Ultrastructure of the aloricate bicosoecid *Pseudobodo tremulans*, with revision of the order Bicosoecida

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Summary

The fine structure of the colourless aloricate bicosoecid *Pseudobodo tremulans* has been studied in detail. The anterior flagellum bears two rows of tripartite tubular mastigonemes, and the posterior one is smooth. The cell has no other distinguishable surface decoration. The internal structure reveals typical features of bicosoecids. The largest rootlet of *P. tremulans* (R3) is a broad microtubular band passing from the basal body of anterior flagellum towards the cytostome region, which is presented by a lip. At its proximal end, rootlet R3 has a characteristic L-shape cross section and is normally associated with fibrillar material. The posterior basal body is oriented to the left. A mitochondrion with tubular cristae is associated with the middle part of rootlet R3. Small numbers of extrusomes are present. Small microbodies are located close to the nucleus. There is a structure similar to concentric rings in the transition zone of each flagellum. The taxonomy of bicosoecids (including pseudodendromonads) is discussed, and a revision of the order is proposed. The four included families are classified on the basis of characters associated with the cell surface and the feeding apparatus.

Key words: *Pseudobodo tremulans*, heterotrophic flagellate, ultrastructure, bicosoecids, pseudodendromonads

Introduction

Bicosoecids are heterokont, bacterivorous, heterotrophic flagellates that live in marine and freshwater habitats. Until recently, only loricate, sedentary flagellates of the genus *Bicosoeca* (=*Bicoeca*) have been considered to be members of the group (Zhukov, 1978). However, in the past two decades, a number of nonloricate species and genera have been added (Fenchel, 1982, Fenchel and Patterson, 1988, Larsen and Patterson, 1990, Teal et al., 1998, O'Kelly and Nerad, 1998, Karpov et al., 1998, Guillou et al., 1999). Molecular phylogenies suggest that the bicosoecids are monophyletic and represent one of several basal lineages in the stramenopile group of protists, diverging before the acquisition of chloroplasts in photosynthetic members of the group (Leipe et al. 1994, 1996; Cavalier-Smith et al. 1995/1996; Karpov et al. 1998; Guillou et al. 1999). The relationships among these basal lineages are unresolved. The absence of ultrastructural and molecular data from many species belonging to, or thought to be related to, the bicosoecids has prevented a better understanding of bicosoecid taxonomy and phylogeny.

Pseudobodo tremulans (Griessmann, 1913) Fenchel, 1982 is the first nonloricate flagellate to be referred to the bicosoecids (Fenchel, 1982). The brief report of its ultrastructure (Fenchel, 1982) contains few details, and no molecular sequence data are available. Consequently, its relationships with other bicosoecids are not well understood. In this, the first of two reports on *P. tremulans*, the ultrastructural features of this species are described and compared with other bicosoecids.

Material and Methods

Pseudobodo tremulans (clone 0–13) was obtained from the culture collection of the Institute of Inland Water Biology, Russian Academy of Sciences, Borok, Russia. This clone was isolated and identified by Dr. A.P. Mylnikov from brackish water (salinity 1.0–1.2%) sample collected on the littoral of White sea near the Marine Biological Station of Zoological Institute, Russian Academy of Sciences (Kartesh), in August, 1986. Cultures were grown in

artificial sea water and periodically supplied with suspensions of a single bacterial strain of *Klebsiella aerogenes*.

For light microscopy (LM), both living and fixed cells were studied. Observations were made using a Leitz Ortholux II microscope equipped with bright-field and differential interference contrast optics. Black and white photographs were taken using Agfa APX 25 film.

For electron microscopy (EM) of whole mounts, a suspension of cells was placed on a formvar-coated grid, stained with 2% aqueous uranyl acetate, and air-dried.

For EM of sections, 1 ml of cells was mixed with 1 ml of a solution containing 4% glutaraldehyde, 0.05M cacodylate buffer and 0.24M sucrose. After fixation for 2 H on ice, the pellet was collected by centrifugation and rinsed for 15 min in 0.025M cacodylate buffer with 0.1M sucrose. After postfixation with 1% osmium tetroxide in 0.05M cacodylate buffer for 1 H at 4° C, the pellet was dehydrated in an alcohol series and embedded in Epon resin. Blocks were serially sectioned with a diamond knife on a Reichert Ultracut ultramicrotome, mounted on formvar-coated slot grids, and post-stained with uranyl acetate and lead citrate. Whole mounts and sections were viewed on a Philips CM 10 electron microscope operating at 80 kV.

