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MICROMASTIGOTES SCOTTAE SP. NOV. (PARABASALIDA: SPIROTRICHONYMPHINA): A NEW TWIST ON THE HYPERMASTIGONT CONDITION.

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This study redescribes the genus *Micromastigotes*, Hollande and Carruette-Valentin, 1971, a parabasalid flagellate symbiotic in termites, on the basis of light and electron microscopy and erects a new species, *M. scottae*. The genus *Micromastigotes* is characterised by possessing flagella bands which spiral around the anterior portion of the cell, the location of the nucleus and Golgi bodies at the base of the anterior flagellated area, and an axostyle which runs the length of the cell. Individual flagella are derived from the central axis of the cell exiting perpendicularly, each is offset from the preceding flagellum by 12 — 18° thus forming a spiral band. Ultrastructurally, the flagella bases form a structure resembling a spiral staircase. Peltoaxostylar and preaxostylar fibres arise from the anterior most kinetosomes and the striated lamina forms a weakly undulating sheet directed posteriorly from each flagellum. Dense lamina and parabasal fibres are absent. *Micromastigotes* was originally classified as part of the Spirotrichonymphina and there are some similarities to other genera in this group, but *Micromastigotes* lacks a flagellar gutter, a U-shaped band at the base of the flagella composed of the striated and dense lamina, which is diagnostic of the spirotrichonymphines. The spiralisation pattern in *Micromastigotes* is not consistent with previous schemes for the development of a polymastigont condition in spirotrichonymphines suggesting that *Micromastigotes* may represent an independent derivation of a polymastigont condition from a trichomonad-like ancestor.❐ *Parabasalia, Spirotrichonymphina, Micromastigotes, Privileged-Flagella Hypothesis, Schedorhinotermes*.

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The flagellate phylum Parabasalida includes the most diverse ranges of cell structures of any protist group. Most species occur as anaerobic symbionts in a range of hosts including mammals, reptiles and birds but the greatest diversity of species occurs within termites and wood-eating cockroaches (Yamin, 1979). Much of this diversification has been associated with increases in cell size and in the complexity of the flagellar structures and their associated fibrous support organelles. The simplest parabasalids, the Trichomonadida have 3 anterior flagella and a recurrent flagellum, and this arrangement, 3 + R, forms the basis of the 'privileged flagella hypothesis' which considers that the more complex arrangements are all derived states arising by addition and elaboration of one or more of the 'privileged' elements (Brugerolle, 1991). The more complex parabasalids usually have vastly greater numbers of flagella and have been collectively classified as the Hypermastigida although this taxon is almost certainly a polyphyletic collection of groups which have independently adopted a multiflagellated or polymastigont condition (Brugerolle & Patterson, 2001). Even though molecular phylogenies of the parabasalids consistently recover the polymastigont Trichonymphidae as the earliest diverging branch of the parabasalid tree, this result is almost certainly artefactual and it should not be interpreted that the ancestral parabasalid was polymastigont (Hampl et al., 2004).

Recent molecular phylogenies of the parabasalids (Gerbod et al., 2001, 2002; Hampl et al., 2004; Keeling, 2002; Okhuma et al., 2000, 2005) have suggested that there have been up to five acquisitions of the polymastigont condition, six if Calonymphidae is polyphyletic (Gerbod et al., 2002) (Fig. 1). Four groups have been traditionally united as the Order Hypermastigia: the Trichonymphidae, Eucomonymphidae and Staurojoeninidae (collectively the Suborder Trichonymphina); the Suborder

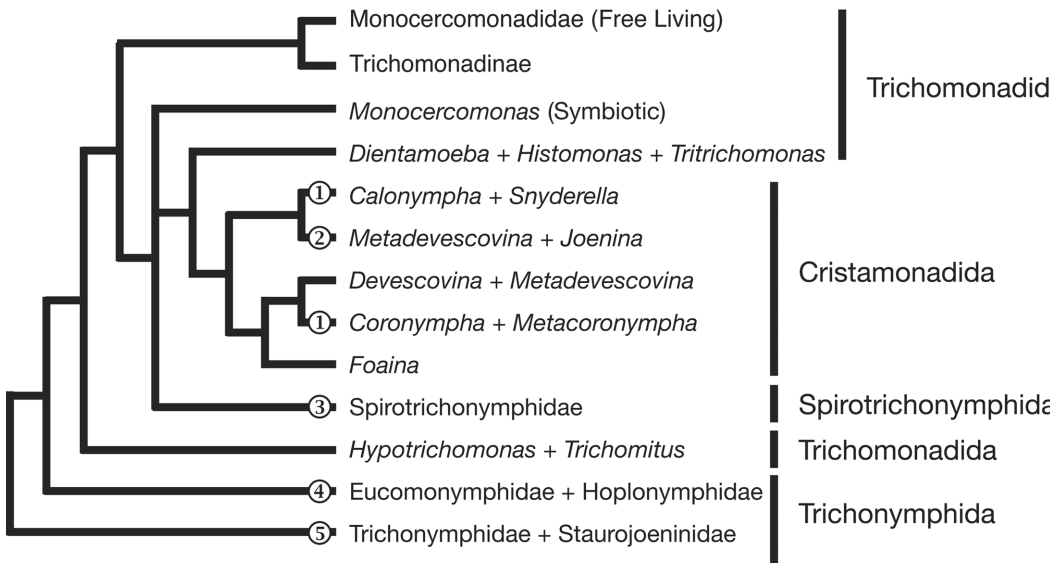

FIG. 1. Phylogeny of the Parabasalia and acquisitions of a multimastigont condition, redrawn after Gerbod et al., (2002) & Ohkuma et al. (2005). 1, Calonymphid type: replication of a karyomastigont; 2, Lophomonad type: replication of flagellum 1 into an anterior flagella plate; 3, Spirotrichonymphid type: spiral flagella bands, flagellar gutter surrounding the kinetosome base; 4, Eucomonymphid type: slightly spiralised flagella bands, each kinetosome base connected to a striated, sinusoidal root; 5, Trichonymphid type: meridional flagella bands, derived from a complex anterior rostrum.

