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Immunological and ultrastructural characterization of spirotrichonymphid flagellates from *Reticulitermes grassei* and *R. flavipes* (syn. *R. santonensis*), with special reference to *Spirotrichonympha*, *Spironympha* and *Microjoenia*

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Abstract

Five species of spirotrichonymphids representing three genera have been studied by light and immunofluorescence microscopy, and by transmission electron microscopy. The genus *Spirotrichonympha*, represented by *S. flagellata* from *Reticulitermes grassei*, is characterized by a compound axostyle composed of several fibers or subaxostyles. The genus *Spironympha*, represented by *S. kofoidi* from *Reticulitermes flavipes* (syn. *R. santonensis*) and by the two new species *S. verticis* and *S. lanceata*, is characterized by flagellar lines restricted to the anterior area and a simple, tubular axostyle. *Spironympha verticis* and *S. lanceata* are mainly distinguished by ultrastructural details of their flagellar lines and axostyle. These three *Spironympha* species were found in hosts identified as *R. flavipes* or *R. santonensis*, but not in *R. grassei*. This provides additional support for the synonymy of *R. santonensis* with *R. flavipes* recently demonstrated by molecular methods. The generic diagnosis of *Microjoenia* is emended, based on an ultrastructure study of *M. fallax*.

Keywords: Biodiversity; Protozoa; Parabasalia; Termites; Immunofluorescence; Ultrastructure

Introduction

Mapping biodiversity by the identification and description of species is a continuing challenge. This task has not been completed (May 1990), particularly not for microorganisms such as bacteria, archaea (Pace 1997; DeLong and Pace 2001) and protists (Patterson 1999, 2001; Finlay 2001; Dawson and Pace 2002). This is especially true for the symbiotic protozoa living in the hindgut of 'lower termites' that are involved in wood, grass and soil digestion (Inoue et al. 2000; König et al. 2002). These protozoa comprise parabasalid and oxymonad flagellates that were mostly identified by light microscopy in the early 20th century (Grassé 1952; Yamin 1979; Brugerolle and Lee 2001). However, this primary identification of the symbiotic protozoa has not been performed in many termite species (Yamin 1979), or is incomplete or sometimes unusable. Over the last 30 years, electron microscopy has proved an important tool for characterizing the genera (Patterson 1999, 2001; Taylor 1999; Brugerolle and Patterson 2001), but several genera harbored by termites could not be studied by this

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technique (Hollande and Carruette-Valentin 1971; Brugerolle and Lee 2001; Brugerolle and Radek 2006). Molecular identification of the genera and species living in termites is in its early stages (Gunderson et al. 1995; Keeling et al. 1998; Gerbod et al. 2002; Keeling 2002; Moriya et al. 2003; Ohkuma et al. 2000, 2005); many sequences from these works have not been assigned to named taxa. One of the major obstacles to such studies is the lack of primary identification of genera and species by light microscopy, which is necessary to assign a sequence to a species by in situ hybridization. It is also important to point out that termite flagellates often cannot be cultivated but have been successfully isolated with a micromanipulator (Fröhlich and König 1999). Therefore, sequence amplification is performed on isolated organisms, or on the whole content of the termite gut, and the sequences have to be screened and assigned afterwards. Moreover, the symbiotic fauna in a termite is complex; some 'lower termite' species harbor about 10 genera and 20 species of protozoa, which complicates identification by either microscopy or molecular biology.

We have undertaken to better characterize spirotrichonymphids (Brugerolle 2001; Brugerolle and Bordereau 2004) that are symbiotic polymastigote parabasalids largely represented in 'lower termites' (Grassé 1952; Honigberg 1970; Mannesmann 1972; Yamin 1979). In a first study of the *Porotermes* flagellates from Australia, the genera *Microjoenia*, *Spirotrichonympha* and *Spirotrichonymphella* were compared by electron microscopy (Brugerolle 2001). In a second study of *Hodotermopsis sjoestedti* flagellates, the genus *Holomastigotes* was characterized (Brugerolle and Bordereau 2004). The latter study has been completed by the characterization of *Spirotrichonympha*, *Spironympha* and *Microjoenia* species from *Hodotermopsis sjoestedti* (Brugerolle 2005). In the course of these studies, it was discovered that species of these three genera also occur in termites living in Europe, that they had not been studied by electron microscopy, and that some of these spirotrichonymphid species are new to science.

Since the first cytological description of the flagellates of *Reticulitermes lucifugus* by Grassi (1917), many additional studies have contributed to identifying the flagellates of such termites in Europe, as reported by Yamin (1979). One major contribution was that of Duboscq and Grassé (1928) who described *Spirotrichonympha flagellata* from *Reticulitermes grassei* and *Spironympha kofoidi* from *R. santonensis*, a termite first identified by Feytaud in France (Feytaud 1924; Clément 1978; Vieau 2001).

For the present study, we used light-, immunofluorescence and electron microscopy to study the flagellates of termites identified as *Reticulitermes grassei* Clément, 1978, *R. santonensis* (Feytaud, 1924) from France, and as *R. flavipes* (Kollar, 1837), a species reported from the USA (Austin et al. 2002) and also from various areas of Europe (Ye et al. 2004). Our study not only provides new data to characterize the spirotrichonymphid genera *Spirotrichonympha*, *Spironympha* and *Microjoenia*, but also identifies two new *Spironympha* species in *Reticulitermes santonensis* that are also present in *R. flavipes*. This supports the synonymy between *R. flavipes* and *R. santonensis*, which has been long suspected (Feytaud 1924; Vieau 2001) and recently demonstrated by molecular phylogenetic studies (Miura et al. 1998; Clément et al. 2001; Jenkins et al. 2001; Austin et al. 2002, 2004; Marini and Mantovani 2002; Ohkuma et al. 2004; Uva et al. 2004; Ye et al. 2004).

Material and methods

The termite *Reticulitermes grassei* from the southwest of France (Clément et al. 2001; Uva et al. 2004), treated as *R. lucifugus grassei* by other authors (Jenkins et al. 2001; Marini and Mantovani 2002), was collected in the area of Les Eyzies; *R. santonensis* (Clément et al. 2001; Uva et al. 2004) was collected on the Ile d'Oléron and cultured at the laboratory of the Université de Bourgogne in Dijon. Both were identified by morphological criteria (Clément 1978). Specimens of *R. flavipes* were provided by Prof. König from the University of Mainz (Germany); they originate from the Bundesanstalt für Materialforschung und Materialprüfung (Berlin, Germany). The hindgut of each studied termite was opened with a pair of tweezers, and the fluid content was mixed in a drop of Ringer's solution. Protozoa were observed and photographed under either phase contrast or differential interference contrast, using a Leica DMR microscope equipped with a Q-Fish Light Station.

For immunofluorescence, cells were permeabilized in 0.5% Triton X-100 in Tris-maleate buffer for 1 min, and air-dried on immunofluorescence slides previously coated with 0.1% poly L-lysine solution (Sigma). Alternatively, the Triton-permeabilized cells were fixed with 3.7% formaldehyde for 5 min before drying on the poly L-lysine-coated immunofluorescence slides. Slides were stored at −20 °C. After two on-hour washes in PBS, the cells were blocked with 1% bovine serum albumin for 15 min, and incubated overnight at 4 °C with the undiluted supernatant of the monoclonal antibodies (MAb). After three on-hour washes in PBS, the cells were incubated with a 1/200 dilution of the secondary antibody, an anti-mouse Ig:IgG/M antibody conjugated with fluorescein isothiocyanate (FITC) (Sigma). After washing twice in PBS for 1 h, the slides were mounted in 1/1 PBS/glycerine solution containing 10 mg/ml DABCO (Sigma) as an antifading agent. The MAb used were: the anti-tubulin MAb IG10, the anti-parabasal fiber MAb IE10, and several 3H5, 24E3, 2G5, and VIF1 MAbs produced against cytoskeletons of trichomonads such as *Trichomonas vaginalis* or *Tetratrichomonas gallinarum* by the first author according to the procedure described by Brugerolle and Viscogliosi (1994).