Conventions of cell orientation and nomenclature of cell components are those of O'Kelly and Patterson (1996).

Results

Light microscopy

Trophic cells of *Pseudobodo tremulans* are pear-shaped or egg-shaped, with a broad posterior end (Figs 1, 2). Two flagella of unequal length emerge from the base of a small apical papilla or "collar" (Fig. 1). The posterior flagellum emerges from a pit or small pocket located at the base of the papilla, while the anterior flagellum emerges from the other (flat) side of the papilla.

Cells are 5–8 µm long. Anterior flagellar length is 12–15 µm and posterior flagellar length is 8–10 µm (Figs 1, 2). Normally trophozoites attach to the substratum with its posterior short flagellum, which emerges to the left side of the cell (Fig. 1) and then bends around the cell. Adhe-

sion occurs at the very tip of posterior flagellum. The anterior flagellum is directed to the opposite side and beats with a sinusoidal wave. Attached feeding cells look slightly triangular with the cytostome region directed laterally. This region looks very translucent through LM, and manifests a lip that is supported with a prominent cytoskeletal element, sometime being visible even with LM (Fig. 1).

Free-swimming cells (Figs 2, 4) normally appear during exponential phase of culture growth. These swarmers move rather quickly and straight. Their anterior flagellum is directed forward, and the posterior flagellum is abutting the cell surface at its proximal end (Figs 2, 4). The swarmers look more rounded and just be temporarily detached sessile cells. In both sessile cells and swarmers, the nucleus is located next to the flagellar bases, and food vacuoles with bacteria inside are visible in the posterior part of the cell (Fig. 1, 2).

Cell division has not been studied in details. Cells divide longitudinally, forming 4 flagella at an early stage, corresponding to late prophase (Fig. 3). The nucleus looks rounded at this stage and the nucleolus is inconspicuous in comparison to that at interphase (Fig. 1).

Ultrastructure

The anterior flagellum of *P. tremulans* has two opposite rows of tubular hairs or mastigonemes, which appear to be tripartite (Fig. 4). There are two thin terminal filaments of unequal length at the distal end of each mastigoneme. These hairs are the only decorations found on either cell or flagellar surfaces (Figs 4, 5, 14). Both flagella lack acronemes.

Basal bodies are orientated at an obtuse angle to each other, which can vary essentially to an antiparallel position in different cells (Fig 17). Some, if not all, C-tubules have longitudinal septae (Figs 10, 11). Similar septae also occur in B-tubules at the flagellar transition zone (Figs 8, 9). Structures in the transition zone are not complex (Figs 6–9). There is a slightly curved transverse plate at the level of the cell surface. The central tubules of the axoneme terminate in a small depression at the centre of this plate, which is associated with a prominent axosome (Figs 6, 8).

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[1] All other rootlets and cytoskeleton in general will be discussed in a separate paper.

Figs 1–3. LM of different stages of *Pseudobodo tremulans*. **1** – attached cell (arrowheads show a cytostomal band of microtubules), ➤ **2** – swimming cell slightly pressed under the cover slip, **3** – dividing cell with 4 flagella.

