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## Electron microscopic identification of the intestinal protozoan flagellates of the xylophagous cockroach *Parasphaeria boleiriana* from Brazil

Received: 15 November 2002 / Accepted: 12 December 2002 / Published online: 19 March 2003  
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**Abstract** Flagellate protozoa of the hindgut of the xylophagous blattid *Parasphaeria boleiriana* were examined by light and electron microscopy. This species harbours two oxymonad species of the genera *Monocercomonoides* and *Polymastix*, the latter bearing *Fusiformis* bacteria on its surface. A diplomonad was present and has features of the genus *Hexamita* rather than *Spiroucleus*. In addition, two trichomonads of the genera *Monocercomonas* and *Tetratrichomastix* were identified. A precise comparison with species of blattids and other insects was difficult because most of these flagellates have been described only by light microscopy after cell staining and there are few electron microscope studies and no molecular studies. None of the flagellates contained wood fragments in their food vacuoles and so evidently do not participate in the digestion of wood or cellulose.

### Introduction

As reported by Yamin (1979), lower termites and the roach *Cryptocercus* harbor about 434 species and subspecies of parabasalids and oxymonad flagellates in their

intestines. We must also add several neglected retortamonad and diplomonad flagellates found in termites and in several cockroaches (Cleveland et al. 1934; Grassé 1952; Brugerolle and Lee 2000b). Certain of these symbiotic flagellates have vacuoles containing pieces of wood in their cytoplasm and, of course, these species are presumed to digest wood and cellulose (Honigberg 1970, 1978; Yamin 1978; Odelson and Breznak 1985). The others are saprophytic flagellates that phagocytise bacteria and various food particles and utilise the end-products of food digestion. Wood and cellulose are also digested by symbiotic bacteria or fungi (Bauchop 1977; Wenzel et al. 2002), but it has recently been demonstrated that termites can produce their own cellulases (Watanabe et al. 1998; Ohtoko et al. 2000).

Flagellates of cockroaches were partially identified by light microscopy in the first part of the last century (Grassé 1952) and more modern techniques, such as electron microscopy, have mostly focused on the extraordinarily rich fauna of *Cryptocercus* (Cleveland et al. 1934; Hollande and Carruette-Valentin 1971). Owing to difficulties in collecting and cultivating the tiny flagellates occurring in cockroaches, these have not received much attention. Also, these flagellates belong to genera that are more abundant in other anaerobic intestinal habitats such as the intestinal cloaca of fish, amphibians and reptiles, the coecum of rodents or the hindgut of tipulid and melolonthoid insect larvae (Grassé 1952; Kulda and Nohynkova 1978; Brugerolle and Müller 2000). The identification by molecular techniques of species of protozoa devoid of medical or economic importance is still in its infancy (Cavalier-Smith 1995; Gunderson et al. 1995; Fröhlich and König 1999; Ohkuma et al. 2000; Keeling 2002).

The discovery of a new xylophagous cockroach species, *Parasphaeria boleiriana* (Grandcolas and Pellens 2002) from the Brazilian Atlantic forest, prompted us to explore its intestinal fauna of flagellate protozoa. Our first observations showed numerous species of small flagellates whose identification by light microscopy proved difficult (Pellens et al. 2002). Electron microscopy is

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better suited for identifying these flagellates and is necessary in order to determine whether they participate in the digestion of wood or cellulose, the main food of this cockroach.

## Materials and methods

*P. boleiriana* were collected in Brazil (Grandcolas and Pellens 2002) and grown in the laboratory in boxes containing wet paper and rotten wood. The hindgut was opened with tweezers and the content mixed in two drops of Ringer's solution. Protozoa were centrifuged and fixed in a solution of 1% glutaraldehyde in 0.1 M phosphate buffer for 1 h and, after washing in buffer, the cells were post-fixed in 1% osmium tetroxide in phosphate buffer for 1 h. After washing and pre-embedding in 1% agar, the cell pellet was stained en bloc in saturated uranyl acetate in 70% ethanol. After dehydration in an alcohol series, the cell pellet was embedded in Epon 812 resin. Sections were cut on a Reichert Ultracut S microtome, stained with lead citrate for 15 min, carbon-coated, and examined under a JEOL 1200EX electron microscope at 80 kV.

Living cells were observed under a phase contrast and differential contrast microscope (Leica) equipped with a Q-Fish video-station (Leica), and were photographed after fixation.

