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ORIGINAL ARTICLE

Molecular phylogeny of Spirotrichonymphea (Parabasalia) with emphasis on *Spironympha*, *Spirotrichonympha*, and three new genera *Pseudospironympha*, *Nanospironympha*, and *Brugerollina*

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Abstract

Spirotrichonymphea, one of the six classes of phylum Parabasalia, are characterized by bearing many flagella in spiral rows, and they occur exclusively in the guts of termites. Phylogenetic relationships among the 13 described genera are not well understood due to complex morphological evolution and a paucity of molecular data. One such understudied genus is *Spironympha*. It has been variously considered a valid genus, a subgenus of *Spirotrichonympha*, or an "immature" life cycle stage of *Spirotrichonympha*. To clarify this, we sequenced the small subunit rRNA gene sequences of *Spironympha* and *Spirotrichonympha* cells isolated from the hindguts of *Reticulitermes* species and *Hodotermopsis sjostedti* and confirmed the molecular identity of *H. sjostedti* symbionts using fluorescence in situ hybridization. *Spironympha* as currently circumscribed is polyphyletic, with both *H. sjostedti* symbiont species branching separately from the "true" *Spironympha* from *Reticulitermes*. Similarly, the *Spirotrichonympha* symbiont of *H. sjostedti* branches separately from the "true" *Spirotrichonympha* found in *Reticulitermes*. Our data support *Spironympha* from *Reticulitermes* as a valid genus most closely related to *Spirotrichonympha*, though its monophyly and interspecific relationships are not resolved in our molecular phylogenetic analysis. We propose three new genera to accommodate the *H. sjostedti* symbionts and two new species of *Spirotrichonympha* from *Reticulitermes*.

KEYWORDS
coevolution, Metamonada, SSU rRNA, symbiosis, termite

INTRODUCTION

NUMEROUS insects have established intimate mutualistic relationships with microorganisms, which play key roles in the hosts' adaptation to various environments (Ohbayashi et al., 2019; Takeshita et al., 2015). Symbiotic associations with microorganisms have had a profound impact on the adaptation to xylophagy (wood-feeding) and this association has contributed to the expansion of the termite niche in terrestrial ecosystems (Brune & Ohkuma, 2011; Ohkuma & Brune, 2011). "Lower" termites (all termite families except the apical Termitidae) harbor at least one, usually multiple, species of symbiotic protists in their hindguts (Kitade et al., 1997). These cellulolytic protist species are unique to termites and wood-feeding *Cryptocercus* cockroaches and are never found in other environments (Honigberg, 1970). This symbiotic relationship is essential for the digestion of lignocellulose consumed by the termites. The community composition of the gut protists is usually specific to the host species,

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and closely related host species tend to have closely related protist symbiont species (Kitade, 2004; Kitade & Matsumoto, 1993). In some cases, co-cladogenesis has been observed among the protists, their endosymbiotic bacteria, and host termites (Ikeda-Ohtsubo & Brune, 2009; Noda et al., 2006, 2007, 2018; Taerum et al., 2018), which must have resulted from the strict vertical transmission of gut symbionts. This transmission mode stems from a social behavior of the host termite called proctodeal trophallaxis (anus-to-mouth transmission of hindgut fluid), and it enables the survival and vertical transmission of these anaerobic protists (Inoue et al., 2000; Michaud et al., 2020).

The class Spirotrichonymphea (Parabasalia) comprises 13 genera: *Cononympha*, *Cuppa*, *Fraterculus*, *Holomastigotes*, *Holomastigotoides*, *Microjoenia*, *Micromastigotes*, *Rostronympha*, *Spiromastigotes*, *Spironympha*, *Spirotrichonympha*, *Spirotrichonymphella*, and *Uteronympha* (Čepička et al., 2017; Taerum et al., 2020). They are characterized by many flagella in single rows that form right-handed helices, patchy parabasal bodies along with the flagellar rows, and axostylar fibers that originate at the anterior end of the cells and pass around the nucleus. Flagellar bands may be tightly wound at the cell apex, forming a protruding spiral staircase-like structure called a pseudo-rostrum with an internal columella, or the cell apex may be blunt. The nucleus may be positioned in the center or at the extreme anterior of the cell. Axostylar fibers may end at the level of the nucleus or continue posteriorly, in which case they may form a single axostylar trunk or continue as a bundle of fibers. These variable characters have been used to delimit genera, though not always clearly or appropriately, leading to a certain degree of taxonomic confusion (Brugerolle & Lee, 2000; Čepička et al., 2010).

The genera *Spirotrichonympha* and *Spironympha* exemplify the challenges of morphology-based genus definitions. *Spirotrichonympha* was first fully described in 1917 (though named in 1911) and has an apical pseudo-rostrum with columella, helical flagellar bands that proceed to the posterior end of the cell, and axostylar fibers that form a loose bundle posterior to the nucleus (Grassi, 1917; Grassi & Foà, 1911). The type species is *Spirotrichonympha flagellata* Grassi from *Reticulitermes lucifugus* (Grassi, 1892, Grassi, 1917). *Spironympha* was first described in 1917 (with an English translation published in 1921) and has an apical pseudo-rostrum with columella, helical flagellar bands that cover only the anterior portion of the cell, and a robust axostylar trunk posterior to the nucleus (Koidzumi, 1917, 1921). The type species is *Spironympha porteri* Koidzumi from *Reticulitermes flaviceps* (or possibly *R. kanmonensis*, see below). While *Spirotrichonympha* cells are typically elongated and fusiform in shape (Gile et al., 2018; Grassi, 1917), *Spironympha* cells are variable in shape, even within a single species. They can be fusiform or rounded to pyriform with a prominent posteriorly protruding axostyle (Koidzumi, 1917, 1921). However, Duboscq & Grassé treated these genera as variations of the same organism, arguing that all *Spirotrichonympha* and *Spironympha* cells have an axostyle, though not always clearly visible, and that the remaining character, the extent of the flagellar bands, was insufficient for distinguishing genera (Duboscq & Grassé, 1928). This led later authors to treat *Spironympha* as a subgenus of *Spirotrichonympha* (Grassé, 1952; Yamin, 1979). More recently, immunofluorescence and electron microscopic studies revealed that the axostyle of *Spirotrichonympha* is composed of multiple fibers, each in the form of a partially rolled microtubular sheet, while the axostyle of *Spironympha* is a single tube (Brugerolle, 2001, 2005, 2006a; Brugerolle & Bordereau, 2006). This characteristic is correlated with the extent of the flagellar bands, that is, cells with a single axostylar tube also have flagellar bands restricted to the anterior portion of the cell, and cells with a fibrous axostyle also have flagellar bands extending to the posterior. This supports the notion of two distinct genera and clarifies their delimitation. These studies also described new species of both *Spirotrichonympha* and *Spironympha*, effectively reinstating the generic status of these taxa (Brugerolle, 2005, 2006a; Brugerolle & Bordereau, 2006).

Molecular data, on the other hand, have demonstrated that host identity is perhaps the most important characteristic for distinguishing spirotrichonymphid genera. Recent SSU rRNA gene phylogenies indicated that the genus *Spirotrichonympha*, as morphologically defined, is polyphyletic, with *Spirotrichonympha* species from distinct host lineages branching separately (Gile et al., 2018; Jasso-Selles et al., 2017). Because the type host of *Spirotrichonympha* is *Reticulitermes*, *Spirotrichonympha* species from other lineages should be transferred to new genera. So far the genus *Cononympha* has been reinstated for the "*Spirotrichonympha*" symbiont species of *Coptotermes* and *Heterotermes* hosts (Rhinotermitidae), and the new genus *Cuppa* has been established for "*Spirotrichonympha*" from *Paraneotermes* (Kalotermitidae; Jasso-Selles et al., 2017; Taerum et al., 2020). Some of these differences are clear without molecular data, for example "*Spirotrichonympha*" *africana* from *Hodotermes mossambicus* has been transferred to *Uteronympha* on the basis of morphological and ultrastructural characteristics (Brugerolle, 2006b). *Spironympha* species have also described from multiple host lineages, including *Hodotermes mossambicus* and *Hodotermopsis sjostedti*, in addition to their type host lineage *Reticulitermes*. No molecular data are yet available for *Spironympha*, so it is unknown whether the genus is monophyletic or if species from distant hosts will branch separately. For a complete list of *Spironympha* and *Spirotrichonympha* species, see Table S1.