Figs 4–13. Ultrastructure of *Pseudobodo tremulans*. **4** – whole mount preparation of swimming cell showing posterior smooth (p) and anterior flagellum with mastigonemes (mn), **5** – longitudinal section (LS) of cell showing a disposition of organelles, **6** – LS of flagellar transition zone with concentric rings (arrows) and basal body (b), **7–11** – TS of flagellum at different levels of transition zone **(7–9)**: **7** – upper part of transition zone with 2 central tubules and concentric rings (cr), **8** – section through axosome with one central tubule, **9** – transition zone just under transverse plate; and basal body **(10–11)**. Arrows show septae in B and C tubules. **12** – TS of R3 showing its L-shape, composition of 8 (between two bars) and 3 (between 2 another bars) microtubules; and association with fibrillar material (asterisk), **13** – structure of kinetocyst (k). Abbreviations: a-anterior flagellum, b-basal body, bp-basal body of posterior flagellum, c-collar or papilla, cr-concentric rings, fv-food vacuole, m-mitochondrion, n-nucleus, p-posterior flagellum, pn-perinuclear space containing mastigonemes, t-transverse plate, R3-cytostomal rootlet.
Scale bars: 2 (for figs 1–3) – 3.7µm, 4 – 2µm, 5 – 0.5µm, 7 (for figs 6–11) – 0.1µm, 12 – 0.1µm, 13 – 0.3µm.
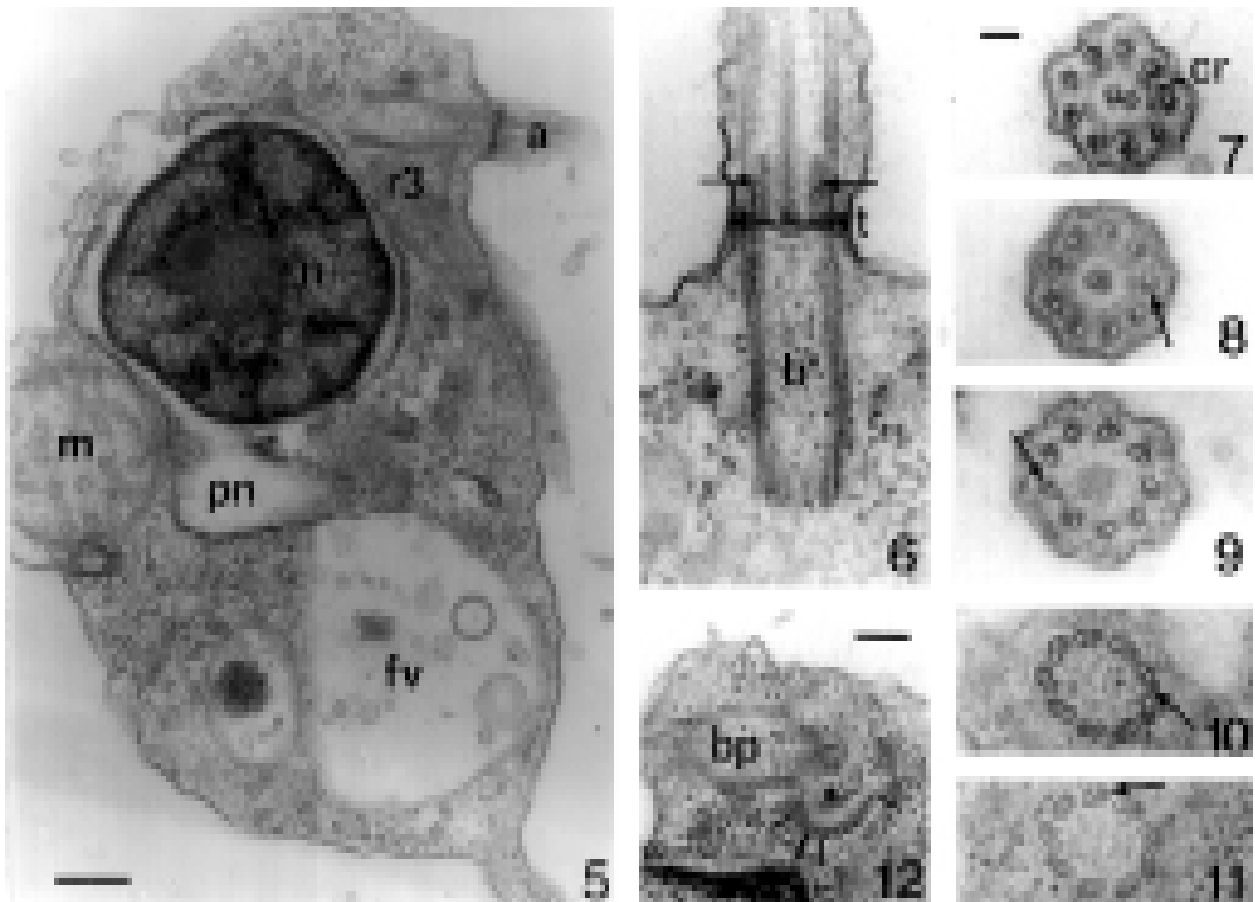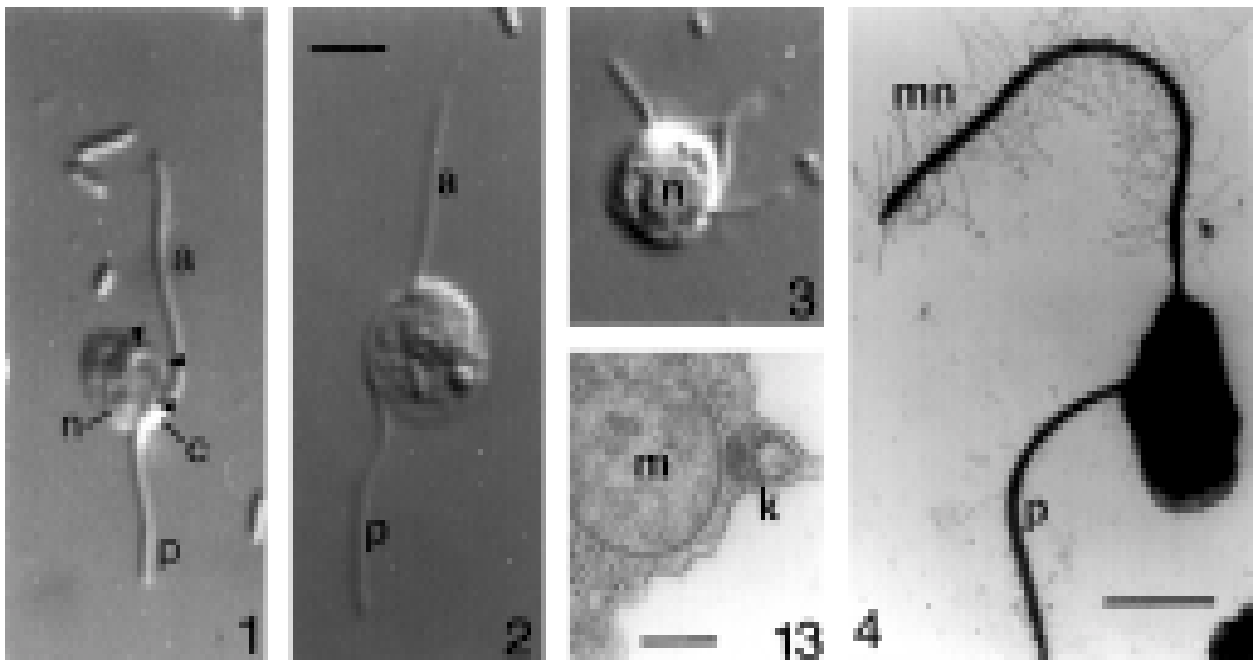
One of these central tubules seems to terminate earlier than another (Fig. 8).