## Results and discussion

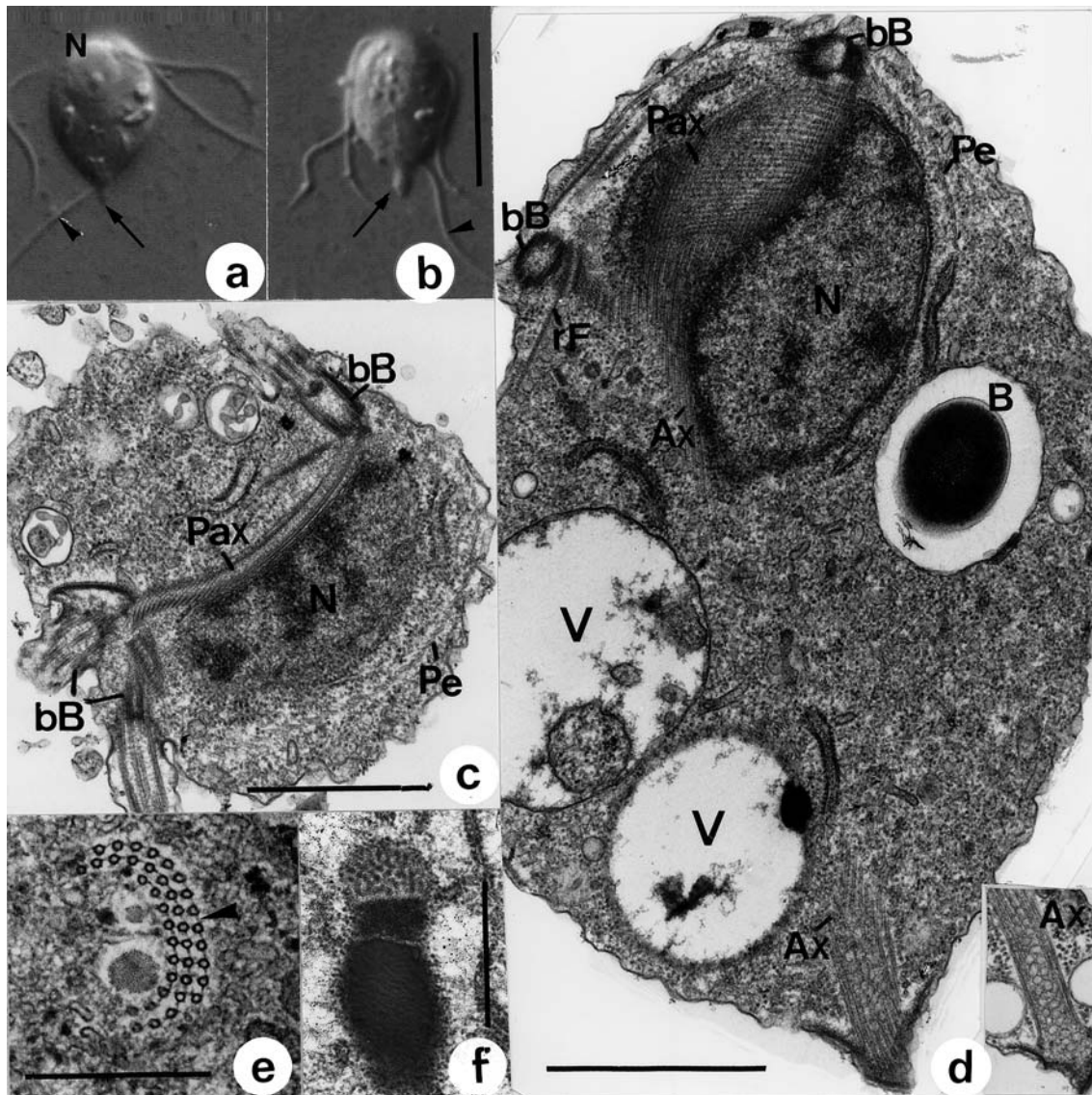
The tiny flagellates composing the fauna were not concentrated and were mixed with bacteria and many wood fragments. Oxymonad species of the genera *Monocercomonoides* and *Polymastix* were always present and were the most abundant flagellates in the cockroaches. A diplomonad species of the genus *Hexamita* was also regularly present, but at low concentration, and, two trichomonad species of the genera *Monocercomonas* and *Tetratrichomastix* were identified by light microscopy, but were lacking in some cockroaches. One or two additional species were noted, but their small number prevented reliable identification either by light or electron microscopy.

### The oxymonads *Monocercomonoides* and *Polymastix*

The flagellate with an ovoid body bearing four anterior flagella bent back posteriorly and arranged in two pairs at each side of a large nucleus belongs to the genus *Monocercomonoides* (Fig. 1). The flagella are longer than the body, one is longer than the others and an axostyle protrudes slightly posteriorly (Fig. 1a, b). Electron microscopy confirmed the presence of one species of the genus *Monocercomonoides*. The structure of the preaxostyle is revealed on the anterior part; it joins the two pairs of basal bodies and gives rise to the axostyle microtubules (Fig. 1c, d). This crystalline axostyle, which traverses the cell axially, is composed of three rows of microtubules interconnected by bridges (Fig. 1e). Also, an anterior ribbon of microtubules representing the pelta covers the nucleus (Fig. 1c, d). A recurrent fibre of about 12 microtubules is also observed under the plasma membrane in front of the recurrent

flagellum (Fig. 1d). The cell has no mitochondria and no Golgi apparatus. The cytoplasm contains food vacuoles with rod-shaped bacteria, but no wood fragments were detected (Fig. 1d). Large areas of cytoplasm free of ribosomes or other organelles are filled with glycogen particles, and there are composite vacuoles the content of which resembles lipids (Fig. 1f).