Intergeneric relationships in the Spirotrichonymphea are not well-resolved. Monophyly of each of the genera *Cononympha*, *Holomastigotes* and *Holomastigotoides* is strongly supported in SSU rRNA phylogenies, as is a

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sister group relationship between *Cuppa* and *Fraterculus*. However, the relationships among *Cononympha*, *Holomastigotoides*, *Holomastigotes*, *Spirotrichonympha*, and the *Cuppa*-*Fraterculus* clade remain unresolved. Representatives of the other Spirotrichonymphea genera have not yet been included in molecular phylogenetic analyses. In order to improve the ambiguous phylogeny and systematics of Spirotrichonymphea, molecular phylogenetic analysis based on a wide range of taxa combined with careful morphological observations are necessary. In this study, we obtained sequences from six species of the genus *Spironympha* and three species of *Spirotrichonympha* from the guts of *Reticulitermes* spp. and *H. sjostedti*. Additionally, we collected molecular data from the genera *Cononympha* and *Holomastigotoides* in the gut of an Australian *Coptotermes* sp. and inferred the phylogenetic relationships among Spirotrichonymphea. Based on these analyses and observations of stained specimens, we propose three new genera. We also describe two new species of *Spirotrichonympha* from *Reticulitermes* termites.

MATERIALS AND METHODS

Sampling of host termites

Host termites and symbiotic protists investigated in this study are listed in Table 1. The termite colonies were collected with nest wood and maintained in plastic boxes at room temperature. Host termites were identified based on phenotypic traits of soldier castes (Takematsu, 1999). In addition, identifications were confirmed for *Reticulitermes flavipes* and *Reticulitermes tibialis* by sequencing the mitochondrial 16S rRNA gene as described previously (GenBank accession numbers MK118730 and MK118726; Taerum et al., 2019).

Light microscopy

For specific identification and morphological investigation of the *Spirotrichonympha* and *Spironympha* species in termites from Japan, we carried out microscopic observation of protargol-stained specimens as described previously (Kitade et al., 1997, 2012; Radek et al., 2018). The hindgut contents of worker termites were suspended in 0.4% NaCl and fixed with Schaudinn's fixative prior to protargol staining (modified from procedure A; Honigberg & Davenport, 1951). The slide mounts were observed with a BX-50 light microscope (Olympus, Tokyo) equipped with differential interference contrast or phase contrast. Images were recorded with a DP-27 digital camera (Olympus, Tokyo). Spirotrichonymphea protists were identified to species level for *Hodotermopsis sjostedti* and *Reticulitermes* spp. based on Brugerolle (2005) and Koidzumi (1921). For the specific identification of living protists, we observed termite intestinal contents in advance under a differential interference or phase contrast microscope. Then we carefully compared

TABLE 1 Host termite species and protist species characterized in this study.

Termite	Sampling location	Protist[a]	Cell code
Reticulitermes amamianus	Amamioshima Is., Japan	*Spirotrichonympha ryukyuensis* sp. nov.	RaS1
R. okinawanus	Okinawa Is., Japan	*Spirotrichonympha ryukyuensis* sp. nov.	RoS1
R. yaeyamanus	Okinawa Is., Japan	*Spironympha porteri*	RyS1
R. miyatakei	Amamioshima Is., Japan	*Spironympha porteri*	RmS11
R. kanmonensis	Yamaguchi, Japan	*Spironympha porteri*	RkS9
R. kanmonensis	Yamaguchi, Japan	*Spironympha* sp.	RkSs5
R. tibialis	Arizona, USA	*Spirotrichonympha tibialis* sp. nov.	RD-230, RD-184, SL-ST, RDC-322
R. tibialis	Arizona, USA	*Spironympha kofoidi*	RTI-675, RTI-662, RSL-SPN
R. flavipes	New Jersey, USA	*Spironympha kofoidi*	FPS11
Hodotermopsis sjostedti	Yakushima Is., Japan	*Nanospironympha obtusa* gen. nov	M45[b]
	Yakushima Is., Japan	*Pseudospironympha oblonga* gen. nov.	M2[b]
	Yakushima Is., Japan	*Brugerollina cincta* gen. nov	M5[b]
	Tam Dao, Vietnam	*Nanospironympha obtusa* gen. nov	HOD7-M, HOD3-Obl
	Tam Dao, Vietnam	*Pseudospironympha oblonga* gen. nov.	HOD3-405
Coptotermes sp.	Atherton, Australia	*Holomastigotoides* sp.	CoptoH3
	Atherton, Australia	*Cononympha* sp.	CoptoS2

[a] Species names before revision see also Table S1.

[b] These sequences are obtained from the whole protistan community. See Materials and Methods.

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morphology of the living protists with the protargol specimens. Measurements of the new *Spirotrichonympha* species in *Reticulitermes okinawanus* were taken from the protargol specimens.

Fluorescence in situ hybridization (FISH) on *Spironympha* and *Spirotrichonympha* from *Hodotermopsis sjostedti*

FISH was performed to identify the protist morphotypes that correspond to each rRNA phylotype, using candidate sequences for *Spironympha* obtained from the whole gut contents of *H. sjostedti*. Briefly, the termite gut contents were fixed in 4% paraformaldehyde; the fixed cells were then spotted onto a silane-coated glass slide. After dehydration in ethanol, the slides were incubated with the hybridization solution (0.9 M NaCl and 0.1 M Tris–HCl) containing fluorescently labeled probes for 2 h at 48°C (Noda et al., 2003, 2006; Sato et al., 2014). Specific probes for the protist species designed in this study were HsS-M45-803 (5′-GGCATCGTTTAAGGCGGTAA-3′) for *Spironympha obtusa*, HsS-M2-817 (5′-TGACAAGACTCCGTCGGCA-3′) for *Spironympha oblonga*, and HsS-M5-831 (5′-TTAGAACCCCACCAAAAGATG-3′) for *Spirotrichonympha cincta*. Each of these probes was 5′-labeled with 6-carboxyfluorescein (6-FAM) and used for FISH simultaneously with a probe for all eukaryotes (Ohkuma et al., 1998) 5′-labeled with Texas Red. The specimens were washed for 20 min in a washing buffer (0.2 M NaCl and 0.1 M Tris–HCl) at 48°C. They were then mounted using SlowFade Diamond antifade reagent (Thermo Fisher) and observed under an epifluorescence microscope.

Collection of SSU rRNA gene sequence data using micromanipulation

Protist cells of the genera *Spironympha*, *Spirotrichonympha* and *Holomastigotoides* in the hindgut suspension of *Reticulitermes* spp. and *H. sjostedti* from Japan and *Coptotermes* sp. from Australia were isolated manually and washed extensively under a microscope equipped with a micromanipulator (Cell Tram, Eppendorf), as described elsewhere (Noda et al., 2012). Pools of about 10 isolated cells were used for the following amplifications.

Four to ten cells of each protist species showing a typical morphology were isolated and used as a template for isothermal whole-genome amplification (WGA) or cDNA synthesis, as previously described (Noda et al., 2012). The small subunit ribosomal RNA (SSU rRNA) genes and EF-1α genes were amplified from the WGA product by PCR, using the previously described primers (Noda et al., 2012). If the EF-1α gene was not sufficiently amplified by the first PCR, a second PCR was performed using the primers EF1F2 (5′-CGDGGDATYACNATYGAYAT-3′) and EF1R1 or oligo-dT primes for EF-1α gene by using the first PCR product as a template. In the case of protist species from *Hodotermopsis*, a new second PCR primer set specific for Spirotrichonymphea was designed (EF1FSpi: 5′-AGGCNGAGCGNGAGCGNGG-3′, EF1RSpi: 5′-TGRCARTCRAANACNGGCTGG-3′). After purification, the amplification products were cloned using the Zero Blunt pCR4-TOPO PCR cloning kit for sequencing (Invitrogen) or T-vector pMD19 (Takara) and Competent Quick DH5α (Toyobo). Clones containing inserts of the expected size were partially sequenced, and the complete DNA sequence of each representative clone was obtained. Partial DNA sequences of the clones were sorted into phylotypes with >98.0% sequence similarity.

For termites collected in Vietnam and USA, *Spironympha* and *Spirotrichonympha* 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 cell was washed twice in fresh Ringer's solution (8.5 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$, 0.1 g $NaHCO_3$ per liter, HiMedia Laboratories) and ejected, either singly or in pools of 2–6 cells, into a 0.5 mL tube for DNA extraction using the MasterPure DNA Purification Kit (Epicenter), using half quantities of the manufacturer's protocol and resuspending purified DNA in 5 μL of TE buffer. The SSU 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 described previously (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 *Escherichia coli* (Invitrogen), following the manufacturer's protocols. Inserts from 2 to 8 positive transformant colonies per template were amplified using standard sequencing primers M13F and M13R, purified using the GeneJet PCR purification kit (Thermo Fisher), and sequenced on both strands using an Applied Biosystems 3730 capillary sequencer (Applied Biosystems). For details of isolated cells, see Table S2.

The sequences obtained in this study have been deposited in the DNA Databank of Japan under accession numbers LC722510–LC722521, LC722567–LC722575, and in the US NCBI GenBank under accession numbers OP289677–OP289688, OP294994, and OP293350.