Spirotrichonymphina (Spirotrichonymphidae) and the Suborder Lophomonadina. The fifth group, the Calonymphidae, has long been classified within the Trichomonadida but has recently been grouped with the devescovinids and lophomonads as the Order Cristamonadida on the basis of molecular phylogenies and reassessment of morphological structures (Brugerolle & Patterson, 2001). Each of these five acquisitions of the polymastigont condition has been accompanied by a distinctive subcellular architecture of tubules and fibres to support the profusion of locomotory flagella. The simplest system is seen in the calonymphids where polymastigery is achieved by simple multiplication of the privileged karyomastigont, the privileged flagella plus associated nucleus (Dolan et al., 2000a, 2000b). The simplicity of this structure and its' clear relation to flagella structure in the monomastigont devescovinids is what has lead to the grouping of calonymphids initially within Trichomonadida (Brugerolle & Lee, 2000) and more recently within Cristamonadida (Brugerolle & Patterson, 2001). Even if the calonymphids are polyphyletic as suggested by Gerbod et al. (2002), the two calonymphid groups have both achieved the polymastigont condition in the same way, by replication of the karyomastigont, and constitute an example of parallel evolution (Dolan et al., 2000a, 2000b; Dolan & Kirby, 2002). The lophomonads also retain the privileged flagella and the polymastigont condition is achieved by vast replication of flagellum 1 into an anterior flagella plate (Brugerolle & Patterson, 2001; Brugerolle & Bordereau, 2003). More complex arrangements which are less readily interpreted in terms of modification of the privileged flagella occur in the other three groups. Eucomonymphids are characterised by their flagella being grouped into slightly spiralised, longitudinal rows and each flagellum possesses a striated, sinusoidal microtubular root which was termed the parabasal filament by Hollande & Carruette-Valentin (1971) although its relationship to the parabasal filament of trichomonads is unclear (cf. Brugerolle, 1999, 2000; Cameron & O'Donoghue, 2003). Trichonymphids have a very complex anterior rostrum from which meridional lines of flagella arise, the rostrum itself is a very complex structure with multiple interacting layers of fibrils (Hollande & Carruette-Valentin, 1971). Spirotrichonymphina, as the name suggests, have spiral rows of flagella radiating from the anterior end, each row has as its base a structure termed the 'gutter of the flagella band' by Brugerolle (2001) composed

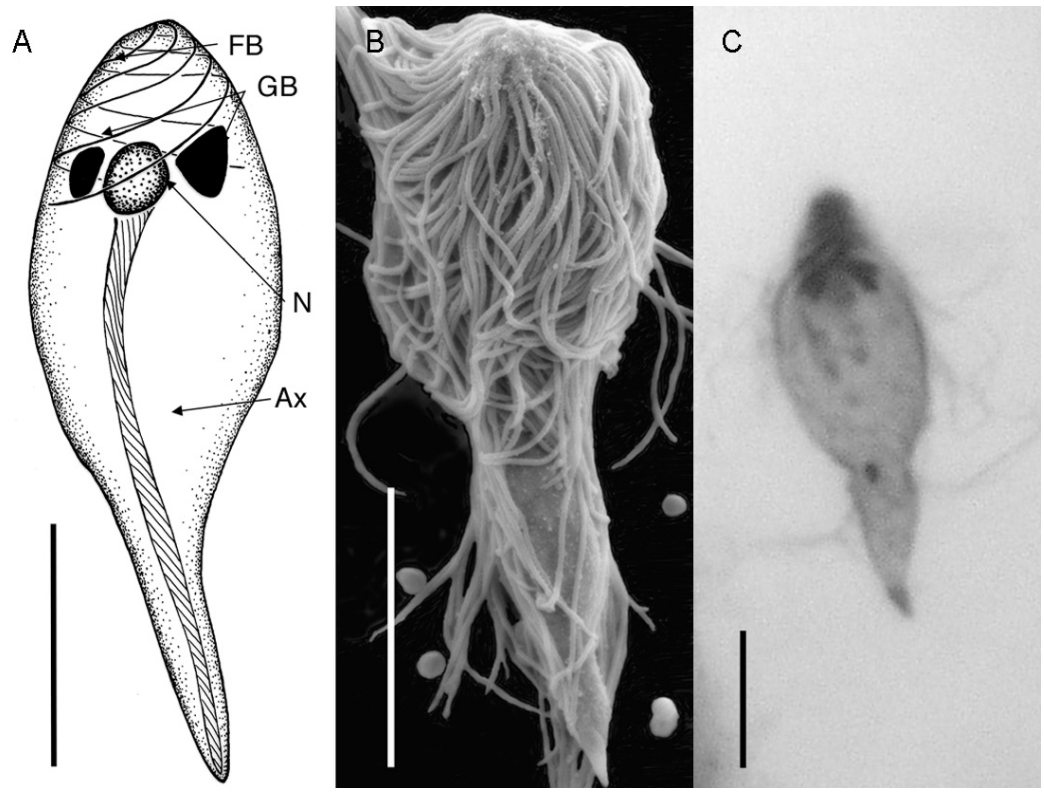

FIG. 2. Morphology of *Micromastigotes scottae* sp. nov. A, Line diagram. B, Scanning electron micrograph. C, Light micrograph of protargol stained specimen. Scale bars = 10 μm. Ax, axostyle; FB, flagella band; GB: Golgi body; N, nucleus.

