomastigotoides* cells we isolated from *P. simplex* is well within the levels of variation documented for other single parabasalid species, including the new *Spirotrichonympha* species described here. We were therefore unable to find evidence for multiple *Holomastigotoides* species in *P. simplex*.

Heterotermes tenuis is reported to contain four or five species of *Holomastigotoides*, *H. campanula, H. globosus, H. hemigymnum, H. oswaldoi,* and *H. sphaeroidalis,* though only one out of two Brazilian populations studied contained *H. sphaeroidalis* (De Mello 1954). The species-specific characteristics are difficult to distinguish under the conditions used for single cell isolation, and our data suggest only three *Holomastigotoides* species in our Colombian isolate of *H. tenuis*, so no attempt was made to assign sequences to species.

All seven new *Holomastigotoides* sequences from three termite species branch together along with the *Holomastigotoides* sequences from *C. formosanus* (Fig. 2). Interestingly, the two *Holomastigotoides* sequences from *C. testaceus* branch specifically with the two *Holomastigotoides* sequences from *C. formosanus* in a pattern that is suggestive of co-speciation: an ancestral pair of *Holomastigotoides* could have each speciated as their *Coptotermes* hosts did. (Note that the two distinct *C. formosanus* symbionts are labelled *H. mirabile* because only one morphospecies has been described from this termite (Xie et al. 2011).) Co-speciation between parabasalids and their termite hosts has been demonstrated before, between *Pseudotrichonympha* and termites in the Rhinotermitidae (Noda et al. 2007), so it would be reasonable to expect that *Holomastigotoides* might show a similar pattern. However, the three new *Holomastigotoides* sequences from *H. tenuis* do not conform to this pattern, and more *Holomastigotoides* sequences from additional *Coptotermes* species will be needed to clarify this.

Overall, our data support the monophyly of the genus *Holomastigotoides*, at least within the Rhinotermitidae, though they also show that the genus is in need of major taxonomic revision. This is notable because although *P. simplex* is part of the Rhinotermitidae, it branches separately from *Coptotermes, Heterotermes,* and *Reticulitermes* in mitochondrial genome trees (Bourguignon et al. 2015). This suggests that the genus *Holomastigotoides* is ancestrally present in the Rhinotermitidae. This provides polarity to the observed differences between *Reticulitermes*-type fauna and *Heterotermes/Coptotermes*-type fauna: the *Reticulitermes*-type is therefore the odd one in the family. These observations, previously made on the basis of morphology alone, led to the suggestion that *Reticulitermes* somehow replaced its hindgut fauna with that of *Hodotermopsis* (Kitade 2004). This is a difficult hypothesis to test, given that the split between *Reticulitermes* and the *Heterotermes/Coptotermes* clade is estimated to have occurred 60 million years ago (Bourguignon et al. 2016). It is also difficult to reconcile with the distinct historical biogeographies of the hosts (Bourguignon et al. 2016; Inward et al. 2007). Perhaps additional symbiont data from relatives of *Hodotermopsis*, such as *Archotermopsis, Stolotermes*, and *Porotermes*, will shed light on this mystery.