For transmission electron microscopy (TEM), the entire fauna of the termite gut was fixed in a solution of 1% glutaraldehyde (Polysciences) in 0.1 M phosphate buffer at pH

7 for 1 h at room temperature. Following centrifugation and a buffer wash, cells were post-fixed in 1% osmium tetroxide in the phosphate buffer for 1 h. After a water rinse, cells were pre-embedded in 1% agar (Difco), stained 'en bloc' with saturated uranyl acetate in 70% ethanol for 1 h, completely dehydrated in an alcohol series, and embedded in Epon 812 resin (Merck). Sections were cut on a Reichert Ultracut S microtome, stained with lead citrate for 15 min and examined under a JEOL 1200 EX electron microscope at 80 kV.

Results

Observations

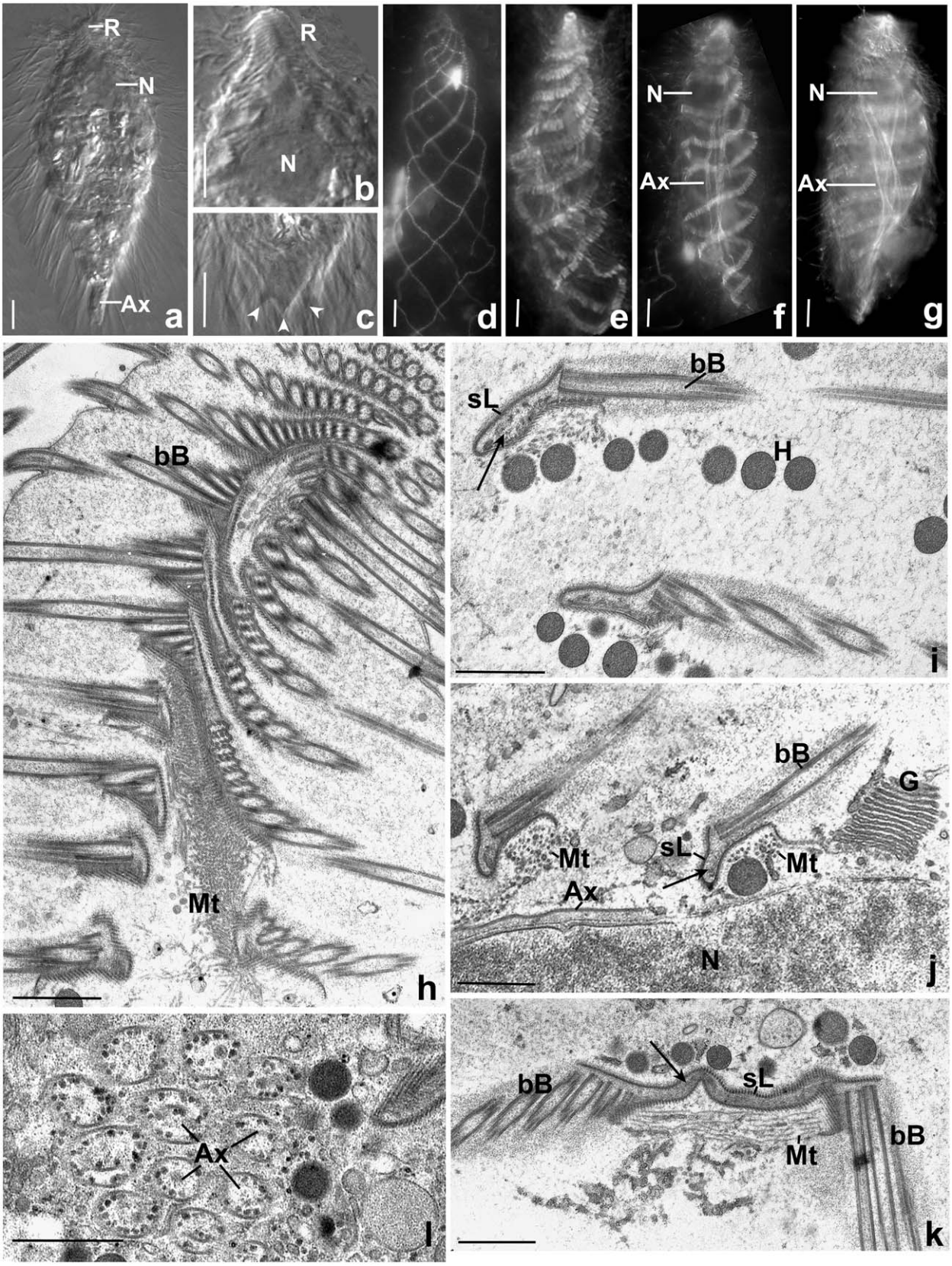
The genus *Spirotrichonympha*

There is only one *Spirotrichonympha* species in the termites examined in this study; it occurs in *Reticulitermes grassei* and has been described clearly as *Spirotrichonympha flagellata* by Grassi (1917) and Duboscq and Grassé (1928). In this species, the cell is large and spindle-shaped with a marked rostrum; flagellar lines extend over the whole cell body, and the axostyle protrudes posteriorly in either one major or several individual spikes (Figs. 1a–c). The cell has an average length of 78 μm (60–90 μm), an average width of 28.6 μm (25–30 μm), and contains abundant wood particles in the posterior part. Several antibodies, MAb 3H5 or 24E3, revealed the spiralled flagellar lines that are closer in the rostrum than in the rest of the body (Figs. 1d–f). Flagellar lines present in the anterior region nearly reach the posterior part in large spirals. Most of the cells have three flagellar lines, but cells with two, four or five flagellar lines are present in the population. Using the MAbs 24E3, VIF1 or a mixture of antibodies comprising the anti-tubulin MAb IG10, allowed us to label both the flagellar lines and the axostylar fibers in the same cell (Figs. 1f and g). The axostyle is composed of about 10–15 fibers that surround the nucleus to form a kind of sleeve around it. Curving backward to the nucleus, these fibers progressively group to form a large axostyle bundle that tapers toward the posterior end (Fig. 1g). Longitudinal sections observed by electron microscopy showed two or more flagellar lines arising at the anterior end (Fig. 1h), which is covered by the double microtubule row of the pelta–axostyle lined by sigmoid fibers as in other spirotrichonymphids (not shown). Transverse sections at the nucleus level indicate that the number of flagellar lines varies from two to six. The organization of the structures in the flagellar lines is quite the usual one, and each row of basal bodies is accompanied by a well-developed striated lamina that recurves to cover the proximal end of the basal bodies (Figs. 1i–k). On its posterior face, the striated lamina is associated with microtubules (Figs. 1j and k). The columellar space is filled with disorganized microtubules, which disappear near the nucleus (Fig. 1h). Transverse sections showed that the nucleus is surrounded by many microtubular rows of about 15–20 microtubules (Fig. 1j). Posterior to the nucleus, each row rolls back on itself, creating an incompletely zipped tube or subaxostyle (Fig. 1l) corresponding to the axostylar fibers observed by immunofluorescence microscopy (Fig. 1g). Several tubes/fibers group to form an axostylar bundle that protrudes at the posterior end of the cell. Golgi bodies are associated with the flagellar lines in the zone around the nucleus (Fig. 1j), but were not revealed by immunofluorescence.