of outer dense lamina and an inner striated lamina. The flagellar gutter thus has the form of a U-shaped ribbon spiraling around the cell close to the base of the kinetosomes. This arrangement occurs in the widespread and speciose genera of spirotrichonymphines, *Spirotrichonympha* and *Holomastigotoides* (Brugerolle, 2001; Lingle & Salisbury, 1995) but variations occur in the simpler genera *Microjoenia*, *Spirotrichonymphella* and *Micromastigotes* Hollande & Carruette-Valentin (Brugerolle, 2001; Hollande & Carruette-Valentin, 1971). In *Microjoenia*, the flagellar gutter is poorly formed, the dense and striated lamina being frequently separated and not forming a continuous ribbon connecting adjacent kinetosomes (Brugerolle, 2001). The flagella of *Spirotrichonymphella* are derived from a central core or columella, each flagellum is connected to the columnella by a pair of striated roots, internal to the striated roots is a striated lamina which spirals downward to follow the path of the flagella band (Brugerolle, 2001; SLC pers observ.). *Micromastigotes* apparently has the simplest morphology of all; the flagella are apparently derived directly from a central core, axial within the cell and in the description by Hollande & Carruette-Valentin (1971), lacking both striated roots and the striated lamina. The possibility that *Micromastigotes* represents the simplest spirotrichonymphine led us to undertake a complete redescription of the genus and a detailed study of its flagella structures following the discovery of a new species of this genus in the northern Australian pest termite species *Schedorhinotermes intermedius*.

MATERIALS AND METHODS

Thirteen colonies of *Schedorhinotermes intermedius* were collected from southeastern Queensland (QLD) Australia, from Joyner (3 colonies), Deception Bay (1), Samsonvale (3), Samford (4), Ferny Hills (1) and Moggill Creek (1). Colonies were collected from under

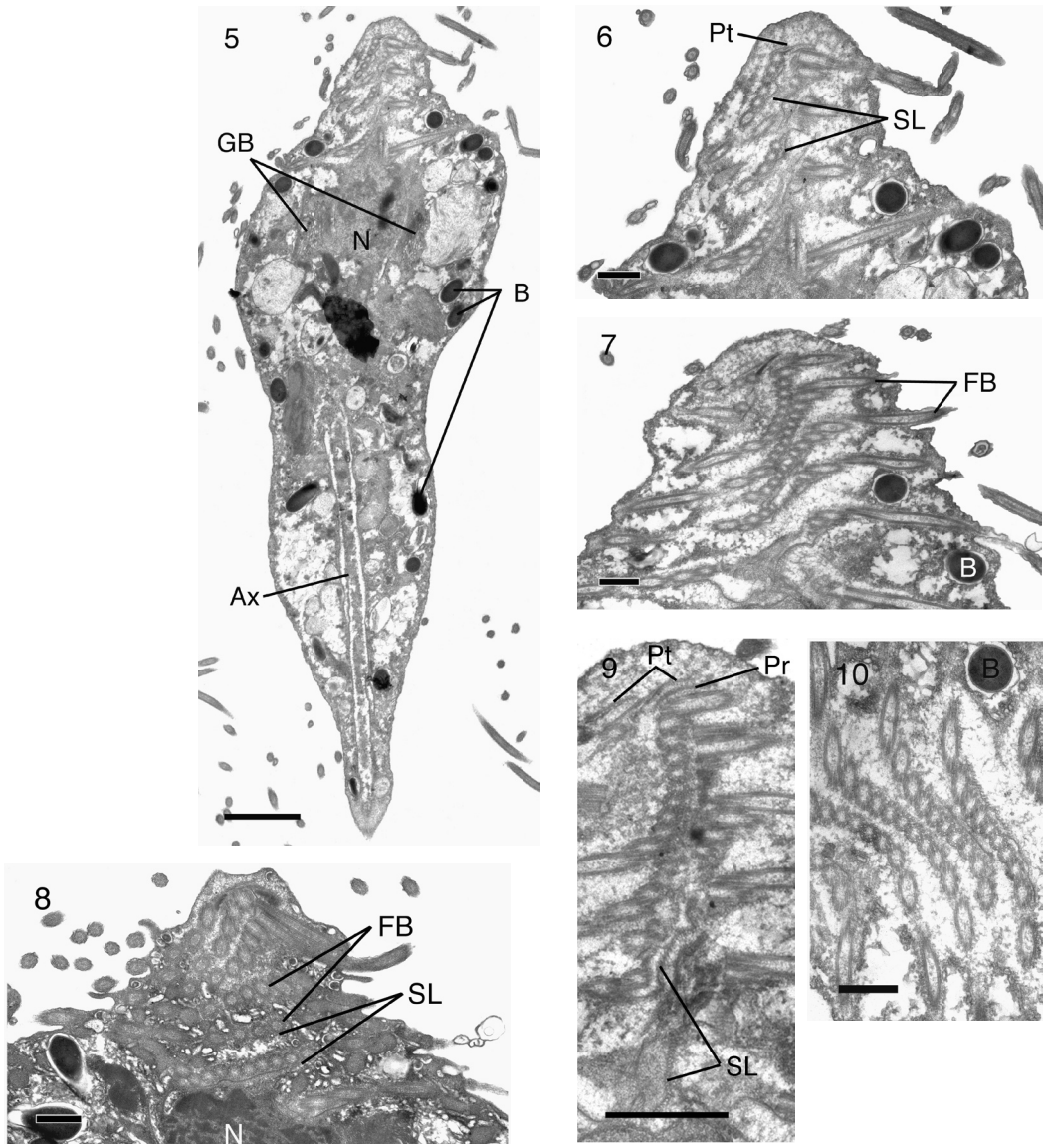

FIG. 3. *Ultrastructure* of *Micromastigotes scottae* sp. nov. A, Longitudinal section, whole cell; B–D, Serial longitudinal sections through a single cell from axial plane (6) to peripheral plane (8); E–F, Anterior mastigont system. Scale bars: 5: 2 µm; 6-8: 500 nm; 9: 1 µm; 10: 500 nm. Ax: Axostyle; B: Bacteria; FB: Flagella bands; GB: Golgi body; Pr: Preaxostyle; Pt: Peltoaxostyle; SL: Striated Lamina.