There is an inconspicuous but easily identified ring-like or spiral structure above the transverse plate close to, and connected with, the peripheral doublets of the axoneme (Figs 6–8). Both flagella contain axonemes of normal 9+2 structure (Fig. 7). No paraxial elements have been found.

The microtubules surrounding the cytostome region originate from the flagellar basal bodies[1] (Fig. 5). They represent root R3, which has 11–13 microtubules. Near its proximal end, root R3 is associated with fibrillar material and is L-shaped in cross section, with its microtubules arranged in an 8+3 pattern (Fig. 12). More distally, the "abc" subunit of this root separates from the rest of the root and turns slightly left. The broadest subunit, initially containing 8 microtubules, increases in number to 10 and passes to the right side of the ventral region forming a loop that supports the cytostome (Figs 15–18). This subunit then passes left and back to make contact with the "abc" subunit (Figs 15–18).

The nucleus is always located next to the flagellar basal bodies, but no structures connecting these organelles are visible (Fig. 5). The interphase nucleus is of the vesicular type, with well-developed heterochromatin. It contains the profiles of tubular mastigonemes in the perinuclear space (Figs 5, 14), as is typical for other bicosoecids.

The mitochondria are always located close to the nucleus at the dorsal side of the cell and in association with the broad rootlet at the ventral side (Figs 5, 14–18). It has true tubular cristae.

One or two dictyosomes of the Golgi apparatus are located close to the nucleus. There are large food vacuoles in the posterior part of the cell (Figs 5, 14). Profiles of small microbodies applying to the posterior side of the nucleus were found. Several extrusomes similar to kinetocysts were observed at the cell periphery (Fig. 13).