The genus *Spironympha*

Three species corresponding to the description of the genus *Spironympha* Koidzumi, 1917 have been observed in *R. santonensis*. They correspond to *Spironympha kofoidi* Duboscq and Grassé, 1928, and to two species described as new below: *S. verticis* and *S. lanceata*.

Spironympha kofoidi. In this species the cell has a spindle shape with a long rostrum and a body terminated by an axostyle that protrudes distinctly (Figs. 2a and b). The cell measures 61 μm (45–73 μm) in length and 22 μm (14–20 μm) in width. Flagellar lines occupy the anterior half of the body. Typically, the nucleus situated at the base of the rostrum is far from the top of the cell, sometimes near the middle of the cell. Several MAb, including IE10 and 2G5, specifically revealed the flagellar lines, which number from two to five (Figs. 2c–e). In the rostral zone, flagellar lines are typically coiled together (Figs. 2b and c); at the nucleus level they spread and terminate near the middle of the cell (Figs. 2c–e). Labeling with MAb VIF1 revealed the flagellar lines, the axostyle and the nucleus after formaldehyde fixation, and gave a more complete view of these structures (Fig. 2f). Axostylar fibers outline the nucleus and group backward to form an axostylar trunk that tapers toward the posterior end. Electron microscopy showed the flagellar lines that arise at the anterior end and spiral in the anterior half of the cell (Fig. 2g). Basal bodies of the flagellar lines are associated with a striated lamina limited in size that contains mildly electron-dense material (Figs. 2g–j). About 10 microtubular rows arise around the nucleus and converge to form the axostylar trunk at the posterior part of the nucleus (Figs. 2g and j). The axostyle is a large tube that contains particles as large as hydrogenosomes (Fig. 2g). In longitudinal sections it is limited by two microtubular rows (Fig. 2g); in transverse sections it is composed of five microtubular rows in its median part and three rows in its posterior parts (Figs. 2k and l). Golgi bodies were not revealed by our antibodies, but electron microscopy observations showed that they are concentrated along the flagellar lines at the nucleus (Figs. 2g and j). The nucleus contains coccoid bacteria with a corrugated cell wall, visible at high magnification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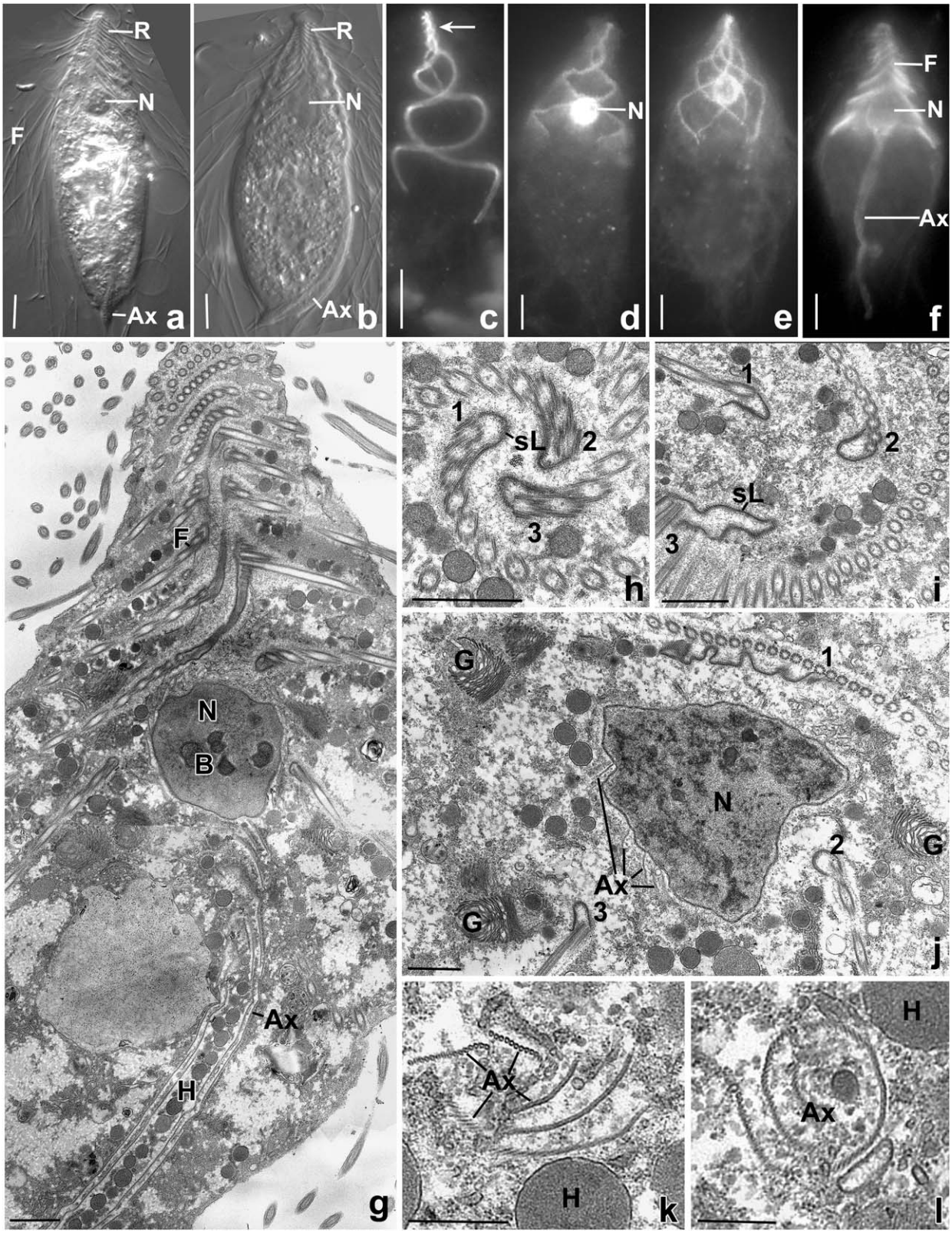
(Fig. 2g). Cells with similar ultrastructural features in the flagellar lines and axostyle were also observed in *Reticulitermes flavipes*.