A second flagellate covered with long *Fusiformis* bacteria is more remarkable and belongs to the genus *Polymastix* (Fig. 2a–d). The spindle-shaped cell of about 10  $\mu\text{m}$  in length bears four anterior flagella, one of which is longer than the body. The axostyle protrudes slightly posteriorly, but is generally masked by the *Fusiformis* bacteria. Electron microscopy reveals the same structures as *Monocercomonoides* such as the flagella arranged in two pairs on each side of the anterior grooved preaxostyle (Fig. 2e). The crystalline axostyle originating from the preaxostyle, is composed of about ten rows of bridged microtubules, and the pelta is present above the nucleus (Fig. 2e, f). The cell has no mitochondria or Golgi body. *Polymastix* cells bear long *Fusiformis* bacteria attached longitudinally or simply stuck on the surface of the protozoa (Fig. 2a). The attachment consists of a narrow isthmus of the cell coat (Fig. 2f, h). The food vacuole contains cocci bacteria (Fig. 1c, f) or *Fusiformis* bacteria still attached to the vacuole membrane by an isthmus (Fig. 2h). It is not clear whether the *Fusiformis* bacteria constitute food for the flagellate, but this is likely as they are found inside the food vacuoles. The cytoplasm also contains composite vacuoles with a lipid-like content (Fig. 2g). The genera *Polymastix* and *Monocercomonoides* were distinguished by light microscopy after staining (Grassé 1952; Brugerolle and Lee 2000a), and later electron microscopy studies confirmed their oxymonad features and provided further arguments for distinguishing them (Brugerolle and Joyon 1973; Brugerolle 1981). *Monocercomonoides* species are endocommensal flagellates present in various anaerobic habitats such as the intestine of vertebrates (Nie 1950; Grassé 1952; Kulda and Nohynkova 1978) and also in the hindgut of many insects as reported by Grassé (1952). Several species have been identified in insects: *Monocercomonoides melolonthae* from melolonthoid larvae of coleopterids such as *Cetonia* (Grassé 1926; Travis 1932), *Monocercomonoides tipulae* from the hindgut of tipulid larvae (Geiman 1932; Ludwig 1946), *Monocercomonoides orthoptera* from *Periplaneta orientalis* and *Ectobius lapponicus* (Bélar 1916; Grassé 1952), *Monocercomonoides panesthiae* from the xylophagous blattid *Panesthia javanica* (Kidder 1936), *Monocercomonoides globus* from the roach *Cryptocercus punctulatus* (Cleveland et al. 1934). Several *Monocercomonoides* species such as *M. melolonthae* from *Oryctes nasicornis*, *M. orthoptera* from *Gryllotalpa vulgaris*, *Blabera cranifer* and *M. tipulae* from *Tipula* larvae have been studied by electron microscopy and have helped to characterise the genus (Brugerolle and Joyon 1973). More recently, the species *Monocercomonoides sinaica* from the termite *Kaloterme*