Phylogenetic analyses

DNA sequences obtained in the current study and publicly available sequences were aligned using ClustalW2 and the alignments were refined manually. Ambiguously

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aligned positions were omitted from the subsequent phylogenetic inference analysis; a final alignment of 54 taxa and 1309 sites for SSU was used. For EF-1α, 269 amino acid positions of 17 taxa were used. The maximum likelihood (ML) analysis was conducted in RAxML 8.2 (Stamatakis, 2014), using the GTR+I+Γ model. Nonparametric bootstrapping values were obtained from 10,000 replicates for the SSU rRNA gene and EF-1α gene. Bayesian analysis was performed in MrBayes 3.2.6 (Ronquist et al., 2012) using the GTR+I+Γ model. Analyses were conducted with two independent runs and trees were sampled every 100 generations. The starting tree was random, and four simultaneous Markov chains were run for 20,000,000 generations in each gene. Log likelihoods stabilized well before 5,000,000 generations, and the remaining generations were used to estimate Bayesian posterior probabilities.

RESULTS AND DISCUSSION

Morphology and phylogenetic position of *Spironympha* from *Reticulitermes*

Spironympha cells are characterized by rows of flagella that originate at an apical pseudo-rostrum with columella and proceed in a right-handed helix covering only the anterior 1/4 to 1/2 of the cell length. They have a single axostylar trunk that might or might not be visible as a protrusion from the posterior end of the cell (Brugerolle & Bordereau, 2006; Koidzumi, 1917, 1921). The type species is *S. porteri* (=*Microspironympha porteri*), symbiont of *Reticulitermes flaviceps* collected in northern Taiwan by Koidzumi (1921). However, a recent study determined that both *R. flaviceps* and *Reticulitermes kanmonensis* occur in this area, so the type host of *Spironympha* could be either species (Wu et al., 2019).

We observed *Spironympha* cells in *R. kanmonensis*, *R. yaeyamanus*, and *R. miyatakei* and identified them as *S. porteri* (referred to as *Spironympha* sp. in Kitade & Matsumoto, 1993). *S. porteri* is variable in size (20–55 μm long) with a nucleus positioned anteriorly, at the base of the apical columella. It has a variably shaped posterior that often includes a tail-like appendage in which a slender axostyle is visible. Its spiraling rows of flagella begin at a somewhat blunt apical pseudo-rostrum and proceed for a few turns, ending at or before the cell's equator (Koidzumi, 1917, 1921). Each of these features can be seen in the symbionts from *R. kanmonensis*, *R. miyatakei*, and *R. yaeyamanus* (Figure 1A–D).

We isolated *S. porteri* cells from these three *Reticulitermes* host species and sequenced their amplified SSU rRNA genes (Table 1). Six or eight clones for each protist sample were sequenced (see Table S2), all of which shared more than 98% nucleotide identity. They formed a moderately supported clade with an unidentified symbiont of *Reticulitermes labralis* (MK541701) in our SSU rRNA phylogeny, indicating that the sequences were all derived from the same protist species (Figure 2). However, the eight clones obtained from *S. porteri* in *R. kanmonensis* (represented by RkS9_1 and RkS9_2) included some that branched separately, with

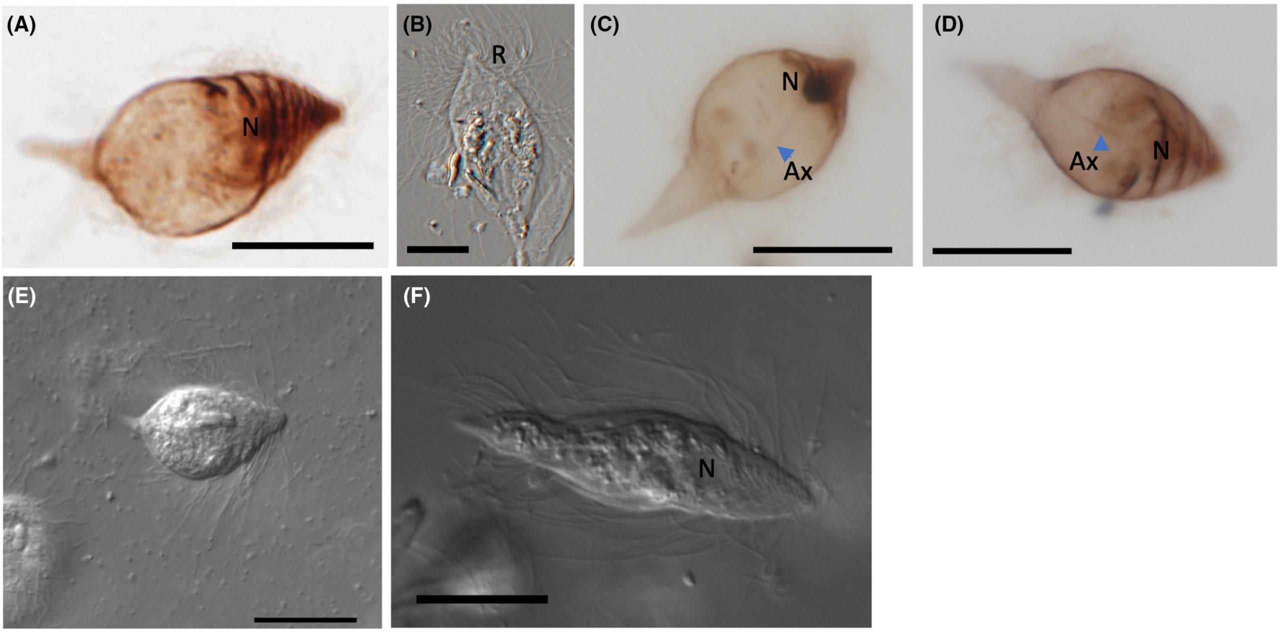

FIGURE 1 Light micrographs of *Spironympha* from *Reticulitermes* hosts. (A–D) *Spironympha porteri*. (A, B) *S. porteri* from *R. yaeyamanus*. (A) Protargol-stained cell. (B) Differential interference contrast (DIC) micrograph. (C) *S. porteri* from *R. miyatakei*, protargol-stained cell. (D) *S. porteri* from *R. kanmonensis*, protargol-stained cell. (E, F) *Spironympha kofoidi* from *R. tibialis*, DIC. axostylar trunk (Ax), nucleus (N), pseudo-rostrum (R). Scale bars = 20 μm.

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an unidentified symbiont of *R. flaviceps* (MK541697). These had lower sequence identity with clones from the other clade (only 96%). These sequence differences might be due to an additional cryptic species in the pool of *S. porteri* cells from *R. kanmonensis*, or they might be due to intraspecific variation. We conservatively consider all sequences of the clade that includes both RkS9_1 and RkS9_2 to derive from *S. porteri*.

An additional, unidentified *Spironympha* phylotype was obtained from *R. kanmonensis* (indicated by RkSs5 in Figure 2). The living cells were smaller than typical *S. porteri*, with a mean length of 12.5 ± 8.3 (n = 5), and the ridged cell surface structures formed by the flagellar rows were more distinct. However, we were unable to identify the equivalent of these cells in stained specimens, possibly due to morphological changes during fixation. The eleven clones derived from this species were sequenced. The sequence similarity was high between these clones (>98.3%) and lower between representative sequence (RkSs5_1) and *S. porteri* sequences (95.4%–96.7%), indicating that these sequences were likely from a distinct species. Future research sequencing many cells of *Spironympha* morphology from East Asian *Reticulitermes* hosts, combined with careful investigation of properly prepared specimens, will help clarify the diversity of this genus.

We also observed cells with *Spironympha* morphology in *R. flavipes* and *R. tibialis*. These cells were variable in size and shape. All exhibited an anterior rostrum with columella and flagellar rows that do not reach the cell posterior, but some had a protruding axostyle while others had rounded or flattened cell posteriors (Figure 1E,F). The mean cell length was 34 ± 13 μm (n = 13). These characteristics are consistent with those of *Spironympha (Spirotrichonympha) kofoidi*, whose type host is *R. flavipes* (=*R. santonensis*) (Duboscq & Grassé, 1928). Although other authors had previously established *Microjoenia*, *Spironympha*, and *Spirotrichonympha* as distinct genera, Duboscq & Grassé considered them as a spectrum of young to mature cells when describing this species. "Young" cells had a *Microjoenia* morphotype, that is, a very small, top-shaped cell, with very short flagellar bands originating from a ring near the cell apex (Brugerolle & Bordereau, 2006; Grassi, 1892). Molecular data have since demonstrated that *Microjoenia* is a distinct, valid genus (Gile et al., 2021). "Intermediate" cells had a similar overall shape to *Microjoenia*, like a toy top, but larger, and with an apical pseudo-rostrum with columella, spiral bands that extend no more than half the length of the cell, and a conspicuous rod-shaped axostyle protruding from the posterior. "Mature" cells also had an apical pseudo-rostrum with columella, spiral bands that extend no more than half the length of the cell, and a rod-shaped axostyle protruding from the posterior, but cells were longer and spindle-shaped (Duboscq & Grassé, 1928). The current morphological diagnosis of *Spironympha* encompasses both the "intermediate" and "mature" morphotypes, due to their single axostylar trunks and flagellar bands extending only partway down the cell (Brugerolle & Bordereau, 2006).