Spironympha verticis n. sp. In this species the cell has a spindle shape with only the anterior quarter covered by flagella (Figs. 3a–d). The cell has an average length of 61 μm (52–70 μm) for an average breadth of 29 μm (20–32 μm). The blunt anterior end contains a less well-defined rostrum. Flagellar lines describe prominent dexiotropic strips which are nearly transverse (Figs. 3a and d). The large and rounded nucleus is situated towards the rear of the flagellar zone and does not penetrate inside the columella of the rostrum (Fig. 3b). Typically, the nucleus contains particles in its peripheral zone (Fig. 3b), that electron microscopy revealed to be bacteria. The remaining part of the cell is free of flagella and is filled with wood particles. The cell terminates in a relatively large area where generally several spikes of the axostyle are observed (Figs. 3a and c). Immunofluorescence microscopy with MAb IG10 revealed the transverse flagellar lines, the nucleus and the axostyle (Figs. 3e and f) that distinguish *S. verticis* n. sp. from the other two *Spironympha* species present in this termite. Typically, the axostyle arises anterior to the nucleus (Figs. 3e and f), moves on one side of the nucleus to form the axostylar trunk that traverses the cell, and protrudes at the posterior end. The anterior part of the axostyle is composed of several branches that spread in some cells, and converge backward to form the axostylar trunk. Electron-microscopical observation of a longitudinal section showed the transverse flagellar lines arising in the anterior zone (Figs. 3g and h). At its periphery, the rounded nucleus contains rod-shaped bacteria with smooth cell walls. The large columellar space free of organelles anterior to the nucleus presents several microtubular rows of the axostyle (Figs. 3g and h). In each flagellar line, basal bodies are accompanied by a striated lamina that is reinforced by two unequal, dense, striated fibers (Figs. 3i–k). One fiber is located inside the groove of the striated lamina, the other is associated with the anterior edge of the striated lamina. These two additional fibers are microfibrillar and striated with a 14 nm periodicity in the longitudinal section (Fig. 3j). Microtubules forming a pony tail are associated with the striated lamina in the anterior area (Fig. 3h). The ultrastructural details of the striated lamina are very apparent in transverse sections of the flagellar lines and are typical of this species (Fig. 3k). Anterior to the nucleus the axostylar trunk is composed of several microtubular rows (Figs. 3h and l). Posterior to the nucleus the axostylar trunk is a tube generally limited by a single microtubular row in its posterior part and large enough to contain hydrogenosomes (Figs. 3m). However, variation in the number of microtubular rows of the axostylar trunk was observed among the cells examined by electron microscopy. The terminal spikes observed by light microscopy (Fig. 3c) probably correspond to the ends of microtubular rows of the axostyle. The typical ultrastructural features of the striated lamina and the axostyle were also observed in a *Spironympha* species from *Reticulitermes flavipes*.

Spironympha lanceata n. sp. In this species the arrow-shaped cell is widest in the terminal part of the flagellar area that only occupies the anterior cell quarter (Figs. 4a–c). Average cell length is 40 μm (32–45 μm), average width 19 μm (16–26 μm). The rostrum is well-defined and the elongated nucleus penetrates into the columella (Figs. 4b and c), in contrast to *S. verticis*, described above. Flagellar rows are oblique and well-defined (Figs. 4a and d). Except for the anterior, flagellated part, the cell body is filled with wood particles. The axostyle is straight and terminates in an arrowhead-shaped part carrying a single spike (Figs. 4b and c). Immunofluorescence microscopy using MAb IG10 revealed the anterior spiralled flagellar lines, the nucleus penetrating into the columella, and the axostyle (Figs. 4d and e). Electron microscopy observation of a longitudinal section showed the anterior flagellar lines arising at the apex, the nucleus penetrating into the columella, and the posterior axostyle trunk (Fig. 4f). The striated lamina associated with the flagellar lines bears a dense cord attached to its anterior edge (Fig. 4g). In addition, the striated lamina is doubled on its posterior face by a layer of thin, dense microfibers 20 nm in diameter carrying a superimposed layer of microtubules (Figs. 4g–i). About 15–20 microtubular rows appear around the nucleus (Figs. 4f and g), and converge posteriorly to form a large axostylar trunk comprising about three microtubular rows (Figs. 4f, j and k). A species with such typical characters as the striated lamina and the axostyle was also observed by electron microscopy in *Reticulitermes flavipes*.

Fig. 1. *Spirotrichonympha flagellata* from *Reticulitermes grassei*. **a**–**c**. Differential interference contrast micrographs. **a**, **b**. Whole cell covered by flagella, rostrum (R), and nucleus (N); Ax = axostyle. **c**. Posteriorly protruding axostyle with several spikes (arrowheads). **d**–**g**. Immunofluorescence micrographs. **d**. Cell labeled by MAb 3H5 that revealed five spiralled flagellar lines. **e**, **f**. MAb 24 E3 revealed three or two flagellar lines and axostylar fibers (Ax). **g**. MAb VIF1 also revealed flagellar lines and axostylar fibers forming compound axostyle (Ax); N = location of nucleus. **h**–**k**. Electron micrographs. **h**. Anterior longitudinal section showing spiralled lines of flagella/basal bodies (bB) and columellar space containing microtubules (Mt). **i**, **j**, **k**. Sections of flagellar lines to show striated lamina (sL) with electron-dense microfibrillar material inside the groove (arrow), as well as external microtubules (Mt), microtubular row of axostyle (Ax) around nucleus (N), Golgi body (G), and hydrogenosomes (H). **l**. Transverse section of incompletely zipped axostylar tubes (Ax) composing axostyle trunk. Scale bars: 10 μm in figures **a**–**g**, 1 μm in figures **h**–**l**.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The genus *Microjoenia*

Microjoenia fallax has been described from *Reticulitermes santonensis*, and *M. hexamitoides* from *R. grassei* (Grassé 1952). Here, we present the features of the former species only. In *M. fallax*, the cell has an upside-down bulb shape, with an anterior tuft of flagella and a posteriorly protruding axostyle (Figs. 5a and b). Its average length is 20 μm (16–28 μm), its average width 10.4 μm (4.1–12 μm). Immunofluorescence labeling with MAb 24E3 showed an average of 10 (6–15) flagellar lines radiating around the anterior zone and the nucleus (Figs. 5c–f). They are oriented either longitudinally or slightly obliquely. At the top of the cell there is a disk stained by antibodies (Figs. 5c and d). MAb IG10 revealed the nucleus outlined by the u-shaped anterior part of the axostyle that continues backward as a straight axostylar trunk (Fig. 5g). Electron-microscopical study showed that the flagellar lines originate subapically with a first basal body #2 bearing sigmoid fibers in contact with the double row of the pelta/axostyle that covers the anterior end of the cell (Figs. 5h–j). The flagellar lines are accompanied by a simple striated lamina closely applied against the basal bodies (Figs. 5i–k). About six microtubular rows outline the nucleus and group at its rear to constitute the axostyle trunk (Figs. 5h and k). The axostylar trunk is composed of about four microtubular rows along most of its length (Figs. 5h and l). The space in front of the nucleus that corresponds to the columellar space in *Spirotrichonympha* contains disorganized microtubules; in some sections a transverse microtubular paradesmosis linking two flagellar lines is observed (not shown). Golgi bodies are situated around the nucleus (Figs. 5h–k). The nucleus presents rod-shaped bacteria (Fig. 5k), and the cytoplasm is filled with food vacuoles containing bacteria and wood particles (Fig. 5h). A species with the same ultrastructural features was also observed in *Reticulitermes flavipes*. The species *Microjoenia hexamitoides*, which occurrs in *R. grassei*, has the same generic features as *M. fallax*.

Diagnoses

The genus *Spironympha* is characterized by means of light- and electron microscopy, to accommodate the two *Spironympha* species described as new above. In addition, a revised morphological diagnosis of the genus *Spirotrichonympha* separates it from *Spironympha* as emended below.

Genus *Spirotrichonympha* Grassi & Foa, 1911

Spirotrichonymphid with spindle-shaped body and well-defined rostrum and columella. Spiralled flagellar lines nearly reaching posterior end of cell comprising a striated lamina with specific fibrillar associations. Compound axostyle composed of several fibers or subaxostyles that posteriorly form a bundle of axostylar fibers. Electron-microscopical description of *S. flagellata* from *Reticulitermes grassei*, *S. cincta* from *Hodotermopsis sjoestedti*, *S. mirabilis* from *Porotermes grandis*; SSU rRNA sequence assigned by in situ hybridization to *S. cincta* from *Hodotermopsis sjoestedti*.