Discussion

Ultrastructure

This study describes in detail the general ultrastructure of *Pseudobodo tremulans*. Fenchel (1982) investigated a few ultrastructural characters of *P. tremulans*, which basically parallel those described in this study. Both strains have an identical, bifurcating rootlet emanating from the anterior basal body that forms the underlying support for the area of food ingestion. Although Fenchel (1982) described unilateral mastigonemes in his strain I interpret the mastigonemes shown in figure 2 of his paper as bilateral, as are those in our strain. No other comparable observations were made, except for the generalization that the overall cell structure was reminiscent of *Bicosoeca maris* and the chrysophytes that had been investigated. These were the first clues that bicosoecids and chryso-phytes shared a common ancestor exclusive of other eukaryotic lineages, and may be part of a coherent phylogenetic assemblage (e.g., stramenopiles).

The ultrastructure of bicosoecids has been examined for several species: in the genera *Bicosoeca*: *B. planctonica* (Belcher, 1975), *B. kepneri* and *B. lacustris* (Mignot, 1974b), *B. maris* (Moestrup and Thomsen, 1976), *B. socialis* and *B. petiolata* (Mylnikov, 1995) and in several aloricate bicosoecids: *Cafeteria roenbergensis* (Fenchel and Patterson, 1988; O'Kelly and Patterson, 1996), *Acronema sippewissettensis* (Teal et al., 1998), *Caecitellus parvulus* (O'Kelly and Nerad, 1998), *Siluania monomastiga* (Karpov et al., 1998), and *Symbiomonas scintillans* (Guillou et al., 1999). The ultrastructural differences among most of them have been discussed in other papers (O'Kelly and Patterson; 1996, O'Kelly and Nerad, 1998; Karpov et al., 1998; Teal et al., 1998). We will not repeat these discussions, but for clarity, summarise the species' characteristics in a Table 1. The description of these additional taxa allows for better delineation of characters common to bicosoecids, allowing the following diagnosis (Karpov et al., 1998):

'Chrysophycean' heterotrophic flagellates without plastids, having one or two flagella, with or without a lorica; feeding apparatus represented by lip or by permanent cytostome with cytopharynx; transitional spiral fibre, if present, located above or under transverse plate; the broadest microtubular rootlet connected with cytostome region, often "L-shaped" in cross section and associated with fibrillar material; sedentary and planktonic, freshwater and marine.

Recent investigations of bicosoecids (O'Kelly and Nerad, 1998; Teal et al., 1998; Guillou et al., 1999; present paper) confirm that the presence of the main rootlet, R3, is a fundamental character possessed by members of this group (see Table 1). It always passes towards, and supports the cytostome region, which may be presented by a lip or a true cytostome with cytopharynx (Moestrup and Thomsen, 1976; O'Kelly and Patterson, 1996; O'Kelly and Nerad, 1998; Teal et al., 1998; Karpov, 2000), and represents 8+3 microtubular pattern (Table 1). In two tiny bicosoecids, *Siluania* (Karpov et al., 1998) and *Symbiomonas* (Guillou et al., 1999), the number of microtubules of R3 are reduced to 3+1 and 6+3, respectively. At its proximal part this rootlet has a characteristic L-shaped cross section and is normally associated with fibrillar material connecting it with both basal bodies. A mitochondrion with vesicular or tubular cristae is usually associated with the middle part of this rootlet.