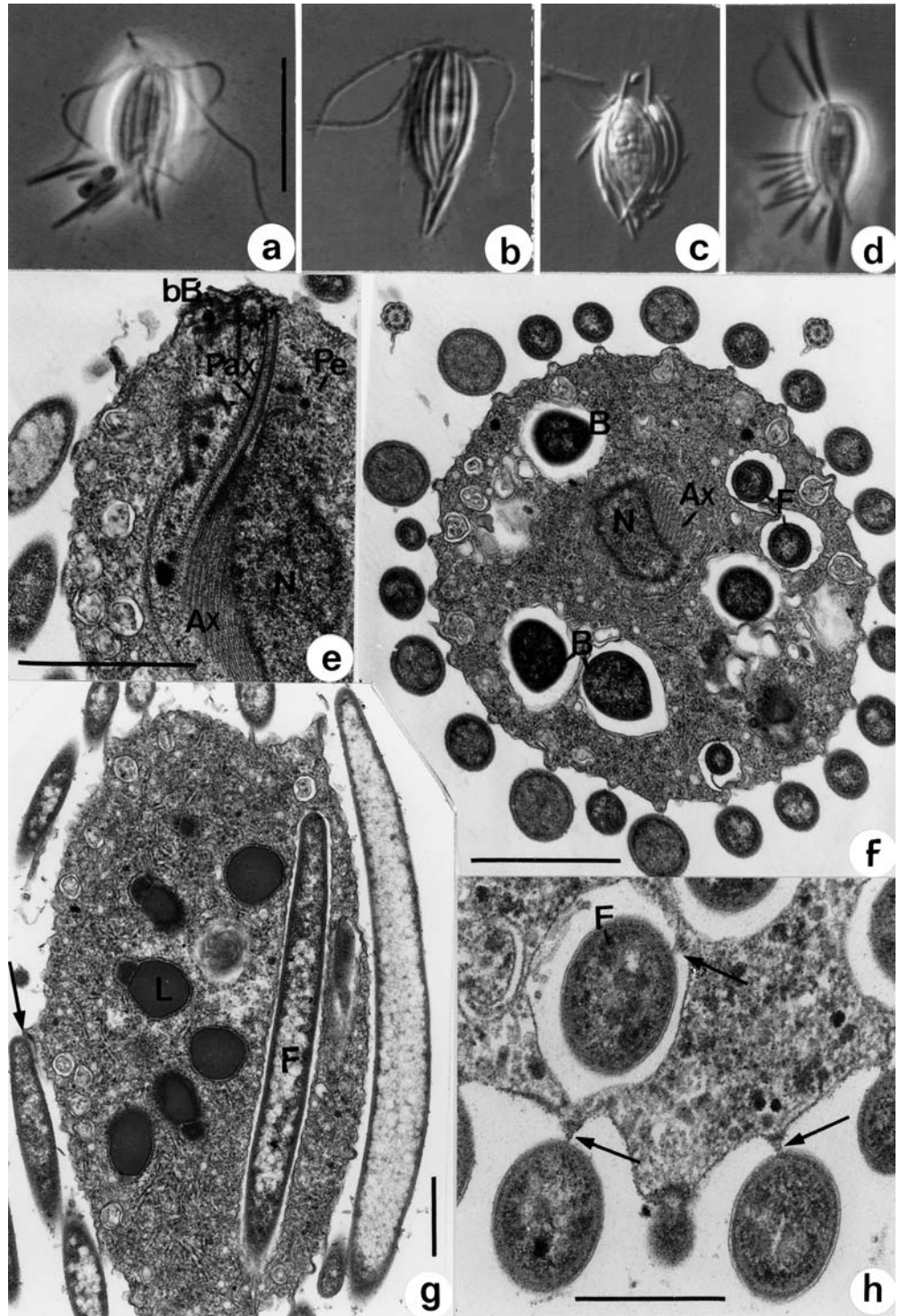
**Fig. 1** Light (a, b) and electron (c–f) micrographs of *Monocercomonoides* sp. a, b Interferential contrast micrographs of *Monocercomonoides* sp. cells showing the ovoid cell shape, the two pairs of flagella inserted on each side of a large anterior nucleus (N), one flagellum (arrowhead) longer than the others and an axostyle (arrow) protruding posteriorly. c Transverse and d longitudinal sections of the cell showing the two pairs of basal bodies (bB) attached on each side of the preaxostyle (Pax) adjacent to the nucleus (N). Microtubules of the axostyle (Ax) arise from the preaxostyle and extend to the posterior end (d inset). There is a recurrent microtubular fibre (rF) under the recurrent flagellum and a microtubular ribbon or pelta (Pe) covering the nucleus; food vacuole (V) with bacteria (B). e Section of the axostyle composed of interlinked rows of microtubules. f Composite vacuole containing electron dense lipid-like material. Bars: a, b 10  $\mu\text{m}$ ; c, d 1  $\mu\text{m}$ ; e, f 0.5  $\mu\text{m}$

*sinaica* confirmed the ultrastructural features of this genus (Radek 1994). A comparison of the *Monocercomonoides* species found in *Parasphaeria* with the three species described in blattids is difficult because we did not use the staining techniques of the earlier authors. These parasitic flagellates are probably not host specific

and the same species might occur in several insect hosts. We have few reliable features to separate the species, for which a molecular identification would be required.

*Polymastix* species have been found in the hindgut of insects and myriapods and all bear *Fusiformis* bacteria on their surface. *Polymastix melolonthae* occurs in many insects such as *Tipula* larvae (Mackinnon 1913a), *Melolontha*, *Rhizotrogus*, *Cetonia*, *Anomala*, *Oryctes*, *Grylotalpa* (Grassé 1926, 1952). Other species such as *Polymastix phyllophagae* from the coleopterid *Phyllophaga* (Grassé 1926), *Polymastix legeri* from the myriapod *Glomeris* (Grassé 1926), *Polymastix nitidus* from another myriapod, *Rhizocrinus*, (Grassé 1952) have also been described. Only one species *P. melolonthae* from *Melolontha* larvae has been studied by electron microscopy (Brugerolle 1981). It has the common oxymonad features but presents several small differences from *Monocercomonoides* species such as an elongated cell body bearing *Fusiformis* bacteria, a narrow and grooved preaxostyle, an axostyle of about ten rows of microtubules at the