Genus *Spironympha* Koidzumi, 1917

Spirotrichonymphid with a spindle-shaped or arrow-shaped body and with variously defined rostrum. Spiralled flagellar lines restricted to anterior third or half of body. Flagellar lines associated with a striated lamina showing specific ultrastructural peculiarities; single axostyle trunk varies in diameter according to species. Electron microscopy descriptions of *S. kofoidi*, *S. verticis* and *S. lanceata* from *Reticulitermes santonensis* or *R. flavipes*, and of *S. obtusa* and *S. oblonga* from *Hodotermopsis sjoestedti.*

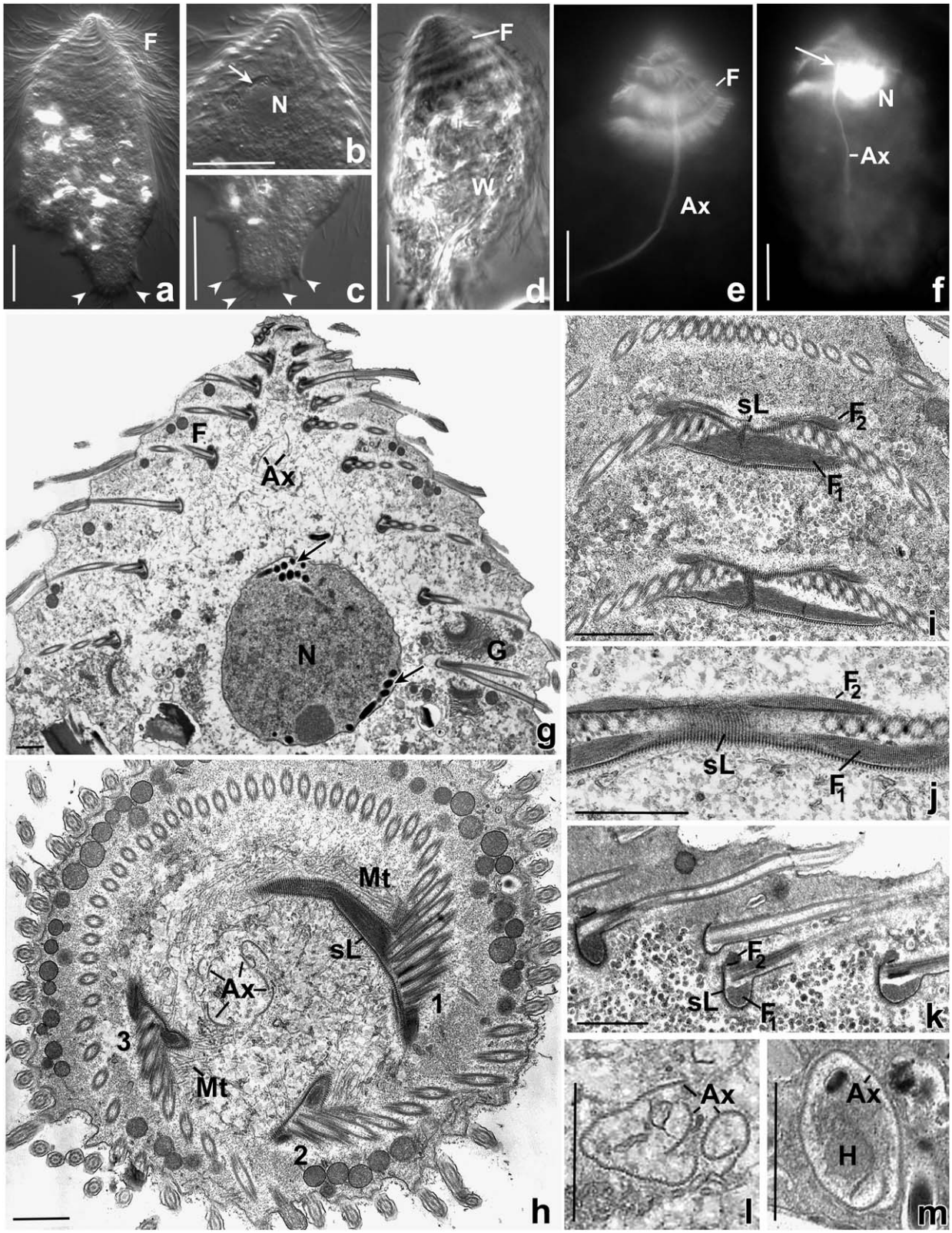
Spironympha verticis n. sp.

Etymology. From Latin vertex meaning summit.

Type material. Hapantotype (sensu ICZN 1999) on two slides of Giemsa-stained intestinal smears and glutaraldehyde/osmium tetroxide fixed intestinal flagellate fauna; deposited at Laboratoire de Biologie Parasitaire, Protistologie, Helminthologie, Muséum National d'Histoire Naturelle, Paris, France; type number MNHN 9 ZS (same slides as hapantotype of *S. lanceata*; respective cells identified by circles on cover slips).

Diagnosis. Symbiontic in termite intestines. Ovoid or spindle-shaped cell 61 μm (52–70 μm) in length and 29 μm (20–32 μm) in width; anterior third with transverse, spiralled flagellar lines and a less-marked rostrum. Two to three flagellar lines associated with a striated

Fig. 2. *Spironympha kofoidi* from *Reticulitermes santonensis.* **a**, **b**. Differential interference contrast micrographs to show flagella (F) in anterior half, twisted flagellar lines in rostrum (R), nucleus (N), and posteriorly protruding axostyle (Ax). **c**–**f**. Immunofluorescence micrographs. **c**. Cell labeled by MAb IE10 revealing two twisted flagellar lines (arrow). **d**, **e**. Cells labeled by MAb 2G5 revealing two or five flagellar lines that spread at the nucleus (N). **f**. Flagellar lines (F), nucleus (N) and axostyle (Ax) stained by MAb VIF1. **g**–**l**. Electron micrographs. **g**. Longitudinal section to show anterior spiralled flagellar lines (F), nucleus (N) with intranuclear bacteria (B), and axostylar trunk (Ax) containing hydrogenosomes (H). **h**, **i**. Transverse section at two levels of anterior part to show three flagellar lines (1, 2, 3), and associated striated lamina (sL). **j**. Transverse section at nucleus level to show three flagellar lines (1, 2, 3), microtubular rows of axostyle (Ax) arising around nucleus (N), and Golgi bodies (G). **k**, **l**. Transverse section of posterior part to show five axostylar rows forming axostylar trunk (Ax), and hydrogenosomes (H). Scale bars: 10 μm in figures **a**–**f**, 1 μm in figures **g**–**l**.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lamina reinforced by two opposite, dense, striated fibers. Large, rounded nucleus separated from flagellated area. Axostylar rows arising anteriorly to nucleus, moving on one side of nucleus to form a posterior axostyle trunk composed of one or two associated microtubular rows. Axostyle terminated by one or several spikes.

Host. Reticulitermes flavipes (Kollar) (distribution: Holarctic), including the junior synonym *R. santonensis* (Feytaud) (France: Saintonge region and Île d'Oléron).

***Spironympha lanceata* n. sp.**

Etymology. From Latin lancea meaning lance.

Type material. Hapantotype (sensu ICZN 1999) two slides of Giemsa-stained intestinal smears and glutaraldehyde/osmium tetroxide fixed intestinal flagellate fauna; deposited at Laboratoire de Biologie Parasitaire, Protistologie, Helminthologie, Muséum National d'Histoire Naturelle, Paris, France; type number MNHN 9 ZS (same slides as hapantotype of *S. verticis*; respective cells identified by circles on cover slips).