Accordingly to this diagnosis *P. tremulans* is a typical aloricate bicosoecid. It belongs to the family Cafeteriaceae (Moestrup, 1995). Like *Cafeteria* it has no lorica and cytopharynx, but there are mastigonemes on the anterior flagellum. The general cell organisation is the same as in *Cafeteria roenbergensis* (Fenchel and Patterson, 1988;
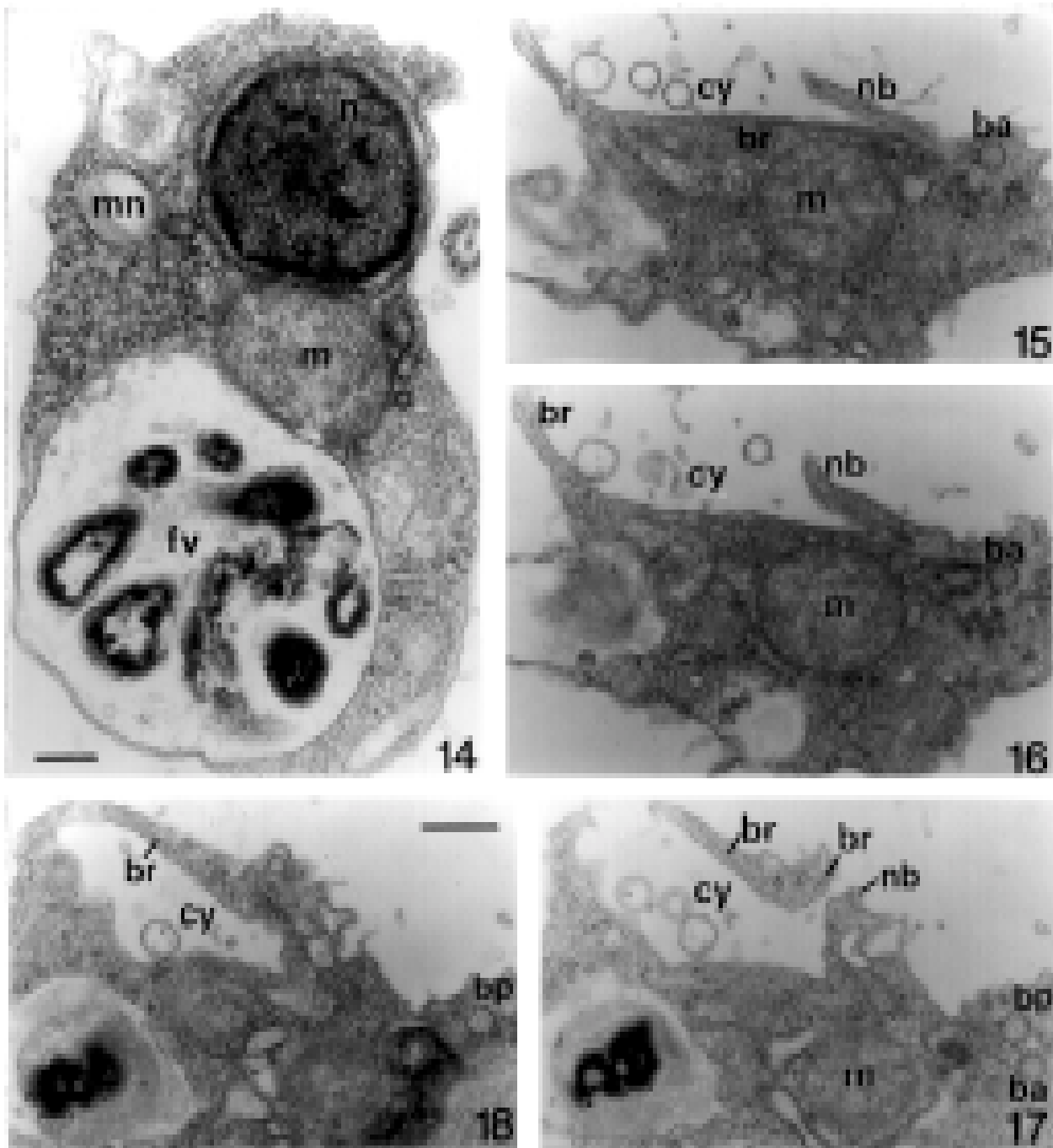
Figs 14–18. Ultrastructure of *Pseudobodo tremulans*. **14** – general cell structure, **15–18** – serial sections showing a pathway of R3 around cytostome. Abbreviations: ba-basal body of anterior flagellum, br-broad subunit of R3, cy-cytostome cavity, nb-narrow band of R3 (*abc*). Other abbreviations are the same as on figs 1–13. Scale bars: 14 – 0.5μm, 18 (for figs 15–18) – 0.5μm.

O'Kelly and Patterson, 1996), therefore it is reasonable to compare two species more closely.