fallen timber, within dead fallen branches and from within tubular galleries within the bark of living trees. Individual termites representing the worker, major soldier and minor soldier castes were collected from each colony. Voucher collections were made of each colony by preserving five of each caste in 70% ethanol which were used to identify the termite species collected. Nest material was collected along with termites and each colony was provided with tissue paper soaked in water as a moisture and food source to maintain the colony in the laboratory. Workers were examined shortly after collection by dissecting the hindgut

into a small drop of invertebrate saline (0.6% NaCl). Some workers were removed directly from the nest and examined, these are referred to as 'dirty' specimens. 'Cleaned' specimens were generated by isolating individual workers from nest material and then rearing them on water-soaked tissue paper for several days to purge them of dirt and coarse wood fibres in their guts.

Light microscopic observations were performed on Giemsa-stained and protargol-impregnated specimens. Giemsa staining was performed on partially air-dried smears fixed with methanol. Slides were examined without coverslips by bright-field microscopy under immersion oil. Specimens from both 'cleaned' and 'dirty' termites were prepared for light microscopy to determine if cleaning caused artefactual changes to the flagellates. Protargol impregnation was performed according to the method of Foissner (1991) on specimens fixed with Schaudin's fluid. Cells were drawn using a camera lucida, and measured in each dimension using a calibrated eye-piece micrometer. Measurements are presented as a range of values followed by the average in parentheses.

Specimens for electron microscopy were collected exclusively from 'cleaned' termites dissected into Locke's fluid (composition in mM: 136 NaCl, 5.6 KCl, 1.2 MgCl2, 2.2 CaCl2, 1.2 NaH2PO4, 14.3 NaHCO3 and 10 dextrose, final pH 7.3–7.4) and fixed in a vast excess, typically at least 10 volumes, of 3% glutaraldehyde in 0.066M cacodylate buffer (pH 7.2) for 30 min. Cells for scanning electron microscopy were washed in 0.1M cacodylate buffer for 1 hour, post-fixed with 1% osmium tetroxide in 1.5% potassium ferricyanide for 1 hour, washed three times in distilled water and stored in 70% ethanol. Specimens were dehydrated in a graded series of ethanol solutions (80%, 90%, 100%, 100%) for 10 min each. Samples were critical point dried in CO2 between 8nm polycarbonate filters in a Millipore Swinnex filter holder. Dried samples were mounted on stubs with double sided tape, sputter coated with platinum and examined in a JOEL 6400 scanning electron microscope. For transmission electron microscopy, fixed samples from several termites were pooled and washed three times in Soerenson's phosphate buffer (pH 6.8) for 30 min each. Cells were post-fixed in 4% osmium tetroxide for 1 hour and washed three times in distilled water (10 min., 10 min. and overnight). Specimens were then dehydrated in a graded series of acetone solutions (5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100%, 100%) for 10 min each. Cells were gradually infiltrated with Epon resin (25%, 50%, 75% Epon in 100% acetone for 1 hour each, 100% Epon overnight) and embedded in fresh 100% Epon, pelleted by gentle centrifugation and cured for 1 day at 60ºC. Semi-thin survey sections were cut with glass knives, stained with 1% toluidine blue and used to orientate sections. Ultra-thin sections (70 nm and 90 nm) were cut with diamond knives, mounted on formvar-coated copper slot grids, stained with 5% uranyl acetate in 50% methanol for 2 min., washed in distilled water for 30 sec. and dried. The sections were then counter stained with Reynold's lead solution (2% lead citrate) for 1 min., washed in distilled water for 30 sec. and dried prior to examination. Sections were examined in a JEOL 1010 transmission electron microscope.

RESULTS

The soldiers and workers from 5 of the 13 colonies (38%) were found to harbour a small hypermastigid flagellate belonging to the genus *Micromastigotes* in addition to other parabasalid flagellate species. This *Micromastigotes* species appeared to be novel and its morphology and ultrastructure are described below.

Micromastigotes scottae sp. nov.

TYPE HOST. *Schedorhinotermes intermedius* (Isoptera: Rhinotermitidae)

HABITAT. Termite hindgut

TYPE LOCALITY. by Moggill Creek, Brisbane, QLD (27°32′S 152°52′E)

OTHER LOCALITIES. Joyner, Pine Rivers, QLD (27°17′S 152°56′E); Ferny Hills, Pine Rivers, QLD (27°23′S 152°56′E)

TYPE MATERIAL. Holotype deposited with the Queensland Museum (Brisbane, Australia), accession number: G463727.

DESCRIPTION. (Fig. 2A–C). Body elongate; rounded anterior rostrum grades into an ovoid mid-body, tapers posteriorly into a long tail; 16-40 (28) µm long by 6.4-13 (9) µm wide; shape index (length to width ratio) 2.3-4.4 (3.1). Anterior rostrum 1.6-6.4 (4) µm long, bears 4 flagella bands which spiral clockwise around the rostrum, approximately 1.5 gyres per band; flagella 8-16 (9.8) µm long, not adherent to the cell, confined to the rostrum giving the cell an

anterior tuft-like appearance. Single nucleus, spherical 1.6-3.2 (2.1) µm diameter, consistently located centrally in the cell at the level of base of the rostrum, 1.6-6.4 (4.2) µm from anterior of cell. Two parabasal bodies, ovoid to spherical flank the nucleus, level with base of the rostrum. Axostyle extends from nucleus to posterior end of cell, slightly curved along its length, non-projecting.