Diagnosis. Symbiontic in termite intestines. Arrow-shaped cell 55 μm (39–65 μm) in length and 22 μm (16–28 μm) in width; anterior quarter partly covered by flagella. Conical anterior end with marked rostrum; posterior end tapering toward protruding axostyle with one spike. Obliquely spiralled flagellar lines and oval nucleus penetrating into columella. Four flagellar lines accompanied by a striated lamina. Striated lamina associated with one dense microfibrillar cord attached to its anterior edge, with a layer of thin microfibers and a superimposed layer of microtubules on its posterior face. Microtubular rows of axostyle arising around nucleus and grouped backward to form one stout axostylar trunk of about three microtubular rows.

Host. Reticulitermes flavipes (Kollar) (distribution: Holarctic), including the junior synonym *R. santonensis* (Feytaud) (France: Saintonge region and Île d'Oléron).

***Microjoenia fallax* Grassé, 1952, emended**

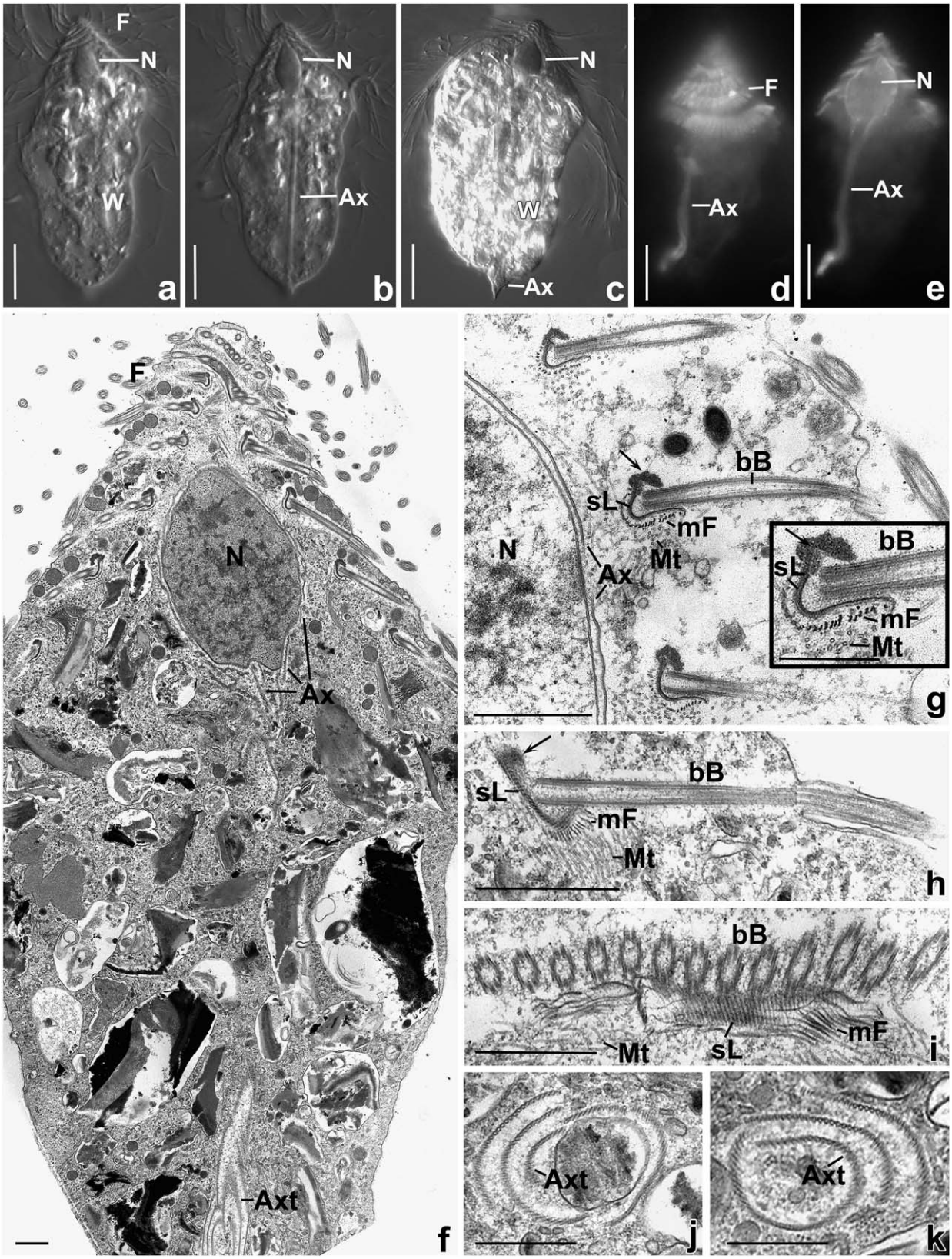
Diagnosis. Upside-down bulb-shaped cell 24.8 μm (15–29 μm) in length and 13.2 μm (10–15.5 μm) in width, with anterior radiating flagella deflected posteriorly. About eight to 15 longitudinal flagellar lines associated with a limited and simple striated lamina. About two to five microtubular rows around nucleus, forming a straight axostylar trunk composed of two to five rows. Golgi bodies situated around posterior end of nucleus.

Host. Reticulitermes flavipes (Kollar) (distribution: Holarctic), including the junior synonym *R. santonensis* (Feytaud) (France: Saintonge region and Île d'Oléron).

Discussion

This study provides new data for the cytological characterization of the three genera *Spirotrichonympha*, *Spironympha* and *Microjoenia*, and is the first ultrastructural study of the genus *Spironympha*. The genus *Spirotrichonympha* has been characterized by light microscopy based on the description of about 30 species (Grassé 1952; Radek 1974, and references therein), but only three electron-microscopical studies have dealt with this genus (Hollande and Carruette-Valentin 1971; Radek 1974; Brugerolle 2001). In an earlier study (Brugerolle 2001), I compared the ultrastructure organization of the flagellar lines of several *Spirotrichonympha* species to the homologous structures in *Microjoenia*, *Spirotrichonymphella* and *Holomastigotoides*; however, the study of the structure of the axostyle was not fully developed. The present study shows that the latter is an important feature to consider in generic taxonomy and cytological evolution of spirotrichonymphids. In *Spirotrichonympha flagellata* from *Reticulitermes grassei* as in other species described so far, e.g. *S. mirabilis* from *Porotermes* (Brugerolle 2001) and *S. cincta* from *Hodotermopsis sjoestedti* (Brugerolle 2005), the axostyle is composed of several fibers or subaxostyles, most of them grouping to constitute the major axostylar trunk. Each subaxostyle arises from a row of about 15–20 microtubules that roll back to form an incompletely zipped tube, like the simple axostyle of trichomonads (Brugerolle 1991). The component microtubules arise around the nucleus, although no well-defined microtubule organizing centers (MTOCs) were detected at their origin. Either these microtubules polymerize and assemble in rows from very discrete MTOCs, or the disorganized microtubules present in the columella assemble to form the rows. An origin from the