We isolated a single cell with "mature *Spironympha*" morphology from *R. flavipes* (FPS11) and combined pools of cells with "intermediate" (675, RSL-SPN) and "mature" (662) morphology from *R. tibialis* (Figure S1; Table S2). All clones showed at least 98.5% pairwise sequence identity and formed a strongly supported clade (Figure 2). This indicates that the morphology of *S. kofoidi* is indeed variable, though whether these variations represent distinct life cycle stages remains a matter of speculation. Two other *Spironympha* species have been described from *R. flavipes* on the basis of morphological and ultrastructural data: *Spironympha verticis* and *Spironympha lanceata* (Brugerolle & Bordereau, 2006; Table S1). Our molecular data did not indicate additional *Spironympha* species in either *R. flavipes* or *R. tibialis*, though we only characterized one cell from *R. flavipes*. Whether *S. verticis* and *S. lanceata* represent distinct species or simply misinterpreted morphological variability in *S. kofoidi* remains to be confirmed by molecular data.

The *S. kofoidi* sequences obtained here branched sister to *Spirotrichonympha virginica*, which was recently described from *Reticulitermes virginicus* (Gile et al., 2018). This was a mistaken identification, as the cell images in that publication clearly show a single axostylar trunk and a lack of flagellar rows on the posterior half of the cell. Because the long flagella can lie appressed along with the cell, it can appear in unstained material that the flagellar rows are extending past the cell equator. For example, fusiform *Spironympha* cells in *R. flavipes*, *R. virginicus*, and *R. hageni* have been misidentified as *Spirotrichonympha flagellata* (type host: *Reticulitermes lucifugus*) despite the clearly protruding axostylar trunk (Lewis & Forschler, 2004). Our observations and previously published data indicate that there are no *Spirotrichonympha* species in *R. flavipes* (Brugerolle & Bordereau, 2006), and specifically not *S. flagellata* (Duboscq & Grassé, 1928). The *S. virginica* clones (MF065849 and MF065850) share 97.8%–98.4% sequence identity with the *S. kofoidi* clones obtained in this study. Based on these morphological and molecular data, we here transfer *S. virginica* to the genus *Spironympha* as *Spironympha virginica* (see taxonomic summary).

Spirotrichonympha from *Reticulitermes*

Spirotrichonympha are characterized by rows of flagella that originate at an apical pseudo-rostrum with columella and progress in a right-handed helix nearly to the cell's posterior. There is a fibrous compound axostyle and the cell posterior is typically rounded or somewhat flattened, not pointed (Brugerolle & Bordereau, 2006). The type species is *S. flagellata* from *R. lucifugus* for which no molecular data are currently available, though

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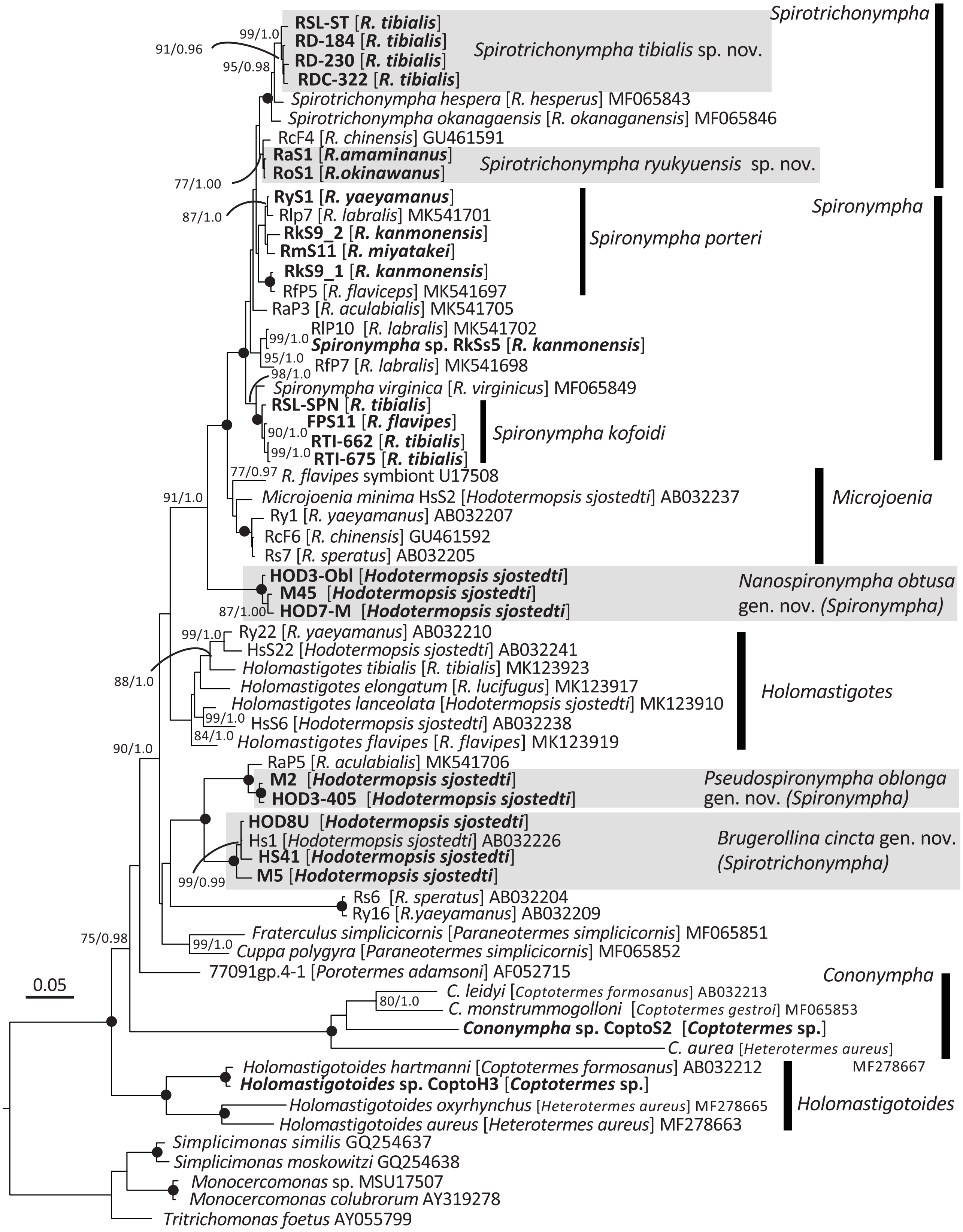

FIGURE 2 Phylogenetic relationships of Spirotrichonymphea based on SSU rRNA sequences. New sequences obtained in this study are indicated by bold text. New taxa proposed in this study are indicated by gray boxes. Host termite names are given in square brackets. Support at nodes is shown where greater than 70% out of 10,000 bootstrap replicates and greater than 0.9 Bayesian posterior probability. Filled circles at nodes indicate full support (100/1.0). The scale bar corresponds to 0.05 nucleotide substitutions per site.

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molecular data are available for *Spirotrichonympha* species from *Reticulitermes hesperus* and *R. okanaganensis* (Gile et al., 2018).

We observed *Spirotrichonympha* cells in the hindgut contents of *R. amamianus* and *R. okinawanus* (reported as *Spirotrichonympha* sp. in Kitade & Matsumoto, 1993). These host termites are closely related (Dedeine et al., 2016) and we could not find morphological differences between the protists in the two host species. Cells are fusiform or elongated pyriform, similar to *S. flagellata*, but with a greater mean cell length (81.7 ± 23.4 μm, *n* = 12). The nucleus is located at the position between the center to one-third position from the anterior end. The pseudo-rostrum is well-developed. At its anterior end is a flagella-free, cap-like operculum of about 1 μm in length. Inside the pseudo-rostrum, the base of the flagellar bands stain heavily, forming a columella (Figure 3).

There are two to six spiral flagellar bands that arise below the operculum and continue along with the body, extending to the posterior end of the cell. The flagellar bands are widely spaced and represent right-handed helices. The flagella emerging near the posterior end of the cell have a length of about 30 μm. They arise from rows of long, needle-like, intensively stained basal bodies constituting flagellar bands, about 5 μm in length at the posterior half of the cell (Figure 3E). The parabasal bodies are located underneath the flagellar bands and are spaced at regular intervals. They appear as slightly irregular spherical or rectangular structures of about 1.5 μm in length at the center or posterior part of the cell, but those located nearer to the anterior end are smaller (Figure 3B,D,F,G). Axostylar fibers do not stain with protargol, and they do not bundle into a single trunk.