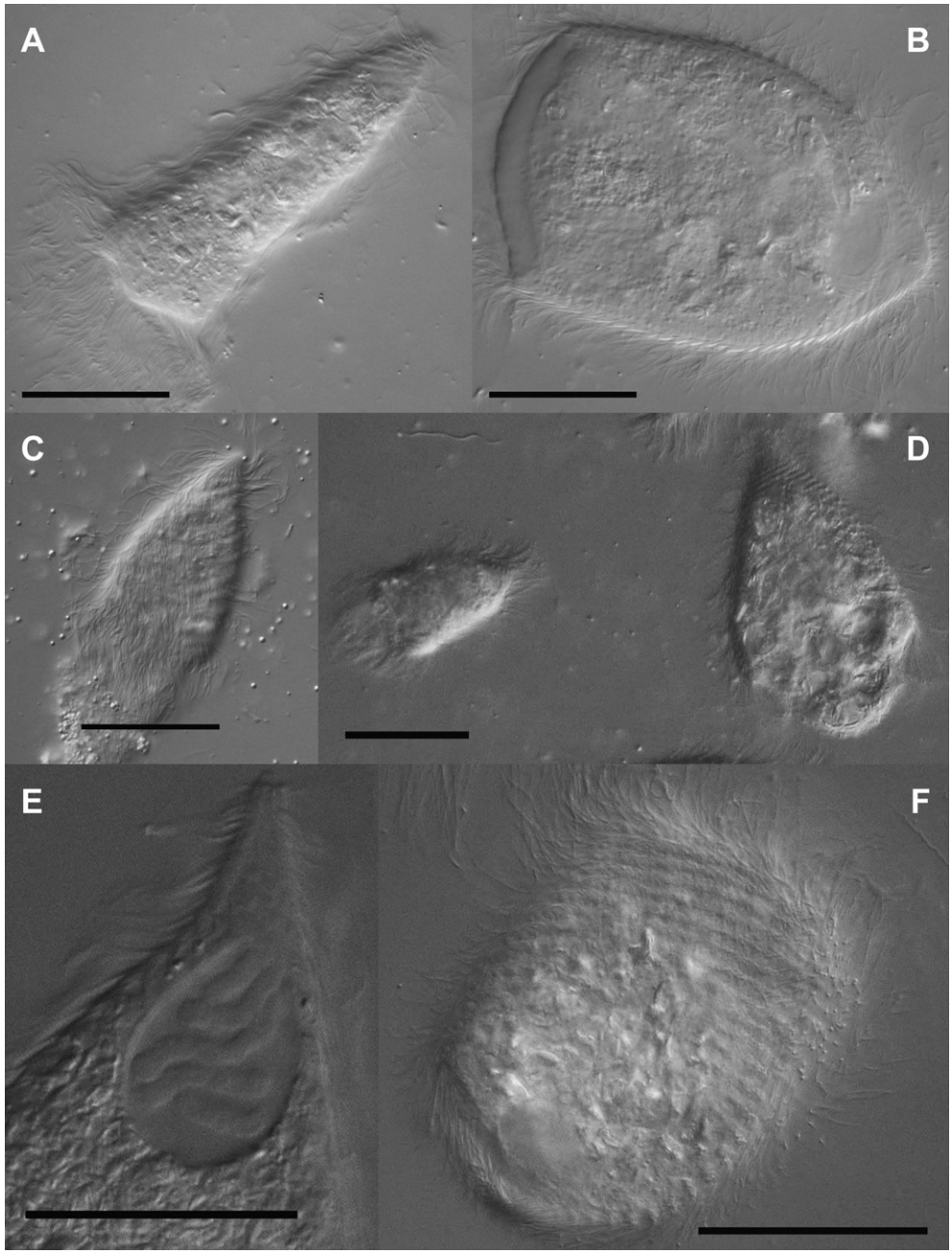

Figure 3 Differential interference contrast light micrographs of *Holomastigotoides*. (**A, B**) Two distinct *Holomastigotoides* morphotypes from *Heterotermes tenuis*. (**C**) *Holomastigotoides* symbiont of *Coptotermes testaceus*, possibly the type species *H. hertwigi*. (**D**) Small (left) and large (right) morphotypes of *Holomastigotoides* from *Prorhinotermes simplex*. (**E**) Detail of nucleus with condensed chromosomes near the cell apex in the large *Holomastigotoides* morphotype from *P. simplex*. (**F**) Distinctive square-shaped *Holomastigotoides* from *Coptotermes gestroi*. Scale bars = 50 μm except D = 100 μm.

CONCLUDING REMARKS

Here, we have determined the molecular phylogenetic position of *Spirotrichonympha* species from *Reticulitermes* hosts. We find that they form a distinct clade from the *Spirotrichonympha* species in *Coptotermes* hosts and from environmental sequences that likely belong to *Spirotrichonympha* species in *Paraneotermes*. The genus *Spirotrichonympha* is therefore likely polyphyletic and defined by plesiomorphic characters, but confirmation of this will require sequences in the intervening clades (e.g. clones HsS22, Ry22, Rsa22, and Hs6) to be definitively attributed to other genera. *Holomastigotoides*, on the other hand, was found to be monophyletic and to form the sister lineage to all other available Spirotrichonymphea sequences. *Holomastigotoides* is represented by multiple

sequence types indicative of multiple species in *H. tenuis* and *C. testaceus* but only one sequence type in *P. simplex*.

TAXONOMIC SUMMARY

Phylum Parabasalia Honigberg 1973
Class Spirotrichonymphea Grassé 1952
Order Spirotrichonymphida Grassé 1952
Family Spirotrichonymphidae Grassi 1917 emend Brugerolle 2001
Genus *Spirotrichonympha* Grassi & Foà 1911

Spirotrichonympha virginica Gile and Keeling sp. nov.