**Fig. 2** Light (a–d) and electron (e–h) micrographs of *Polymastix* sp. **a, d** Phase contrast and **b, c** interferential contrast micrographs showing the cell bearing two pairs of flagella inserted at the anterior end. The cell is covered by *Fusiformis* bacteria that: **a–c** adhere throughout their length or **d** are simply stuck on the cell body; **c** presence of cocci bacteria in the cell. **e** Oblique section showing one pair of basal bodies (*bB*) attached to the preaxostyle (*Pax*), the axostyle (*Ax*) adjacent to the nucleus (*N*) and the microtubular row of the pelta (*Pe*). **f, h** Transverse and **g** longitudinal sections showing the *Fusiformis* bacteria adherent to the cell surface. The connection is made by an isthmus of the cell coat (arrows). Food vacuoles contain cocci (*B*), or *Fusiformis* (*F*) bacteria still adherent to the vacuole membrane (arrow, **h**); lipid vacuoles (*L* in **g**) Bars: **a–d** 10  $\mu\text{m}$ ; **e–g** 1  $\mu\text{m}$ ; **h** 0.5  $\mu\text{m}$



nucleus level, a very short fibre under the recurrent flagellum, which is not adherent, a microfibrillar attachment of the nucleus to one pair of basal bodies and an axostyle not protruding posteriorly (Brugerolle 1981). Given the presence or the lack of *Fusiformis* and some other small differences, it is clear that *Parasphaeria* harbours two oxymonad species, one belonging to the genus *Monocercomonoides* and the other to the genus *Polymastix*. Light and electron microscopy reveal several

small differences between *P. melolonthae* (Brugerolle 1981) and the species found in *Parasphaeria* that might justify the creation of a new species, but here too, the species can not be compared using the same techniques, and a molecular study would be necessary. The description of *Fusiformis* bacteria on the surface of parasitic flagellates is not new, having been described in the oxymonad *Streblomastix* from the termite *Zootermopsis angusticollis* (Hollande and Carruette-Valentin 1970) and

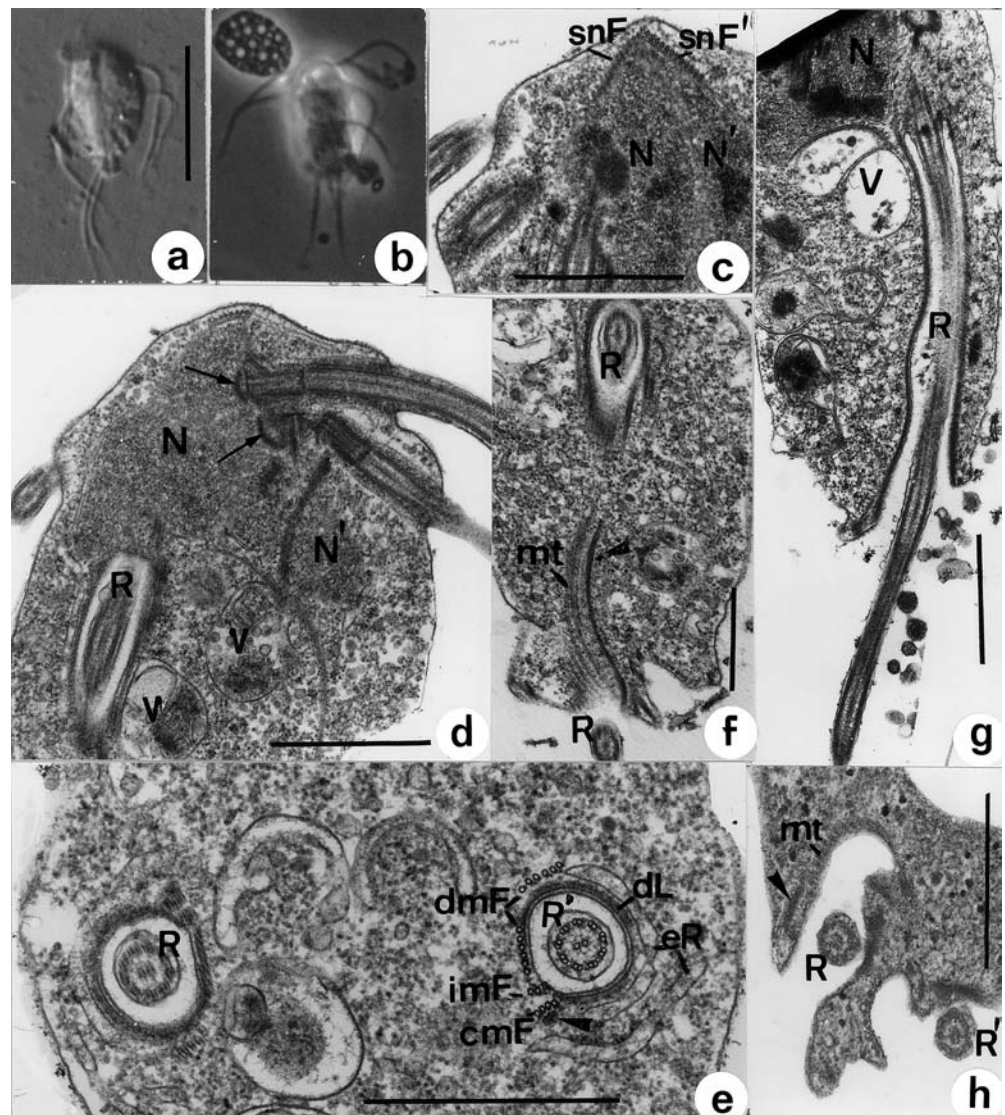
in several parabasalids such as *Lophomonas striata* from blattids (Hollande and Carruette-Valentin 1972) and in *Hoplonympha* from *Postelectrotermes* (Brugerolle unpublished).