SSU sequences from pools of isolated cells from both hosts (RaS1 and RoS1) showed high sequence similarity (99%) and formed a supported clade in our phylogeny along with an unidentified symbiont sequence from *R. chinensis* (GU461591; Figure 2). This clade branched sister to the clade of *Spirotrichonympha* spp. from North American *Reticulitermes* hosts, albeit with weak support (Figure 2). Judging from the phylogeny and complete isolation of its host termites from the other *Reticulitermes* species, *Spirotrichonympha* sp. in *R. amamianus* and *R. okinawanus* should be treated as a distinct species. We here describe this protist as *Spirotrichonympha ryukyuensis*. This species was observed in the gut of *Reticulitermes amamianus* and *Reticulitermes okinawanus* collected from the archipelago of southern Japan: Tokara Islands (Kuchinoshima Is., Nakanoshima Is., Suwanosejima I, Akusekijima Is., Takarajima Is.), Kikaijima Is., Amami-Oshima Is., Tokunoshima Is., Okinoerabujima Is., Yoron Is., Okinawa Is., Kumejima Is., Miyakojima Is., Irabujima Is., Shimojishima Is., Ikemajima Is. (unpublished data by O.K.). These southern islands in Japan are called Ryukyu archipelago.

We also observed *Spirotrichonympha* cells in the hindgut of *R. tibialis* (Figure 3H). No *Spirotrichonympha* cells were observed in *R. flavipes*, in agreement with prior studies (Brugerolle & Bordereau, 2006). The mean length of *Spirotrichonympha* cells in *R. tibialis* was 68 ± 12 μm (*n* = 16). Cells were ovoid to pyriform, with a well-developed apical pseudo-rostrum and a rounded posterior. Flagellar bands were widely and somewhat unevenly spaced, giving the cells a lumpy appearance. The nucleus was centrally positioned, and the helical flagellar bands continued from the rostrum nearly to the posterior pole of the cell (Figure 3H; Figure S1).

We isolated a single cell and three pools of 4–5 cells each matching this morphology from *R. tibialis* collected in two locations (Figure S1; Table S2). The 23 clones sequenced from these templates shared at least 98.8% sequence identity and they formed a supported clade in our SSU phylogeny (Figure 2). This clade branched sister to *Spirotrichonympha hespera* from *R. hesperus*, with which our sequences shared 97.9%–98.4% identity. *S. hespera* has a size range similar to the *R. tibialis* symbiont (53–88 μm in length), but it is characterized by distinctive anterior flagella that consistently and sharply recurve ahead of the cell rather than extending out perpendicularly or lying posteriorly along with the cell surface, as in other *Spirotrichonympha* species (Gile et al., 2018). For these morphological and molecular reasons, and because it inhabits a distinct host from previously described species, we consider the *R. tibialis* symbiont to be a new species: *Spirotrichonympha tibialis*. Note that *R. hesperus* is the sister species to *R. tibialis* (Austin et al., 2008), so it is perhaps unsurprising that their *Spirotrichonympha* symbionts are also sister species.

The type species of the genus *Spirotrichonympha*, *S. flagellata*, was described in *Reticulitermes lucifugus*. It was later also reported to occur in *R. hesperus*, *R. tibialis* and *R. virginicus*, though these were not taxonomy-focused reports (Yamin, 1979). This species is reported to be quite large, ranging from 70 to 100 μm in length, with an anteriorly positioned nucleus (Grassi, 1917). *S. hespera* and the *R. tibialis* symbiont are smaller, 53–88 and 37–81 μm in length, respectively, and both have a more centrally located nucleus, excluding them from the type species (Figure S1; Gile et al., 2018). It, therefore, seems likely that the SSU sequence from *S. flagellata*, when it is obtained, will not be highly similar to those of *S. hespera* or the *R. tibialis* symbiont. Its host is geographically and phylogenetically distant from both *R. hesperus* and *R. tibialis* (Austin et al., 2008; Dedeine et al., 2016).

Spirotrichonymphea from *Hodotermopsis sjostedti*

Two *Spironympha* species have been described from *H. sjostedti*, *Spironympha obtusa* and *Spironympha oblonga*

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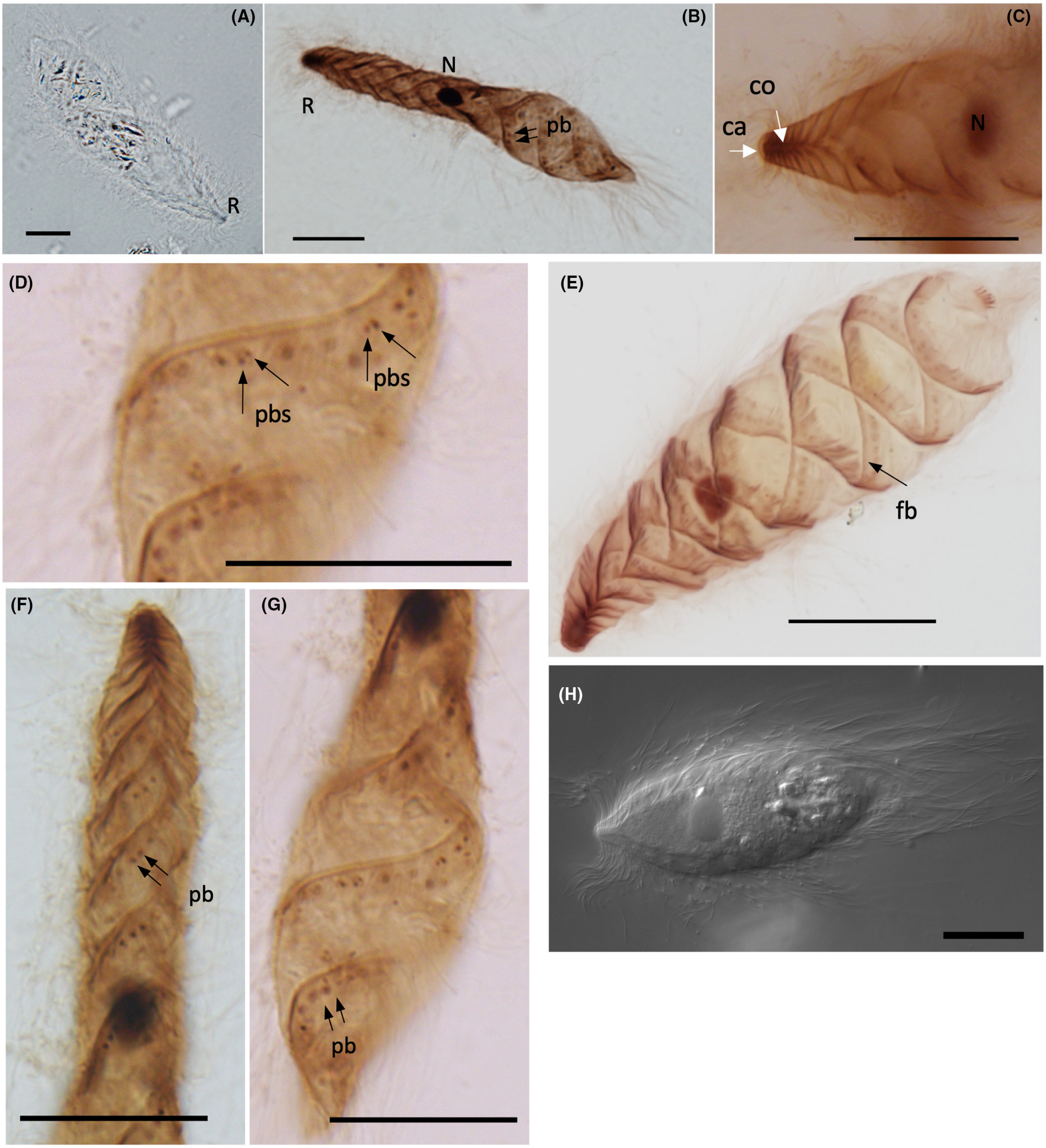

FIGURE 3 Light micrographs of *Spirotrichonympha* from *Reticulitermes* hosts. (A–G) *Spirotrichonympha ryukyuensis*. (A) *S. ryukyuensis* from *R. amamianus*, DIC. (B–G) *S. ryukyuensis* from *R. okinawanus*, protargol staining. (H) *Spirotrichonympha tibialis* from *Reticulitermes tibialis*, DIC. apical cap (ca), columella (co), flagellar band (fb), nucleus (N), parabasal body (pb), stained area in parabasal body (pbs), pseudorostrum (R). Scale bars = 20 μm.

(Brugerolle, 2005). Although *H. sjostedti* is phylogenetically distant from *Reticulitermes*, the type host for the genus *Spironympha*, these species were included in *Spironympha* because their spiral flagellar bands occur only on the anterior half (or less) of the cell, and because they each possess a single axostylar trunk that often forms a point protruding from the cell posterior (Brugerolle, 2005). We observed these characteristics in cells from *H. sjostedti* collected in both Japan and Vietnam (Figure 4). Previously, no molecular data have been available for either of these species.