Fig. 3. *Spironympha verticis* from *Reticulitermes santonensis*. **a–d**. Differential interference contrast micrographs. **a–c**. Cell-bearing flagella (F) in anterior part, nucleus (N) with bacteria at periphery (arrow), posterior axostyle with several spikes (arrowheads). **d**. Phase contrast micrograph showing prominent circular flagellar lines (F) and posterior part filled with wood particles (W). **e–f**. Immunofluorescence micrographs. Cells labeled by MAb IG10 that stained anterior flagellar lines (F) and axostyle (arrow), which arises anterior of nucleus (N) and continues posteriorly as a thin fiber (Ax). **g–m**. Electron micrographs. **g**. Anterior longitudinal section to show flagellar lines (F) arising at anterior end, rounded nucleus (N) containing bacteria at periphery (arrows), large columellar space with axostylar rows (arrowhead), and Golgi bodies (G). **h**. Transverse section anterior of nucleus to show three spiralled flagellar lines (1, 2, 3) with their associated striated lamina (sL) and microtubules (Mt), and microtubular rows of axostyle (Ax) in columellar space. **i**, **j**, **k**. Striated lamina (sL) of flagellar lines are associated with a dense striated fiber (F_1) inside the groove, and with a second fiber (F_2) attached to its anterior edge. **l**, **m**. Sections of axostyle (Ax), both anterior and posterior to nucleus, to show component microtubular rows, and hydrogenosome (H). Scale bars: 10 μm in figures **a–f**, 1 μm in figures **g–m**.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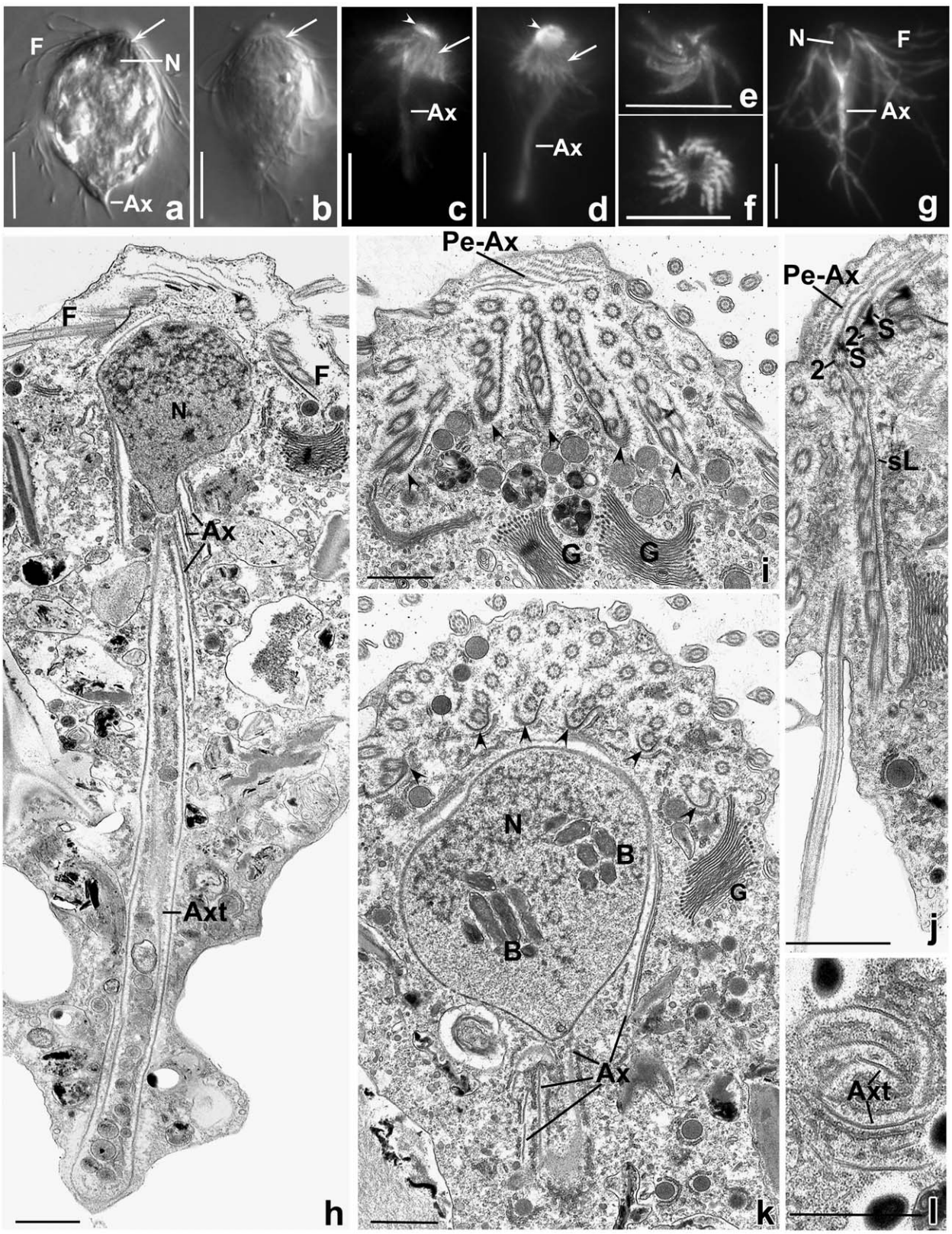
microtubules attached to the flagellar lines seems unlikely, because these microtubules follow the flagellar lines far from the nucleus. Moreover, they are lacking in certain species. This kind of organization in *Spirotrichonympha* has only been described by light microscopy and haematoxylin staining in *S. flagellata* from *Reticulitermes lucifugus* by Grassi (1917) and from *R. grassei* by Duboscq and Grassé (1928), as well as in *S. decipiens* from *Anacanthotermes ochraceus* (Duboscq and Grassé 1943). The organization of the axostyle has been partially shown by electron microscopy in *S. flagellata* (Hollande and Carruette-Valentin 1971); it is well-documented in *S. cincta* from *Hodotermopsis sjoestedti* (Brugerolle 2005) and in *S. mirabilis* from *Porotermes* (Brugerolle 2001). Actually, such axostylar organization is a common feature of the genus *Spirotrichonympha*. Only two *Spirotrichonympha* species, *S. cincta* and *S. leydi*, have been identified by an SSU rRNA sequence (Ohkuma et al. 2000); since *S. cincta* has been studied by electron microscopy it is a good reference for the characterization of the genus.

The genus *Spironympha* was created by Koidzumi (1917) for spirotrichonymphids that have flagella lines restricted to the anterior quarter or third of the cell. The name *Spironympha* was latter changed to *Microspironympha* by Koidzumi (1921), only to be re-established by Brown (1931) and Yamin (1979). However, Grassé (1952) considered *Spironympha* only as a subgenus of *Spirotrichonympha*. Four species have been described by light microscopy, including *S. porteri* from *Reticulitermes speratus* (Koidzumi 1921), *S. kofoidi* (also named *Spirotrichonympha kofoidi*) from *Reticulitermes flavipes* or *R. santonensis* (Duboscq and Grassé 1928; Grassé 1952), and *S. ovalis* from *Reticulitermes hesperus* (Brown 1931). The two species *Microspironympha elegans* from *Reticulitermes tenuis* (Mackinnon 1927) and *Spirotrichonympha polygira* from *Neotermes simplicicornis* (Cupp 1930) were also considered to belong to the genus *Spironympha* by Yamin (1979).

Spironympha kofoidi is described by electron microscopy for the first time in the present study. Two additional species have not been reported from *R. santonensis* or *R. flavipes* in either France or America, thus are described as the new species, *S. verticis* and *S. lanceata*, above. The three *Spironympha* species previously known from *R. santonensis* and two *Spironympha* species described from *Hodotermopsis sjoestedti* by light and electron microscopy (Brugerolle 2005) allowed us to characterize the genus *Spironympha* and to distinguish it from the genus *Spirotrichonympha*. In *Spironympha*, flagellar lines are located in the anterior third or half of the cell, and the axostyle is composed of a single axostylar fiber that distinguishes it from the genus *Spirotrichonympha* (see above). *Spironympha kofoidi* has a long rostrum with two to six coiled flagellar lines that spread at the nucleus level, and a nucleus that is situated far from the anterior end of the cell. *Spironympha verticis* has a blunt rostrum with three flagellar lines that differ from other species described to date by a striated lamina reinforced by two dense, striated fibers. *Spironympha lanceata* has two to four prominent flagellar lines that differ from other species described to date by several ultrastructural features of the striated lamina. The axostyle trunk of *S. kofoidi* and that of *S. lanceata* originate from 10 to 20 microtubular rows close the nucleus that converge posteriorly to form an axostylar trunk of about two to five rows. The axostyle of *S. verticis* arises anterior of the nucleus and moves around it to constitute a posteriorly protruding axostylar trunk of one to three assembled rows. Also, the large rounded nucleus containing peripheral bacteria distinguishes *S. verticis* from the other species living in the same termite.