DIFFERENTIAL DIAGNOSIS. Whilst the original description of *M. grassei* Hollande & Carruette-Valentin, 1971 is quite lacking in detail as to what are the diagnostic features of the species, the accompanying photos clearly show that it is a squat species almost oval in outline with a short, narrow posterior tail. In contrast *M. scottae* is elongate, the cell being approximately 3 times as long as it is wide, and has a posterior tail almost as wide as the anterior portion of the cell. Additionally, in *M. grassei* the flagellar bands extend posterior to the nucleus, are connected to parabasal bodies and individual flagella are adherent in the proximal portion. None of these features occur in *M. scottae*.

ETYMOLOGY. *M. scottae* is named in honour of a friend whom I (SLC) failed, Kirsten Scott.

Ultrastructure. (Figs 3A–F). Individual flagella are derived from a central core which runs axially within the cell, each flagellum is angled approximately 12-18° relative to the previous flagellum in the band (Figs 3B, E, F), one full rotation around the cell thus consists of about 20-30 flagella with increasing numbers of flagella in the posteriormost bands (Fig. 3F), each band wraps around the cell about one and a half times and there are 4 independent bands. Each flagellum extends out perpendicularly or nearly so from the central core, thus more posteriorly located flagella are surrounded by more cytoplasm than anterior ones. Each kinetosome does not appear to be anchored to any particular structure, each is close to a centre tubular structure but not connected to it nor intimately associated with the striated lamina. Peltoaxostylar and preaxostylar fibres arise from the anterior most kinetosomes and are directed towards the anterior membrane of the cell (Fig. 3E). The peltoaxostyle is external to the preaxostyle and is much longer. The dense lamina is absent. A striated lamina is derived from the bottom edge of the kinetosomes and extends posteriorly, approximately paralleling the course of the flagellar band towards the nucleus (Figs 3D, E). The striated lamina surrounds the nucleus and gives rise to the axostyle (Fig. 3D). The axostyle proper runs posteriorly from the base of the nucleus and is composed of a rolled sheet of microtubules surrounding a low-density cytoplasm (Fig. 2A). It extends almost to the posterior end of the cell but does not project beyond the cell (Fig. 2A). There are 2 Golgi bodies which flank the nucleus, posterior to the bottom-most flagella; the parabasal fibre which connects the Golgi bodies to the kinetosomes is absent (Fig. 2A). Electron-dense bacteria appear to be scattered randomly within the cell, several are located near the flagellar bands whereas others occur throughout the body (Figs 3C, F). Food-vacuoles are mostly present within the posterior 'tail-like' portion of the cell and do not appear to contain whole wood fibres (Fig. 2A).

REVISED GENERIC DIAGNOSIS. *Micromastigotes* Hollande & Carruette-Valentin, 1971. Polymastigont flagellates with flagella arranged into bands which spiral around an anterior rostrum in a clockwise direction. Flagella bands are derived from the centre of the cell and radiate like a spiral stair-case. A flagellar gutter connecting individual flagella in each band is absent. Axostyle present, derived from the striated lamina which extends from the posterior of each kinetosome. Nucleus and Golgi bodies located immediately posterior to the flagella bands.

DIFFERENTIAL GENERIC DIAGNOSIS. It is difficult at this time to definitely assign *Micromastigotes* to any parabasalid family and we propose to leave it as a Spirotrichonymphina *insertae sedis* pending a better understanding of relationships amongst these groups. *Micromastigotes* is most readily confused with members of the Spirotrichonymphinae Grassi, 1917; namely *Spirotrichonympha*, *Microjoenia* and *Spirotrichonymphella*. *Micromastigotes* is readily distinguished from the first two genera by the absence of the flagellar gutter and the origin of the flagella in the central axis of the cell. *Micromastigotes* is distinguished from *Spirotrichonymphella* by the restriction of the flagella bands to the anterior portion of the cell; in *Spirotrichonymphella* the bands cover the whole cell.

DISCUSSION

TAXONOMIC STATUS

The original description of *Micromastigotes* by Hollande & Carruette-Valentin (1971) provided a basic description of the species and of what set

this genus apart from other parabasalid flagellates. There was no line diagram of the whole cell but the series of photographs of silver-stained cells and several transmission electron micrographs serve to illustrate the most significant features of the genus, i.e. flagella arising from an anterior rostrum and their derivation from a central spiral within the core of the cell. The features of *Micromastigotes* grassei included:

- spiral flagellar bands arising on an anterior rostrum and which do not form an anterior column or converge in the posterior of the cell;
- large dictyosomes which are widely separated;
- individual flagella are proximally adherant;
- nucleus is apical within the cell;
- axostyle resembling a compact rod and projects posteriorly;
- anteriormost region has a clear cytoplasm, without inclusions, fibrils or tubules;
- flagellar bands, under the cap are enclosed by morphoplasm and are interconnected with each other in 2 independent groups. On its origin each band has the same structure as the those in *Spirotrichonympha*. An ergoplasmic cistern (reticulum?) runs along the flagellar bands and it is between them, where the dictyosomes are in place.
- basal bodies have their cavity filled with glycogen and are connected by electron dense material
- the anterior basal bodies give rise to a preaxostyle that induces the microtubules of the pelta and the axostyle
- axostyle fibrilles enclose a cytoplasm that contains glycogen
- dictyosomes are piled up in a dozen very long cisterns. These contain an electron dense mass. Frequently there are multiple successive sacks, which contain a dense material at the same level, 6-8 saccules are present and are associated with each other by a dense substance.