Spironympha obtusa is a relatively small cell, measuring 19–39 μm, with a blunt anterior end that houses a well-defined columella, five or six flagellar bands that extend about a quarter the length of the cell, terminating at around the level of the nucleus, just posterior to the

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columella, and a posteriorly protruding arrow-shaped axostyle (Brugerolle, 2005). Our protargol-stained *Spironympha obtusa* specimens agree well with this description (Figure 4D–F). We observed an ovoid cell shape with a distinct rostrum-like structure at the anterior end of the cell. Flagellar bands occupy the anterior quarter of the cell. The root of the basal bodies that constitute the outer edge of the columella is intensely stained with protargol (Figure 4E). A well-stained nucleus is located at the posterior base of the columella. The distance between the anterior end of the cell and the nucleus is almost the same as the nuclear length (Figure 4D,E). From the posterior base of the columella, 5–6 intensely stained lines, the basal body roots, extend diagonally backward with a slight winding, and terminate at around the nucleus. Near the terminus of each line, well-stained spherical parabasal bodies form a circle around the nucleus (Figure 4F). Each parabasal body contains one densely stained particle. Axostyle fibers are bundled behind the nucleus into a robust, rod-like, unstained axostyle, which protrudes from the posterior end of the cell in most individuals.

Spironympha oblonga is slightly larger, measuring 32–45 μm in length, with flagellar lines extending the anterior third of the cell; the rostrum is short and has a barely defined columella (Brugerolle, 2005). Our protargol-stained *Spironympha oblonga* specimens agree well with this description (Figure 4J–L). The cell of this species has a spindle shape. The pseudo-rostrum at the anterior end of the cell is not well-defined (Figure 4J,K). A well-stained nucleus is located in contact with the posterior base of the columella-like structure. The distance between the anterior end of the cell and the nucleus is shorter than the nuclear length (Figure 4J,K). Four flagellar bands forming a right-handed helix occupy the anterior one-third of the cell. The rod-shaped basal bodies are weakly stained with protargol. The roots of the basal bodies are intensely stained, constituting spiral cords. Weakly stained spherical parabasal bodies, each containing two intensely staining rod-like areas, are arranged along with the spiral cord (Figure 4L). Axostyle fibers are bundled behind the nucleus into a robust, rod-like, unstained axostyle, which protrudes from the posterior end of the cell in most individuals. The posterior tip of the axostylar protrusion is usually slightly curved (Figure 4K).

In order to link SSU sequence to morphology for these species, we carried out fluorescence in situ hybridization (FISH) and single cell PCR. For FISH, we obtained several SSU sequences expected to belong to Spirotrichonymphea from our previous analyses of hundreds of clones of the SSU rRNA gene amplified by RT-PCR from the whole gut community of *H. sjostedti* (unpublished data). We designed probes specific for these expected Spirotrichonymphea sequences and hybridized them with hindgut contents from *H. sjostedti*. FISH experiments revealed that the sequences M45 and M2 were assigned to *S. obtusa* and *S. oblonga*, respectively (Figure 4A–C,G–I). These results agreed with our single cell PCR experiments, in which SSU sequences from *Spironympha obtusa* cells (HOD7-M and HOD3-Obl) branched with the M45 clone, and sequences from *Spironympha oblonga* cells (HOD3-405) branched with M2 (Figure 2). Because these species branch distantly from each other and from *Spironympha porteri*, the type species, they should both be transferred to new genera. We here propose *Nanospironympha* gen. nov. for *Nanospironympha obtusa*, n. comb., and *Pseudospironympha* gen. nov. for *Pseudospironympha oblonga* n. comb.

One species of *Spirotrichonympha* has been described from *H. sjostedti*, *Spirotrichonympha cincta* (Brugerolle, 2005). Cells are 35–85 μm in length, pyriform, with a long apical pseudo-rostrum (Figure 4M–R). Flagellar bands emerge from the rostrum and spiral around at least three-fourths of the cell length. Flagellar bands are closely and evenly spaced. The axostyle is composed of several fibers, one or more of which might protrude from the posterior end of the cell (Brugerolle, 2005). Our FISH experiments indicated that the sequence M5 was assigned to *S. cincta* (Figure 4M–O), in agreement with a previous study that linked clone Hs1 (AB032226) with this morphotype by FISH (Ohkuma et al., 2000). We also obtained SSU sequences from this species by single cell PCR, from two cells isolated and amplified separately, that measured 54 and 30 μm in length (Figure S1). All clones branched with the Hs1 clone with full support. Because this species

FIGURE 4 Light and fluorescence micrographs of Spirotrichonymphea from *Hodotermopsis sjostedti*. (A–F) *Nanospironympha obtusa*, n. comb. (A–C) Fluorescence in situ hybridization (FISH). Sequence-specific probe M45 conjugated with 6-FAM and eukaryotic consensus control probe conjugated with Texas red were used simultaneously. Arrows indicate cells exhibiting positive signal from the specific probe. (A) Phase contrast micrograph. (B) Fluorescence image with green wavelength filter, indicating cells positive for specific probe hybridization. (C) Fluorescence image with red wavelength filter, indicating all eukaryotic cells. (D–F) Protargol staining demonstrates the anterior nucleus, deeply staining columella, spiral flagellar bands on the anterior portion of the cell, and rod-like axostyle protruding from the cell posterior. (G–L) *Pseudospironympha oblonga*, n. comb. (G–I) FISH with specific probe M2 (6-FAM) and eukaryotic consensus probe (Texas red) as for A–C. (J–L) Protargol staining demonstrates the anterior nucleus, weakly developed pseudo-rostrum, spiral flagellar bands on the anterior portion of the cell, and rod-like axostyle protruding from the cell posterior. (M–R) *Brugerollina cincta*, n. comb. (M–O) FISH with specific probe M5 (6-FAM) and eukaryotic consensus probe (Texas red) as for A–C and G–I. (P–R) Protargol staining demonstrates the prominent pseudo-rostrum, closely spiraled flagellar bands nearly reaching the posterior end of the cell, and rod-like axostyle protruding from the cell posterior. Axostylar trunk (ax), columella (co), nucleus (n), parabasal body (pb), stained area in parabasal body (pbs). Bar = 20 μm.

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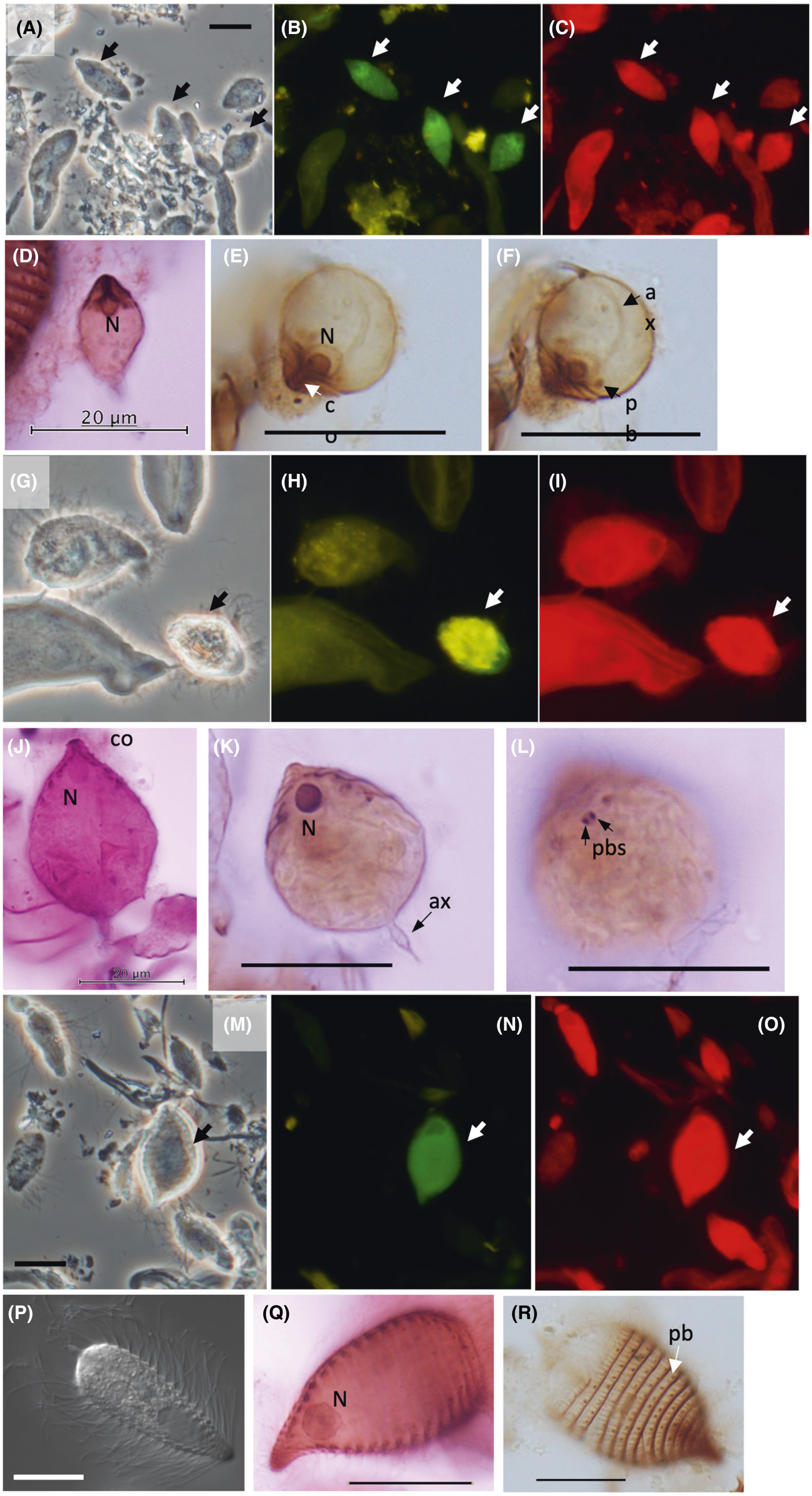