Diagnosis. Multiflagellate, uninucleate parabasalian from the hindgut of *Reticulitermes virginicus*. Cells possess an anterior pseudo-rostrum with columella, a centrally positioned nucleus, bands of flagella forming a right-handed helix that surrounds the cell nearly to its posterior, and ingested wood particles inside the cell (characteristics of the genus). Obligate symbiont of *Reticulitermes virginicus*. Large cells, 63–81 μm in length (median 79 μm), including the axostyle. Cell posterior typically distorted by a protruding axostyle with a distal thickening. SSU rRNA gene sequences with at least 99% identity to 18S rRNA gene sequences MF065849 and MF065850.

Type host. *Reticulitermes virginicus* Banks (Isoptera, Rhinotermitidae), barcode JX975354, University of Florida termite collection accession number FL2261.

Host Collection. Secret Woods County Park, Fort Lauderdale, Florida, USA (26.0857, −80.1800), February 21, 2005.

Etymology. The specific epithet *virginica* refers to the specific epithet of the type host, and means from the state of Virginia, USA.

Zoobank ID. 65563CAE-9EC0-4D37-A805-361818FFF238

Holotype. Permanent microscope slide of ethanol-fixed, unstained protist cells deposited at the Beaty Biodiversity Museum, University of British Columbia, Vancouver, Canada under accession number MI-PR201.

Spirotrichonympha okanaganensis Gile and Keeling sp. nov.

Diagnosis. Multiflagellate, uninucleate parabasalian from the hindgut of *Reticulitermes* haplotype O, which was provisionally named *R. okanaganensis* (Szalanski et al. 2006). Cells possess an anterior pseudo-rostrum with columella, a centrally positioned nucleus, bands of flagella forming a right-handed helix that surrounds the cell nearly to its posterior, and ingested wood particles inside the cell (characteristics of the genus). Large cells, 67–128 μm in length (median 98 μm). Axostylar filaments visible posterior to the nucleus in slightly flattened live cells, not forming a coherent or protruding axostylar rod. Obligate symbiont of *Reticulitermes* haplotype O. SSU rRNA gene sequences with at least 99% identity to MF065846 and MF065847.

Type host. *Reticulitermes* haplotype O (provisionally named *R. okanaganensis* but not formally described) (Isoptera, Rhinotermitidae, barcode KJ438377)

Host collection. Santa Clara county, California, USA (37.2040, −121.9922), March 17, 2009.

Etymology. The specific epithet *okanaganensis* represents the original collection location for the type host haplotype, and means from the Okanagan valley, British Columbia, Canada.

Zoobank ID. 113628BE-998D-4680-B9DB-10ED24A71990

Holotype Fig. 1A

Spirotrichonympha hespera James and Keeling sp. nov.

Diagnosis. Multiflagellate, uninucleate parabasalian from the hindgut of *Reticulitermes hesperus*. Cells possess an anterior pseudo-rostrum with columella, a centrally positioned nucleus, bands of flagella forming a right-handed helix that surrounds the cell nearly to its posterior, and ingested wood particles inside the cell (characteristics of the genus). Large cells, 53–88 μm in length (median 74 μm), typically with recurved anterior flagella surrounding the cell apex and meeting or crossing at their distal ends to form an apical tuft. Obligate symbiont of *Reticulitermes hesperus*. SSU rRNA gene sequences with at least 99% identity to MF065843 and MF065844.

Type host. *Reticulitermes hesperus* Banks (Isoptera, Rhinotermitidae), barcode KJ438376, Beaty Biodiversity Museum accession number MI-PR208.

Host collection. Galiano Island, British Columbia, Canada (48.9544 −123.5368), July 30, 2015.

Etymology. The specific epithet *hespera* represents the specific epithet of the type host, which means western.

Zoobank ID. 4E4ECCCE-EEAD-4799-888F-F46E06528777

Holotype. Permanent microscope slide of ethanol-fixed, unstained protist cells deposited at the Beaty Biodiversity Museum, University of British Columbia, Vancouver, Canada under accession number MI-PR209.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Maximum likelihood phylogenetic analysis of mitochondrial 16S rRNA gene sequences from Rhinotermitidae.
Table S1. Numbers of individual spirotrichonymphid cells isolated and PCR product clones sequenced from each termite.