### The diplomonad *Hexamita*

A diplomonad species was identified by light microscopy (Fig. 3a, b). The cell, about 10  $\mu\text{m}$  long, bears six anterior flagella and two posterior or trailing flagella (Fig. 3a, b). Several features of the genus *Hexamita* are revealed by electron microscopy. First, the two adjacent anterior nuclei are covered by two rows of the supranuclear microtubular fibres (Fig. 3c). There are two depressions on the nuclear surface in front of the basal bodies (Fig. 3d). The proximal portion of the axoneme of the anterior flagella is intracytoplasmic (Fig. 3d). A transverse section in the middle of the cell clearly shows the organisation of

the fibres around the two cytopharyngeal channels that contain the posterior flagella (Fig. 3e). By comparison with the free-living *Hexamita inflata*, we can recognise four cytostomal rows of microtubules associated with the cytopharynxes: two rows of six microtubules or direct fibres, one row of four microtubules lined by a dense rootlet and three microtubules with an intermediate position. There is also a striated lamina that overlies the membrane of the cytopharynxes. The cytopharynxes are accompanied by rough endoplasmic reticulum cisternae (Fig. 3e). At the posterior end, the arrangement of the fibres lining the two cytostomal openings is very complex (Fig. 3f–h), and without scanning electron microscopy it is difficult to reconstitute the architecture of this region and compare it with other *Hexamita* or *Spironucleus* species such as *Spironucleus vortens* (Sterud and Poynton 2002). This flagellate ingests food by the posterior openings or cytostomes and digestive vacuoles are formed at the anterior part of the two tube-like cytopharynxes

**Fig. 3** a, b Light and c–h electron micrographs of *Hexamita* sp. a, b Differential and phase contrast micrographs showing the elongated cell body bearing anterior back-bent flagella and b two posterior trailing flagella arising on each side of the terminal posterior point. c Section showing the nuclei (*N*, *N'*) surmounted by two microtubular rows of the supra-nuclear fibre (*snF*). d The basal bodies of two anterior flagella are inserted in front of nuclear depressions (arrows), the recurrent flagellum (*R*) is located inside a cytopharyngeal channel; food vacuole (*V*). e Median transverse section showing the two recurrent flagella inside the cytopharyngeal channels surrounded by endoplasmic reticulum cisternae (*eR*). Around the channels there are a dense lamina (*dL*) and four sets of microtubules: two rows of six microtubules (*dmF*), one row of four microtubules (*cmF*) doubled by a dense rootlet (arrowhead) and an intermediary row of three microtubules (*imF*). f, g Longitudinal and h oblique sections showing the cytopharyngeal channels lined by microtubular (*mt*) and dense fibres (arrowhead), the two posterior openings (h); food vacuole (*V*). Bars: a, b 10  $\mu\text{m}$ ; c–h 1  $\mu\text{m}$





(Fig. 3g). In this species, the food vacuoles contain less well-defined material that seems not to be wood.