branches distantly from true *Spirotrichonympha* from *Reticulitermes* and has distinct characteristics (i.e. occasionally protruding axostyle, closely and evenly spaced flagellar bands), it should be transferred to a new genus. We here propose *Brugerollina* gen. nov. to accommodate *Brugerollina cincta*, n. comb.

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Phylogenetic relationships among the genera in Spirotrichonymphea

In the hindgut of *Coptotermes* sp. collected in Atherton, Australia, we observed *Holomastigotoides* and *Cononympha*. Both of these genera are typically associated with *Coptotermes* and *Heterotermes* hosts (Jasso-Selles et al., 2017, 2020; Yamin, 1979). *Holomastigotoides* are readily identified by an apically positioned nucleus and closely spaced flagellar bands that run the length of the cell. The species we observed additionally had a large mean cell size, 195.6 ± 89.3 ($n = 3$) and a blunt anterior (Figure 5A). *Cononympha* cells superficially resemble *Spirotrichonympha* cells in having an apical rostrum with columella, but they can be morphologically distinguished by their tendency to trail detritus attached to their posterior flagella. The species we observed had a mean cell length of 23.2 ± 13.3 ($n = 3$) and an ovoid shape with an anterior pseudo-rostrum and a blunt posterior (Figure 5B). We obtained the SSU sequence from these cells and included them in a phylogenetic analysis with new sequences described above and previously published sequences to determine the Spirotrichonymphea phylogeny (Figure 2).

In our analysis, the SSU rRNA gene sequences from each of the genera *Holomastigotes*, *Holomastigotoides*, *Cononympha*, *Nanospironympha*, *Pseudospironympha*, and *Brugerollina* formed monophyletic groups with strong support. Although the genera *Spirotrichonympha* and *Microjoenia* formed monophyletic groups, the support values were less strong. Each sequence of *Holomastigotoides* (CoptoH3) and *Cononympha* (CopotoS2) from *Coptotermes* sp. formed an exclusive clade with sequences from congeneric symbionts of other *Heterotermes* and *Coptotermes* host termites. Intriguingly, unidentified symbiont species from *Reticulitermes aculabialis* formed a strongly supported clade with *Pseudospironympha oblonga* and *Brugerollina cincta* from *H. sjostedti*. The protist fauna in the gut of *R. aculabialis* has not been described using morphological methods, only from SSU sequences amplified from whole gut DNA. However, this result indicates that *P. oblonga* or closely related species inhabit in the gut of this termite despite the different host termites. It is necessary to confirm the protist composition in this termite species.

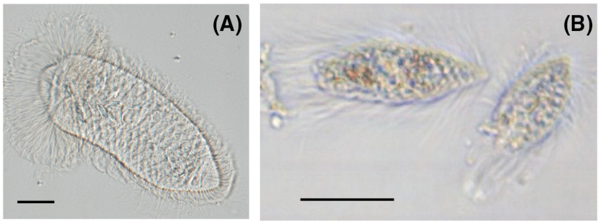

FIGURE 5 Light micrographs of Spirotrichonymphea species from *Coptotermes* hosts. (A) *Holomastigotoides* sp., DIC optics, scale bar = 50 μm. (B) *Cononympha* sp., phase contrast optics, scale bar = 20 μm.

Spironympha and *Spirotrichonympha* together formed a fully supported clade sister to *Microjoenia*. Within this clade, *Spirotrichonympha* were monophyletic, though only with weak support. Within *Spirotrichonympha*, species from North American termites formed a strongly supported clade. Meanwhile *Spironympha* did not form a clade but instead were paraphyletic to *Spirotrichonympha*, with *S. porteri* in a moderately supported sister position. The positions of the other *Spironympha* phylotypes, RkSs5 and *S. kofoidi* are not resolved, so it remains possible that the genus *Spironympha* is monophyletic.

We also obtained EF-1α gene sequences from each of the species studied here (Figure 6). Several clones were sequenced, most of the sequences obtained from single protist species usually shared high sequence similarity (>98.0%). In the case of *Spironympha porteri* from *R. yaeyamanus* and *Cononympha* sp., however, two sequences sharing 89.9% and 82.3% sequence similarity (E1_RyS1 and E1_RyS5, CopS1 and CopS3) were obtained. It is not clear whether these sequences were variations in the genome or intraspecific variations caused by using pooled cells as templates.

Although the number of the sequenced samples is smaller than that of SSU rRNA gene, the analysis using EF-1α gene sequences inferred similar relationships. *Spironympha* and *Spirotrichonympha* from *Reticulitermes* form a supported clade that excludes the *H. sjostedti* symbionts, and *S. cincta* and *S. oblonga* have a strongly supported sister relationship. The genera *Holomastigotoides* and *Cononympha* form a moderately supported clade that branches sister to all remaining Spirotrichonymphea. These genera share the characteristic of flagellar rows that nearly reach the posterior end of the cell and axostyle fibers that are not integrated into a rod-like structure at or near the posterior end of the cell. Although the root position is unclear, these characters are possibly the ancestral characters, and the limited extension of the flagellar rows and rod-like axostyle exhibited by *Spironympha* spp. may be apomorphic. Ultimately, our taxon sampling covered only five out of 13 genera in the Spirotrichonymphea. Analyses based on more intensive taxon sampling and of more marker genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are, therefore, desirable in order to verify the phylogenetic hypothesis presented in this study. Actually, GAPDH sequences have been demonstrated to contain a greater phylogenetic signal and have yielded well-resolved trees largely congruent with the SSU rRNA gene phylogeny (Noda et al., 2012).

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Taxonomic summary

Phylum Parabasalia Honigberg 1973
Class Spirotrichonymphea Grassé, 1952
Order Spirotrichonymphida Grassé, 1952

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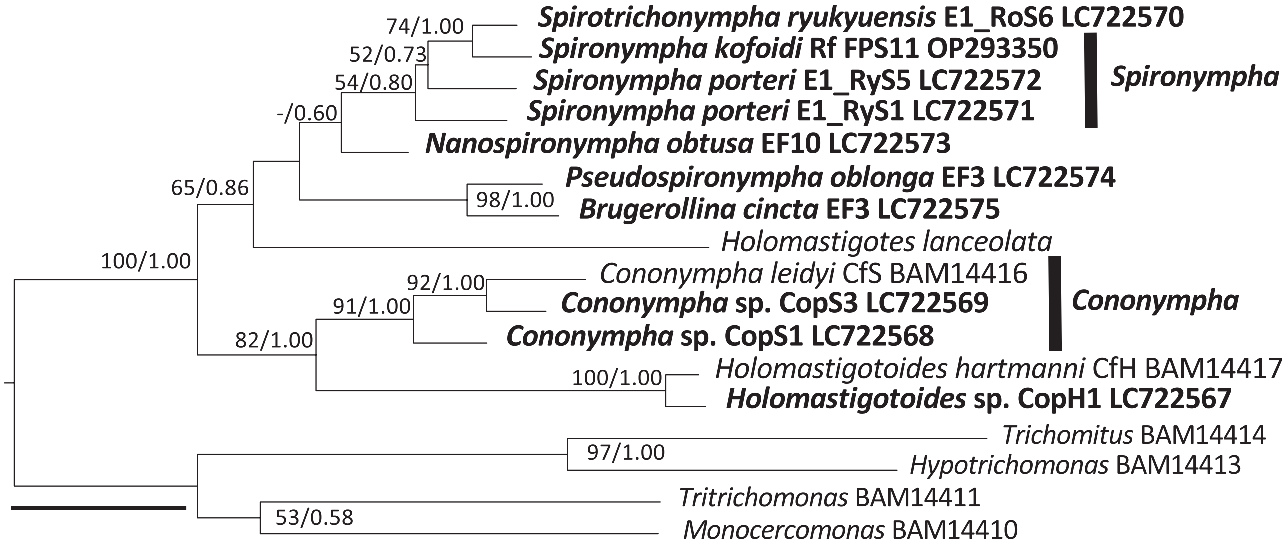

FIGURE 6 Phylogenetic analyses of Spirotrichonymphea based on the partial amino acid sequences of EF1α, using 269 amino acid positions. The trees were inferred by the ML method. The numbers at the nodes indicate ML bootstrap values and Bayesian posterior probability. The scale bar corresponds to 0.05 substitutions per site. New sequences obtained in this study are indicated by bold text. The database accession numbers are indicated after the names of taxa.