There are, however, differences between *M. grassei* and *M. scottae*. The flagellar bands of *M. grassei* extend posterior to the nucleus whereas in *M. scottae* they are anterior to it; the flagella are adherent in *M. grassei* but non-adherent in *M. scottae*; the flagellar bands of *M. grassei* are accompanied by parabasal bodies whereas there is no connection between the parabasal bodies and flagellar bands of *M. scottae*; there is no striated lamina recorded in *M. grassei* but it is prominent in *M. scottae* and finally the basal bodies of each flagellar band are connected by electron dense material in *M. grassei* whereas this doesn't appear to be the case in *M. scottae*. Of these differences the first two, distribution of the flagellar bands and adherant flagella are known to vary within parabasalid genera (Lingle & Salisbury, 1995; Radek, 1997) and so are not sufficient to justify erection of a new genus for *M. scottae*. The published electron micrographs of *M. grassei* (Hollande & Carruette-Valentin, 1971 henceforth H & C-V, 1971) make it difficult to interpret the form of the parabasal bodies. The longitudinal tEM section of *M. grassei* (Fig. 51, H & C-V, 1971) depicts only the anterior of the cell and does not extend to the level of the parabasal bodies which flank the nucleus in *M. scottae*. The written description of *M. grassei*, however, refers to dictyosomes, the form of which is consistent with the bodies found in *M. scottae*. Additionally, in *M. grassei* there is a long cistern of the endoplasmic reticulum which follows the flagellar band (Fig. 52a, H & C-V, 1971). This feature may have been misinterpreted by Hollande & Carruette-Valentin (1971) as it occupies the same position as the striated lamina in *M. scottae* and the published micrographs include ribbon like structures labeled in one figure parabasal lamina (lames parabasales in the original French) which is simply another term for the striated lamina (Fig. 51, H & C-V, 1971) but labelled endoplasmic reticulum in another (Fig. 52a H & C-V, 1971). The electron dense material which connects the basal bodies of *M. grassei* doesn't seem to be present in *M. scottae* but the density of this material seems to vary considerably between the three published micrographs of *M. grassei* so it is hard to determine the significance of this feature.

The key similarity between *M. grassei* and *M. scottae* is the overall structure of the kinety system – flagella bands which spiral from a central core which is oriented axially within the cell. Furthermore, the absence of any similar arrangement in other hypermastigids makes us confident that these two species belong to the same genus. Most of the characters which we have found to vary between these two species are either known to vary between species in other parabasalid genera (i.e. flagellar band length, adherence of

flagella) or are difficult to interpret on the basis of published electron micrographs alone (parabasal body shape and distribution, striated lamina and electron dense connections between the basal bodies). Additionally, none of these features are consistently used for generic level discrimination within parabasalid flagellates whereas the arrangement of the flagella structures has proven to be very valuable and consistent (e.g. Brugerolle, 2001; Brugerolle & Lee, 2000). Whilst it is probable that a thorough reinvestigation of *M. grassei* may clarify many of these issues, at present we prefer to adopt a conservative taxonomic approach, assigning *M. scottae* to *Micromastigotes* rather than erect yet another monotypic genus whose relationships to other parabasalid flagellates is poorly understood.

A complicating factor is *Spirotrichonympha minor,* Radek, 1997. In his revision of the ultrastructure of the Spirotrichonymphidae, Brugerolle (2001) suggested that *S. minor* may be a member of *Micromastigotes* in which case the characteristics of this species must also be taken into account in revision of the latter genus. The description of *S. minor* by Radek (1997) does not include clear evidence for the presence of a flagellar gutter which may give the impression of similarity to *M. grassei*, particularly if one accepts that the striated lamina is absent in *M. grassei*. We have demonstrated the presence of a striated lamina in *M. scottae* in the present study and it is possible that it is also present in *M. grassei* but has been misinterpreted as being part of the endoplasmic reticulum. Furthermore, *S. minor* lacks the arrangement of the flagellar bands which we have found to be diagnostic of Micromastigotes. With the exception of lacking a flagellar gutter the structure of the flagellar bands in *S. minor* is much more similar to that of other *Spirotrichonympha* spp. in that the band is composed of peripheral basal bodies which are interlinked by electron dense material (Brugerolle, 2001; Radek, 1997). Other shared ultrastructural features include peripheral parabasal bodies (*S. mirabilis*, *S. grandis*, *S. elongata*), adherent flagella (*S. grandis*), and flagella bands which extend to the posterior of the cell (*S. mirabilis*, *S. grandis*, *S. elongata*). Of these features, peripheral, ovoid parabasal bodies distributed along the length the flagellar bands are particularly important. In *M. grassei*, the parabasal bodies associated with the flagellar bands are described (if one does not accept our contention that this structure is probably the striated lamina) as a single, narrow cisternae which parallels the flagellar band (Hollande & Carruette-Valentin, 1971) whereas in each of the *Spirotrichonympha* species examined the parabasal bodies are discrete ovoid structures composed of a stack of multiple cisternae, and several separate parabasal bodies are distributed down the length of the flagellar band. *Spirotrichonympha* is, by parabasalid standards, a large genus with 27 recognised species (Radek, 1997; Yamin, 1979) in two subgenera, so there is clearly scope for variation within this genus. Furthermore, *Spirotrichonympha* has recently been shown to be polyphyletic with respect to the genus *Holomastigotes* (Ohkuma et al, 2005). Detailed ultrastructural studies are lacking for the species for which molecular data is available and vice versa, precluding resolution of this matter at the present time, but it seems at least plausible that *Spirotrichonympha* must consist of at least 2 genera with divergent morphologies – one represented by *S. mirabilis*, *S. grandis* and *S. elongata* which possess the features defined by Brugerolle (2001) and the other represented by *S. minor* and defined by the different combination of features outlined in Radek (1997). Additional studies will help to clarify their relationships, but none of the four *Spirotrichonympha* species which have been examined ultrastructurally are sufficiently similar to *Micromastigotes* to warrant their inclusion in this genus or the inclusion of *M. scottae* in *Spirotrichonympha*.