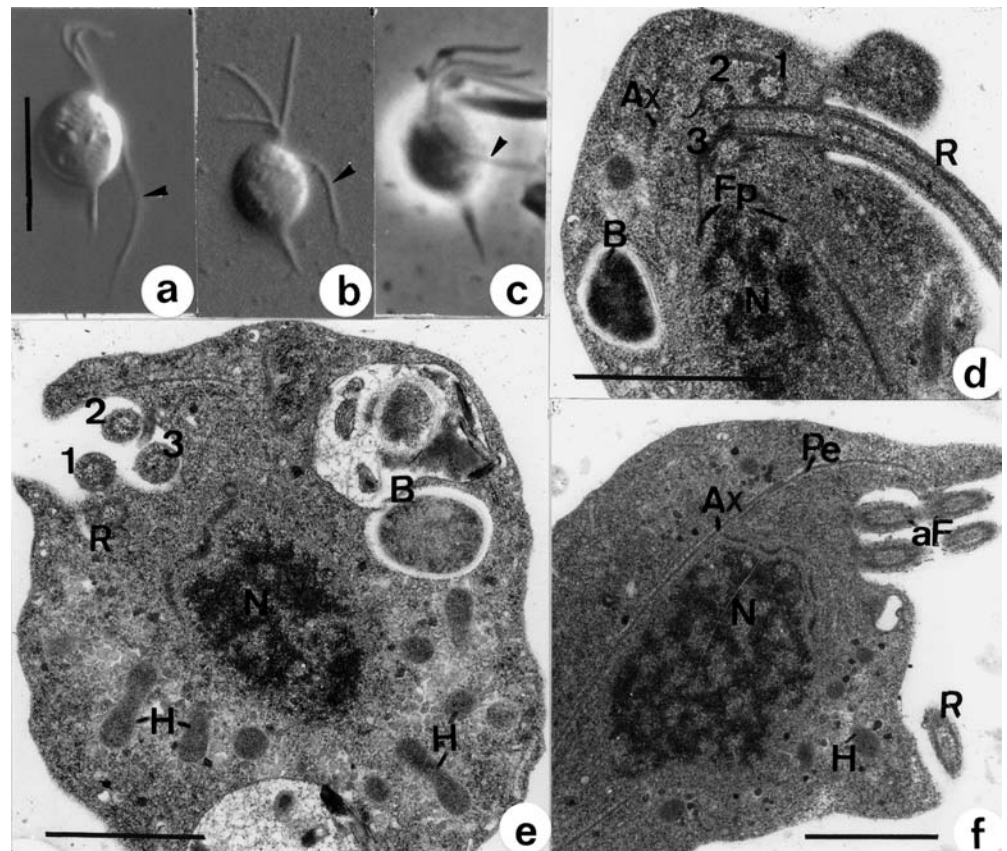
Diplomonads, which have two trailing flagella emerging from two posterior channels, are assigned to three genera: *Hexamita*, *Spiroucleus* and *Octomitus* (Lavie 1936; Grassé 1952). *Hexamita* have two rounded nuclei and posterior flagella lined by dense structures by light microscopy, and comprise free-living and parasitic species (Lavie 1936). In contrast to *Hexamita*, *Spiroucleus* species have a more elongated body, s-shaped nuclei and are parasitic. *Octomitus* species have two anterior adjacent nuclei and two axially posterior flagella but no real posterior openings. The features of these three genera have been redefined after electron microscopy studies (Brugerolle et al. 1973, 1974; Brugerolle 1974; Kulda and Nohynkova 1978; Brugerolle and Lee 2000b) and have been confirmed by several more recent studies of their ultrastructure (Sterud et al. 1997; Sterud 1998; Sterud and Poynton 2002). There is a progressive closure of the cytostomes in passing from the genus *Trepomonas* to the genera *Hexamita*, *Spiroucleus* and *Octomitus* (Brugerolle 1975). However, the distinction between two genera could be quite subtle for certain species which remain to be studied by electron microscopy. Molecular phylogenetic studies seem to confirm the separation between the genera *Spiroucleus* and *Hexamita* (Leipe et al. 1993; Branke et al. 1996; Keeling and Doolittle 1997), but many diplomonad species have not been studied by these techniques. Several parasitic *Hexamita* and *Spiroucleus*

species occur in the intestine of fishes, amphibians, reptiles and mammals (Grassé 1952; Kulda and Nohynkova 1978; Brugerolle and Lee 2000b). Several characters present in the free-living *Hexamita inflata*, such as the direct fibre divided into two rows of microtubules, and the presence of endoplasmic reticulum cisternae along the cytopharynxes, are also shared by the parasitic *Hexamita* of *Parasphaeria*. In addition, in the latter there are no lateral ridges on the surface that mark the path of the cytopharynxes of *Spiroucleus* species (Sterud and Poynton 2002). All of these similarities or differences distinguish the *Hexamita* of *Parasphaeria* from the genus *Spiroucleus*. Several *Hexamita* species have been described in blattids such as *Hexamita periplanetae* (Bêlar 1916) from *Periplaneta* and *Hexamita cryptocerci* from *Cryptocercus* (Cleveland et al. 1934), a species that has also been reported in the cockroach *P. javanica* (Kidder 1936). The lack of electron microscopy or molecular studies of the diplomonads of insects prevent us from comparing the species and clearly identifying the one found in *Parasphaeria*. *Hexamita* species are probably not host-specific and here too we need more comparative microscopic and molecular studies.