The ultrastructural features typical of the three *Spironympha* species from *R. santonensis* were also recognized in spirotrichonymphids living in *R. flavipes*. These observations support the long-suspected synonymy of *R. santonensis* with *R. flavipes* (Duboscq and Grassé 1928; Vieau 2001), recently demonstrated by molecular phylogeny using several markers (Miura et al. 1998; Clément et al. 2001; Jenkins et al. 2001; Austin et al. 2002, 2004; Marini and Mantovani 2002; Ohkuma et al. 2004; Uva et al. 2004; Ye et al. 2004). The protozoan fauna associated with a termite species comprises strictly host-specific and less specific species (Honigberg 1970; Yamin 1979). The flagellate *Spironympha kofoidi* has been identified in termites identified as either *R. flavipes* or *R. santonensis* (Duboscq and Grassé 1928; Grassé 1952; Yamin 1979); the two newly described species, *S. verticis* and *S. lanceata*, also seem specific to this host taxon. Our study provides morphological data indispensable for the identification of spirotrichonymphid

Fig. 4. *Spironympha lanceata* from *Reticulitermes santonensis*. **a–c**. Differential interference contrast micrographs. **a–c**. Cells with a flagellated anterior area (F), nucleus (N) penetrating into columella, and axostyle (Ax) traversing the cell and protruding posteriorly, with wood particles (W). **d**, **e**. Immunofluorescence micrographs of a cell labeled by MAb IG10 revealing anterior transverse flagellar lines (F) and axostyle (Ax), which arises at posterior end of nucleus (N). **f–k**. Electron micrographs. **f**. Longitudinal section to show anterior spiralled flagellar lines (F), nucleus (N) penetrating into columellar space, and axostylar rows (Ax) around and posterior to nucleus forming axostylar trunk (Axt). **g**, **h**, **i**. Basal bodies (bB) of flagellar lines associated with striated lamina (sL), which is reinforced by a dense fiber (arrow) attached to the anterior edge, and doubled by a layer of microfibers (mF) and a layer of microtubules (Mt) on its posterior surface. **j**, **k**. Sections of axostylar trunk (Axt) composed of three to four microtubular rows. Scale bars: 10 μm in figures **a–e**, 1 μm in figures **g–i**, 0.5 μm in figures **g** inset, **j**, **k**.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flagellates and thus for more comprehensive knowledge of protist communities in termites (Lewis and Forschler 2004).

The study of a second species of *Microjoenia*, *M. fallax*, by immunological and ultrastructural techniques contributes to the characterization of this genus previously defined by only one electron microscopy study of *M. anterodepressa* from *Porotermes* (Brugerolle 2001). In this genus, the short flagellar lines radiate around the anterior part of the cell and are longitudinally oriented rather than spiralled, and the rostrum is not well-defined in comparison with *Spirotrichonympha* and *Spironympha.* The axostyle originates in a way similar to *S. kofoidi* or *S. lanceata*, but is relatively thin.

The identification of termite species has greatly improved with the recent use of molecular markers; molecular identification of the protozoan fauna in termites is in early stages as well. The genus *Spironympha* has been identified by two SSU rRNA sequences (HsS6, HsS22) provided by Ohkuma et al. (2000), but in a more recent paper these authors (Ohkuma et al. 2005) assigned these sequences to *Spirotrichonymphella* species that correspond to *Holomastigotes* species according to Brugerolle and Bordereau (2004). The genus *Spirotrichonympha* is also identified by three SSU rRNA sequences provided by Ohkuma et al. (2000). Therefore, no *Spironympha* species has been identified by a molecular marker.

Considering the comparative development of homologous cytological structures to propose hypothetical evolutionary relationships among spirotrichonymphids, the genus *Microjoenia*, with its short flagellar lines and simple axostyle, proved the most simple. It appears close to the genus *Spironympha* by the organization of its axostyle, which is similar to that of *S. kofoidi* and *S. lanceata*. In the genus *Spirotrichonympha* the flagellar area is more extended than in *Spironympha*, and the compound axostyle is composed of several independent microtubular rows. The genus *Holomastigotoides* also shows a compound axostyle similar to that in *Spirotrichonympha* (Grassé 1952; Hollande and Carruette-Valentin 1971; Lingle and Salisbury 1995; Brugerolle unpublished). Although all the spirotrichonymphid genera – *Microjoenia*, *Spironympha*, *Spirotrichonympha*, *Holomastigotoides*, *Spirotrichonymphella*, and *Holomastigotes* – have been described by electron microscopy, several questions remain as to the definition of certain genera. The genera *Spirotrichonymphella* and *Holomastigotes*, for example, are not fully characterized and could be synonyms. The species *Spirotrichonympha minor* (Radek 1974) has an axostyle resembling that in *Microjoenia* or *Spironympha*, and seems close to the genus *Micromastigotes* as incompletely defined by Hollande and Carruette-Valentin (1971). Molecular phylogenetic data have been limited to sequences from the genera *Spirotrichonympha* and *Holomastigotoides* (Keeling et al. 1998; Ohkuma et al. 2000, 2005); other sequences are not unambiguously assigned to *Microjoenia*, *Holomastigotes*, or *Spirotrichonymphella*.

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Fig. 5. *Microjoenia fallax* from *Reticulitermes santonensis*. **a**, **b**. Differential interference contrast micrographs to show anterior flagella (F), longitudinally radiating flagellar lines (arrow), nucleus (N), and posteriorly protruding axostyle (Ax). **c–g**. Immunofluorescence micrographs. **c–d**. Labeling by MAb 24E3 of anterior disk (arrowhead), longitudinal flagellar lines (arrow), and axostyle (Ax). **e**, **f**. Polar view of radiating flagellar lines, variable in number, labeled by MAb 24E 3. **g**. Cells labeled by MAb IG10 revealing flagella (F) and axostylar trunk (Ax) arising around nucleus (N). **h–l**. Electron micrographs. **h**. Longitudinal section showing anterior flagellar lines (F), nucleus (N), and axostylar rows (Ax) arising around nucleus and forming the axostylar trunk (Axt). **i**. Longitudinal flagellar lines (arrowheads) arising at anterior end, covered by microtubular rows of pelta–axostyle (Pe-Ax); G = Golgi bodies. **j**. Anterior section to show flagellar lines originating at anterior basal body #2 bearing a sigmoid fiber (S) lining the pelta–axostyle rows (Pe-Ax); sL = striated lamina. **k**. Section at nucleus level showing axostylar rows (Ax) around nucleus (N) that converge posteriorly to form axostylar trunk, as well as bacteria inside nucleus (B), flagellar lines (arrowheads), and Golgi body (G). **l**. Transverse section posterior to nucleus to show axostylar trunk (Axt) composed of about five microtubular rows. Scale bars: 10 μm in figures **a–g**, 1 μm in figures **h–l**.

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