BIOGEOGRAPHY AND HOST ASSOCIATIONS

The description of a second species of *Micromastigotes* raises an interesting issue about the geographic distribution of this genus. The type species, *M. grassei*, was collected from *Postelectrotermes praecox* on the island of Madeira, off the Atlantic coast of Africa. *Micromastigotes scottae* was found at almost the opposite end of the globe, in *Schedorhinotermes intermedius* in Queensland, Australia. This seemingly widely disparate distribution is easier to understand when the distribution of the respective hosts is taken into account. While both host species have comparatively small distributions in North Africa and adjacent islands and northeastern Australia respectively, the genus *Postelectrotermes* is widely distributed, its 13 species spread over Africa, the Middle East and India, whereas *Schedorhinotermes* broadly overlaps this distribution, its 36 species stretching from Melanesia, to Australia, through the Indonesian archipelago, Asia and into north Africa (Constantino, R. website: http://www.unb.

br/ib/zoo/docente/constant/catal/catnew.html; Myles, T. website: http://www.utoronto.ca/forest/termite/termite.htm). Thus, there is considerable scope for local mixing between one or several species from each genus possibly resulting in host switching. This, combined with the fact that North African and Asian termites have been the least investigated for their flagellate faunas (Yamin, 1979), makes it probable that there are undescribed species of *Micromastigotes* occurring through this intervening area.

Even more interesting than the geographic distribution *Micromastigotes* is the broad taxonomic range of the hosts which it infects; *Postelectrotermes* is a kalotermitid whereas *Schedorhinotermes* is a rhinotermitid. These two families of termites have radically different life-styles. Kalotermitids construct colonies within sound dry wood (including living trees) whereas rhinotermitids are subterranean, the main colony being constructed in fallen timber and foraging trails radiating out under the ground and up trees within tubular galleries made of cemented soil (Watson & Gay, 1991). These differences in host life-style have been frequently mirrored in differences in the taxa of flagellates which occur within them e.g. devescovinids. calonymphids and oxymonads are confined to kalotermids, and eucomonymphids and holomastigotoidids to rhinotermitids (Brugerolle & Lee, 2000; Dolan et al., 2000a, 2000b; Yamin, 1979). Groups which have wider distributions across multiple termite families include the trichonymphids and spirotrichonymphines (Yamin, 1979) suggesting that *Micromastigotes* may be related to one of these.

SYSTEMATIC PLACEMENT OF MICROMASTIGOTES

Hollande & Carruette-Valentin (1971) noted the possible relationship of *Micromastigotes* to other spiral-form hypermastigids and proposed that they formed a natural group without assigning the new genus to an existing or novel family but rather placing it as a genus *insertae sedis* within the suborder Spirotrichonymphina. They did, however, propose several characters upon which the spirotrichonymphines could be split into families (and presumably could be used to place Micromastigotes) including: presence or absence of a rostral column and flagellar bands; numbers of flagellar bands; whose portion is not differentiated out of the parabasal lamellae; position of the nucleus; contingent permanence of the axostyle in cytokinesis; and possible mode of nutrition. Unfortunately, most of these characteristics appear to be of little value in imposing a familial classification upon the spirotrichonymphines. The rostral column, as seen in light microscopy, is an artefact of the cell shape and thus is plastic even at the species level. The columella, a defined fibrillar structure is not always responsible for the appearance of a rostral column under light microscopy. Flagellar bands are a feature of all genera, but if restricted to denote those species which possess a flagellar gutter, this does split *Micromastigotes* and *Spirotrichonymphella* and some from the remaining genera. This feature is, however, variable within *Spirotrichonympha*; flagellar gutters occur in three species examined by Brugerolle (2001), *S. mirabilis*, *S. grandis* and *S. elongata* but are absent in *S. minor* (Radek, 1997). The numbers of flagellar bands are known to be variable within the genera *Spirotrichonympha* and *Holomastigotoides* (Radek, 1997), and it is thus unlikely to be valuable above the generic level. As emphasised above, the differentiation of the parabasal (= striated) lamina is a useful character in understanding the differences between simpler genera such as *Micromastigotes* and the more complex ones. The position of the nucleus is of limited variability within the group and is generally located at the base of the flagellar bands or at the point where the cell expands in diameter. In both cases it is close to the flagellar bases so the fibrous support structures can reach it. The presence of the axostyle throughout cell division has been emphasised as a significant character by Cleveland et al. (1934). It is, however, difficult to apply generally as complete cell cycles are known for only a handful of genera and we currently have no idea how variable this character is within genera. The only characters which show consistent and interpretable variation across the spirotrichonymphines are those related to the mastigont system and its support fibres.

Flagella structure in the spirotrichonymphids was reviewed by Brugerolle (2001) who homologised the anterior end of the spirotrichonymphid flagella band with the privileged flagella. Each band has a complete set of privileged flagella at its anterior end and the majority of the band is composed of the linearly replicated flagellum 3. In addition, each genus within the family could be diagnosed on the basis of modifications of the flagellar gutter – the complex of striated and dense lamella which formed a U-shaped band at the base of the kinetosomes. Brugerolle (2001), however, noted that *Spirotrichonymphella* lacked