P. tremulans is larger than *C. roenbergensis*, which may explain the reduction of C-tubules within the basal bodies of the latter species. Also, there is an electron-dense core inside the basal bodies of *C. roenbergensis* (O'Kelly and Patterson, 1996) that is absent in *P. tremulans*. There is a spiral fibre in the transition zone of *P. tremulans*, which

is absent in *C. roenbergensis*. Both species have extrusomes, but of slightly different structure.

In comparison to other well studied bicosoecids, *P. tremulans* has bilateral mastigonemes on the anterior flagellum, kinetocysts, and microbodies (or paranuclear body), but no cytopharynx have been observed. Additionally, rootlet R3 has a similar structure and traverses through the interior part of the cell in essentially the same way in

Table 1. The features of bicosoecid genera

character genera	lorica	body scales	cytopharynx	mastigonemes	extrusomes	microbody	paraxial rod	bb2 orientation	dense core in bb	concentric rings	R3 composition	habitat	references
Bicocoeca	+	−	−	+	+/−	+/−	+/−	left	+/−	+	8+3	m, fr	Mignot, 1974b, Belcher, 1975, Moestrup, Thomsen, 1976 Mylnikov, 1995
Siluania	−	−	+	+	−	−	−	−	−	+	3+1	fr	Karpov et al., 1998
Adriamonas	−	−	+	−	+	−	−	left	−	+	8+3	so	Verhagen et al., 1994
Caecitellus	−	−	+	−	−	−	−	left	+	−	8+3	m	O'Kelly, Nerad, 1998
Cafeteria	−	−	−	+	+	+	−	left	+	−	8+3	m	Fenchel, Patterson, 1988, O'Kelly, Patterson, 1996
Pseudobodo	−	−	−	+	+	+	−	left	−	+	8+3	m, br	present paper
Acronema	−	−	−	+	−	+	−	left ?	+	?	8+3	m	Teal et al., 1998
Symbiomonas	−	−	−	+	−	−	−	−	−	−	6+3	m	Guillou et al., 1999
Discoselis	−	−	−	−	+	+	−	?	?	?	?	m	Vørs, 1988
Pseudodendromonas	−	+	+	sc	+	−	−	?	−	−	6+1	fr	Mignot, 1974a, Hibberd, 1976, 1985
Cyathobodo	+/−	+	+	sc	−	−	−	left	−	?	6 (7)	fr	Hibberd, 1976, 1985, Strüder-Kypke, Hausmann, 1998

Abbreviations: br – brackish water, fr – freshwater, m – marine, sc – scales, so – soil, "+" – present, "–" – absent, "+/–" – present in not all species of the genus, "?" – data unknown.

C. roenbergensis, *C. parvulus*, and *P. tremulans* (O'Kelly and Patterson, 1996, O'Kelly and Nerad, 1998, this study). The *abc* subunit of R3 is short, and the broad component of R3 is rather long, making a loop around cytostome region and passes back to position adjacent with the distal end of the *abc* subunit. *C. parvulus* has a cytopharynx, but *C. roenbergensis* and *P. tremulans* have not. This indicates that rootlet R3 is very conservative part of the bicosoecid cytoskeleton contrary to the physical presence/absence of a cytopharynx. The reduced number of microtubules in R3 of *Siluania* and *Symbiomonas* may be related to the small dimensions of their cells.

The internal structure of *P. tremulans*' flagellar transition zone is similar to that in most other bicosoecids (Table 1). It differs from the transition helix of chrysophytes, which is thicker and has no connection with the A-tubules of the axoneme. On transverse sections through the transition zone of *P. tremulans*, connections to A-tubules are visible (Fig. 7). This structure is thus more similar to concentric rings or coiled fibres, which occurs in unrelated groups of protists (Andersen et al., 1991, Karpov and Fokin, 1995).

There are septae in B-and C-tubules of the axoneme and basal body. This is the first report of these structures in bicosoecids, but they are well known in the axonemes of some trypanosomatids (Vickerman et al., 1991) and in transition zones and basal bodies of some green flagellates (Moestrup, 1982).