Family Holomastigotoididae Grassi, 1917 emend Čepička et al., 2010

Spirotrichonympha Grassi & Foà, 1911

Spirotrichonympha ryukyuensis Noda & Kitade sp. nov.

Diagnosis: Multiflagellate, uninucleate parabasalian protist. Cells of spindle or elongated pear shape, 81.7 ± 23.4 μm in length (44.8–118.6, n = 12) and 20.9 ± 2.4 μm in width (17.4–26.1, n = 12). Cells possess an anterior pseudo-rostrum with columella. Flagellar bands with well-stained basal bodies forming a right-handed helix extend to posterior end of the cell. Number of flagellar bands 3.2 ± 1.3 (2–5). Nucleus round to oval, located in the anterior half of the body. Longitudinal length of nucleus is 7.0 ± 1.3 μm (3.7–9.8, n = 12). Axostylar fibers not stained with protargol nor bundled into a single trunk. Obligate symbiont of *Reticulitermes okinawanus* and *R. amamianus.*

Etymology: Named after Ryukyu, the region name of Okinawa and Amami in Japan in Late Middle Ages and Early Modern Period.

Gene sequences: SSU rRNA gene accession number LC722510.

Type host: *Reticulitermes okinawanus* Morimoto 1968

Type host locality: The termites were collected in Oku, Kunigami village, Okinawa pref., Japan in 1991.

Hapantotype: A protargol-stained microscopic slide of symbiotic protist of *Reticulitermes okinawanus* is deposited at the National Museum of Nature and Science, Tokyo under accession number NSMT-Pr 463.

ZooBank registration: 9740C6D1-E768-4399-A0C3-25EC1C486384

Spirotrichonympha tibialis Gile sp. nov.

Diagnosis: Multiflagellate, uninucleate parabasalian protist. Cells pyriform, 68.8 ± 10.7 μm in length (37–81, n = 16) and 26 ± 3 μm in width (21–32, n = 16). Cell anterior is a pseudo-rostrum with columella; cell posterior is rounded. Flagellar bands forming a right-handed helix extend to the posterior end of the cell. Nucleus round to oval, located in the anterior half of the body, but not at the apex. No visible axostylar protrusion. Obligate symbiont of *Reticulitermes tibialis.*

Etymology: The specific epithet tibialis matches the host, indicating its obligate relationship.

Gene sequences: SSU rRNA gene accession numbers OP289677–OP289680.

Type host: *Reticulitermes tibialis* Banks

Type host locality: Payson, Arizona, USA.

Hapantotype: Permanent microscope slide of hematoxylin-stained protist cells deposited at the Beaty Biodiversity Museum, University of British Columbia, Vancouver, Canada under accession number MI-PR218.

Zoobank registration: 3E9156FE-507F-4E83-A0DF-A8E5B0E8D4F5

Brugerollina Taerum & Gile gen. nov.

Diagnosis: Spindle-shaped parabasalid protist with strongly developed pseudo-rostrum and columella. Anteriorly positioned nucleus located close to posterior base of columella. Flagellar bands forming a right-handed tight helix extend from the cell apex through three-fourths the cell length. Along with flagellar band, small spherical parabasal bodies rather regularly arranged inside. Axostylar fibers forming several bundles

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that can protrude from the cell posterior in one or more points. Xylophagous.

Type species: *Spirotrichonympha cincta* Brugerolle, 2005

Species assigned: *Brugerollina cincta* Taerum & Gile comb. nov.

Etymology: Fem. N., patronym honoring Guy Brugerolle whose excellent work characterizing the morphology and ultrastructure of Spirotrichonymphea greatly advanced the field.

Brugerollina cincta Taerum & Gile comb. nov.

Description: Morphological and ultrastructural characteristics given in Brugerolle, 2005.

Diagnosis: Obligate symbiont of *H. sjostedti*. Moderate to large sized spirotrichonymphid cells (32–80 μm in length) with long narrow apex. Specimens from Tokunoshima Is., Japan: cell length 41.44 ± 5.3 μm (31.5–52.9, $n = 17$) without axostylar protrusion; 53.58 ± 6.1 μm (43.5–64.5, $n = 17$) including protrusion. Cell width 27.3 ± 4.7 μm (20.8–36.7, $n = 17$). Longitudinal length of nucleus 7.7 ± 1.4 μm (4.6–9.7, $n = 17$). Number of flagellar bands 2 or 4 ($n = 19$).

Gene sequences: SSU rRNA gene accession numbers AB032226 and LC722520.

Zoobank registration: 439CE8BD-3928-4EF6-A8C0-D23EC6C7382F

Spironympha Grassi & Foà, 1911.

Spironympha virginica Gile comb. nov.

Diagnosis: As in Gile et al., 2018 for *Spirotrichonympha virginica* Gile & Keeling. Species incorrectly assigned to the genus *Spirotrichonympha*, here transferred to *Spironympha*.

Gene sequences: SSU rRNA gene accession numbers MF065849–MF065850.

Zoobank registration: 37B1E014-E9CA-4966-9021-CD7BF21BDC5C

Nanospironympha Noda & Kitade gen. nov.

Diagnosis: Parabasalid protist with an apical pseudorostrum housing a columella. Flagellar bands arising from the base of the columella extending less than one fourth the cell length, terminating at the level of the nucleus. Nucleus anterior, in contact with the posterior base of the columella. Distance between cell apex and nucleus approximately equal to nuclear length. Spherical parabasal bodies surround the nucleus in a circle. Axostylar fibers bundled into a rod-like axostyle that protrudes from the cell posterior in a spike. Cytoplasm contains rounded vesicles and ingested wood particles.

Type species: *Spironympha obtusa* Brugerolle, 2005

Species assigned: *Nanospironympha obtusa* Noda & Kitade comb. nov.

Etymology: Gr. *Nanos* = dwarf + *Spironympha* (fem.) = the genus to which this species was originally assigned.

Nanospironympha obtusa Noda & Kitade comb. nov.

Description: Morphological and ultrastructural characteristics given in Brugerolle, 2005.

Diagnosis: Obligate symbiont of *H. sjostedti*. Small spirotrichonymphid protists. Specimens from Tokunoshima Is., Japan: 14.7 ± 2.4 μm in length (10.2–18.2, $n = 15$) and 12.3 ± 2.3 μm in width (9.0–16.7, $n = 15$). Longitudinal length of nucleus is 3.7 ± 0.5 μm (2.9–4.6, $n = 15$). Most individuals with conspicuously pointed posterior end due to thick, protruding axostyle.

Gene sequences: SSU rRNA gene accession number LC722521.

ZooBank registration: 2F3F048E-8A13-4792-9904-17FC56CD8864

Pseudospironympha Noda & Kitade gen. nov.

Diagnosis: Spindle-shaped parabasalid protist with weakly developed pseudo-rostrum and columella. Anteriorly positioned nucleus in contact with posterior base of columella. Distance between cell apex and nucleus shorter than nuclear length. Four flagellar bands forming a right-handed helix extend from the cell apex along with the anterior one-third of cell. Spherical parabasal bodies arranged along with flagellar band, each containing two intensively stained rod-like areas. Axostylar fibers bundled into a rod-like axostyle. Xylophagous.

Type species: *Spironympha oblonga* Brugerolle, 2005

Species assigned: *Pseudospironympha oblonga* Noda & Kitade comb. nov.

Etymology: Gr. *pseudo* = false + *Spironympha* (fem.) = the genus to which this species was originally assigned.

Pseudospironympha oblonga Noda & Kitade comb. nov.

Description: Morphological and ultrastructural characteristics given in Brugerolle, 2005.

Diagnosis: Obligate symbiont of *H. sjostedti*. Moderately sized spirotrichonymphid cells. Specimens from Tokunoshima Is., Japan: 27.7 ± 2.8 μm in length (24.6–34.5, $n = 15$) and 21.8 ± 3.2 μm in width (15.7–28.4, $n = 15$). Longitudinal length of nucleus is 5.8 ± 0.9 μm (4.6–7.6, $n = 15$). Cell of most individuals tightly filled

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with wood particles, with pointed posterior end by axostyle protrusion.

Gene sequences: SSU rRNA gene accession number LC722519.

ZooBank registration: A451C49C-F5D6-40F7-8404-10D84030ACDA

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