#### Trichomonad species

Two species of trichomonad were identified by light microscopy; both have a group of anteriorly directed flagella, a recurrent flagellum and an axostyle protruding

**Fig. 4** a–c Light and d–f electron micrographs of *Monocercomonas* sp. and *Tetratrichomastix* sp. a, b Differential and c phase contrast micrographs of *Monocercomonas* sp. with three anterior flagella (a, b) and *Tetratrichomastix* sp. with four anterior flagella (c) as well as a recurrent non-adherent flagellum (arrowheads). Both species have an axostyle (Ax) protruding posteriorly. d, e Sections of *Monocercomonas* sp. showing the three anterior basal bodies 1–3 orthogonal to the basal body (R) of the recurrent flagellum, two parabasal fibres (Fp) covering the nucleus (N), hydrogenosomes (H) and food vacuoles with bacteria (V). f Longitudinal section of *Tetratrichomastix* sp. showing the four anterior flagella (aF), the recurrent flagellum (R), the pelta-axostyle (Pe-Ax), the nucleus (N) and hydrogenosomes (H). Bars: a–c 10  $\mu$ m; d–f 1  $\mu$ m



posteriorly (Fig. 4a–c). They were assigned to the genera *Monocercomonas*, which has three anterior flagella (Fig. 4a, b) and *Tetratrichomastix*, which has four anterior flagella in addition to the recurrent one (Fig. 4c). Both are small, being less than 10  $\mu\text{m}$  in length. The basal body of the recurrent flagellum is orthogonal to the basal bodies of the three anterior flagella numbers 1, 2 and 3 in *Monocercomonas* (Fig. 4d, e), which are four in number in *Tetratrichomastix* (Fig. 4f). The recurrent flagellum of *Monocercomonas* is non-adherent and that of *Tetratrichomastix* is partially adherent to the cell body, but does not raise an undulating membrane (Fig. 4c, f). The microtubular ribbon of the pelta-axostyle forms an anterior capitulum that surrounds the place of emergence of the flagella and continues posteriorly to give the axostylar trunk (Fig. 4f). Two striated fibres are attached to basal bodies and represent the branches of the parabasal apparatus (Fig. 4d). Hydrogenosomes are present in the cytoplasm and food vacuoles contain bacteria but no wood particles (Fig. 4e).

The taxonomy of trichomonad genera was established from light microscopy studies (Honigberg 1963). This was largely confirmed by electron microscopy studies (Brugerolle 1975–1976; Brugerolle and Lee 2000c) but is now challenged by molecular phylogeny (Gunderson et al. 1995; Delgado-Viscogliosi et al. 2000; Ohkuma et al. 2000; Keeling 2002). *Monocercomonas* species live mostly in the intestines of vertebrates (Nie 1950; Honigberg 1963; Brugerolle and Lee 2000c) and some of these species have been described by electron microscopy (Mattern et al. 1972; Brugerolle 1975–1976). In insects, several species live in termites, cockroaches and coleopterid larvae (Grassé 1952) and the species *Monocercomonas orthopterum* is found in *Blatta orientalis*, but none have been studied by electron microscopy or by molecular techniques. *Tetratrichomastix* was retained as a valid genus by Honigberg (1963) for species living in tipulid and trichopterid larvae such as *Tetratrichomastix parisii* (Mackinnon 1913b; Ludwig 1946). The existence of this genus has been criticised, since tipulid larvae also harbour a *Hexamastix* species whose stages of division evoke *Tetratrichomastix*. Our observations of a *Tetratrichomastix* species in *Parasphaeria* in the absence of *Hexamastix* species and the electron microscopy study confirm the validity of this genus. The species found in *Parasphaeria* is probably the same as *Tetratrichomastix blattidarum* reported in *Blattella* and *Periplaneta* by Young (1935), but new studies are required.

The flagellates identified are the most characteristic species of the fauna of this cockroach, but their occurrence is variable. Additional flagellate species may occur, such as retortamonads, which are frequent in insects. Flagellates are accompanied by bacteria, some of which are as large as flagellates and can have bacterial flagella; and so may have been confused with flagellates, e.g., Fig. 1 in Pellens et al. (2002). It must be emphasized that this is the first record of such a flagellate fauna in a cockroach. Although direct evidence for participation in

the digestion of wood is lacking, this fauna can, however, be related to the xylophagous behavior of this cockroach. This stresses the need for more studies of the intestinal faunas of cockroaches, including poorly known wood-eating species (Grandcolas 1993; Grandcolas et al. 2002). This is also a contribution to the identification of protozoa species and microorganisms in general, which is a continuous challenge for mapping biodiversity (Pace 1997; Corliss 2000; Finlay 2001).

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