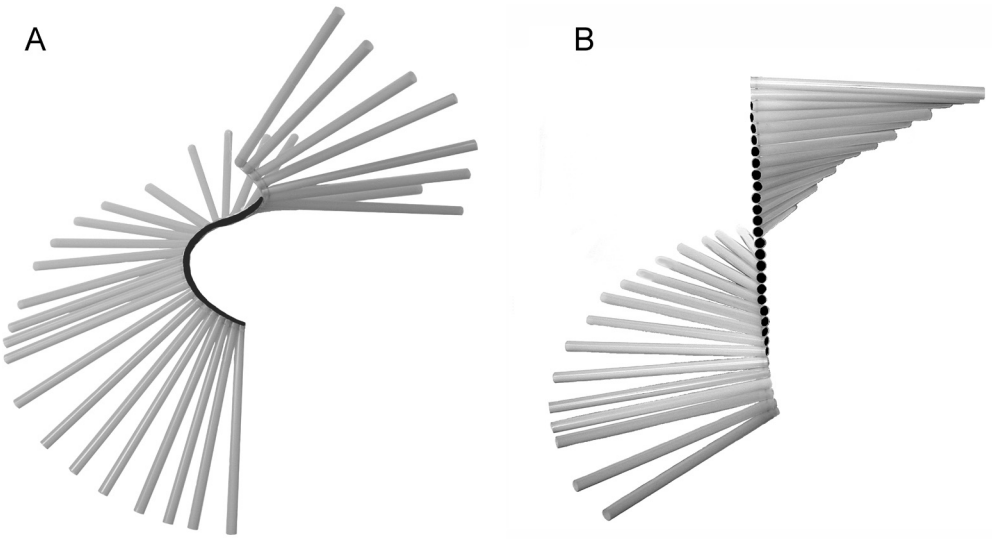

FIG. 4. Schematic diagram of flagellar band structure in the Spirotrichonymphina; each represents a single band for simplicity, all extant species have multiple bands arranged with radial symmetry. A, Spiralised linear band of nearly parallel basal bodies as found in *Spirotrichonympha*, *Microjoenia*, *Holomastigotoides*. B, Spiral stair case, basal bodies each rotated several degrees relative to the preceding body as found in *Micromastigotes*.

a flagellar gutter, the kinetosomes were connected to a central core-like structure, the columella, by striated roots and the striated lamella formed a weak band at the base of the kinetosome. This is a broadly similar arrangement to that seen in *Micromastigotes*, however the striated roots are absent. In both cases, the kinetosomes radiate from a central core, the kinetosome bases are buried deep in the cytoplasm, the dense lamina is absent and the striated lamina forms a sheet rather than a curved half of the U-shaped flagellar gutter. Conversely, *Spirotrichonymphella* lacks an axostyle, flagellar bands extend the entire length of the cell and the basal bodies are not anchored directly together in the axial centre of the cell but rather are anchored to the columella by striated roots. This suggests that *Micromastigotes* should probably be classified within the spirotrichonymphines and that the similarities to *Spirotrichonymphella* are probably not indicative of a close relationship between the two genera.

Brugerolle's (2001) homologisation of the anterior ends of the flagella bands of spirotrichonymphines with the privileged flagella of trichomonads *sensu lato* provides further evidence of the distinction between *Micromastigotes* and the other Spirotrichonymphidae. The arrangement of the flagella bands in most spirotrichonymphines suggests that the privileged flagella are oriented perpendicularly to the cell membrane, the mutliflagellate condition is achieved by serial replication of flagellum 3 and spiralisation is then achieved by torsion of the band (kinetosomes plus flagellar gutter) around the cell, individual flagella are roughly parallel to each other at their bases (Fig. 4A). Spiralisation in *Micromastigotes* has clearly not been achieved in this fashion. Flagella are oriented perpendicularly to the axis of the cell and spiralisation is achieved by rotation of kinetosomes around this central axis, by about 12-18° per flagellum in *M. scottae* (Fig. 4B). The distinction here is not simply related to size. Whilst most spirotrichonymphids are quite large species for which peripheral flagellar bands are the only functional option (flagella radiating from a central core may embed too much of the flagellum shaft in cytoplasm for them to beat effectively) the peripheral location of the flagellar bands occurs in even the smallest spirotrichonymphids. *Microjoenia anterodepressa* is only two thirds the size of *Micromastigotes scottae* and yet displays the classical spirotrichonymphid arrangement of peripheral flagella bands bounded internally by a flagellar gutter (Brugerolle, 2001). The evolutionary relationships of the spirotrichonymphid genera are currently unknown, so it is impossible to discern whether *Microjoenia*

is a small, plesiomorphic genus which already demonstrates the characteristics which were retained in larger genera, or it is a derived genus which has undergone reductive size evolution but still retains features of larger ancestors.

Thus, if *Micromastigotes* is not a spirotrichonymphine, it most likely represents another independent adoption of the hypermastigont condition. This may have occurred by serial replication of the recurrent flagellum. In most trichomonads, the recurrent flagellum is oriented perpendicularly to the long axis of the cell and to the other flagella (Brugerolle, 1999; Cleveland, 1961; Honigberg et al., 1968; Mattern et al., 1967). The recurrent flagellum is thus already in the correct orientation and its formation at an angle to the other flagella may have predisposed it to start a spiralisation process whose end product we see in *Micromastigotes*. Set against this idea is the fact that the recurrent flagellum is frequently modified in trichomonad groups, with the addition of a cresta in devescovinids or as part of the undulating membrane in many trichomonadids, whereas the flagella of *Micromastigotes* are not expanded or modified in any way. For this evolutionary scenario to be tenable, *Micromastigotes* would have to be related to a group with an unmodified recurrent flagellum. Given the difficulties of subjectively resolving ultrastructural homologies, the best solution is probably to generate molecular phylogenies for the groups of interest. Whilst sometimes initially at odds with traditional morphological based classifications, re-evaluation of morphological structures in light of well-resolved, independent phylogenies has proven to be a very useful course of action (e.g. Brugerolle & Patterson, 2001 for the Cristamonadida). Only then can we really address the questions raised by *Micromastigotes*: is it most closely related to the spirotrichonymphines or some other parabasalid group, is its similarity to *Spirotrichonymphella* more than coincidental, does the odd flagella structure of *Micromastigotes* really represent another adoption of the polymastigont condition, and what do parabasalids need with so many extra flagella for it to have evolved independently on so many occasions?

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