Taxonomy

Pseudodendromonads

After the description of 2 bicosoecids possessing a cytopharynx (*Caecitellus* and *Siluania*), one of which lacks the mastigonemes (*Caecitellus*), it became possible to include the pseudodendromonads within the order Bicosoecida (=Bicosoecales) (Karpov et al., 1998; Karpov, 2000). Surprisingly, the rootlet system of the pseudodendromonad *Adriamonas* (Verhagen et al., 1994) is almost identical to that of bicosoecids (Karpov, 2000). Other pseudodendromonads have been less studied, but they also reveal the same main elements of the rootlet system, including an association of mitochondria to the broad rootlet (Mignot, 1974a; Hibberd, 1976, 1985; Strüder-Kypke and Hausmann, 1998). The general organisation of the cell is the same in all studied representatives of bicosoecids and pseudodendromonads. The broad cytostomal rootlet of pseudodendromonads appears to be homologous to R3 of bicosoecids. It originates from both basal bodies, has an L-shaped cross section at the proximal end with an association to fibrillar material, and splits

into 2 branches (broad and narrow) at the distal end; corresponding to the formula 8+3 in *Adriamonas* (Verhagen et al., 1994) or 6+1 in *Cyathobodo* (Strüder-Kypke and Hausmann, 1998). The number of microtubules in the typical pseudodendromonads (*Cyathobodo* and *Pseudodendromonas*) is less (6+1) than in the majority of bicosoecids, but similar to that of *Symbiomonas* (6+3). The absolute orientation of basal bodies in pseudodendromonads is also the same as in bicosoecids (Table 1), and the association of a mitochondrion having tubular cristae with the cytostomal rootlet is likewise a typical character of pseudodendromonads (Mignot, 1974a; Hibberd, 1976, 1985).

Following the description of bicosoecids with a permanent cytopharynx, and the expansion of the circumscription of this order (Karpov et al., 1998), I proposed to unite the pseudodendromonads with bicosoecids in a single order Bicosoecida (Karpov, 2000).

Discocelis Vørs, 1988.

An additional organism may be discussed as a possible member of the bicosoecids. The marine colourless heterokont flagellate *Discocelis saleuta* has been found in interstitial fauna and investigated by N.Vørs (1988). It possesses short anterior and longer posterior flagella, both without mastigonemes. Neither cytopharynx nor lorica has been found in this species. It has a velum or lip at the opposite side of flagellar insertion, which is supported by lateral microtubular rootlets. Unfortunately, the latter have not been investigated in details, and we don't know their composition or the orientation of the basal bodies. Nevertheless, a broad rootlet directed to the lip region has been noted in the description (Vørs, 1988). The general cell morphology reveals mitochondria with tubular cristae, microbody-like organelles, extrusomes; all of which represent the bicosoecid characteristics. The absence of mastigonemes and lorica may be explained by this organism living between sand particles – a very restrictive interstitial habitat. One essential difference between *Discocelis saleuta* and other bicosoecids is the presence of a paracristalline layer under the plasmalemma. This layer may be considered an additional structure supporting the smooth and naked coverings of the cell. Additional investigation of this species is required, but here I presumably place this species among bicosoecids.

Classification of the order Bicosoecida (Grassé) Karpov, 1998

There are 3 discrete characters that vary in bicosoecids: presence/absence of mastigonemes, cytopharynx, and covering structures (i.e., lorica and/or body scales). Therefore the main characters distinguishing species at the family level are: the presence/absence of lorica, body scales and cytopharynx. The flagellar mastigonemes seems to be a character that is pleomorphic in various species of bicosoecids and therefore may not be appropriate for taxonomy at the family level.

All species within the genus *Bicosoeca* possess a lorica. Two genera (*Cyathobodo* and *Pseudodendromonas*) have siliceous scales on the body surface. One species of *Cyathobodo* has thin covering, similar to lorica (Strüder-Kypke and Hausmann, 1998). Therefore, a true lorica may be present among some pseudodendromonads. But, *Bicosoeca* have no scales on the cell surface, and lack a cytopharynx. Thus, these two groups are clearly distinguished from each other and from other bicosoecids, and can be designated into 2 families: Bicosoecidae and Pseudodendromonadidae.

Other bicosoecids are naked and can be divided on the basis of the presence/absence of a cytopharynx. The cytopharynx is a permanent structure supported by special fibrillar sheet. On the other hand, the cytostome or lip designates a cytostome 'region', which may or may not have permanent structures and does not allow for distinguishing groups based on this feature. So, the naked bicosoecids with cytopharynx form a 3^d family, and those without cytopharynx are grouped into a fourth.

On this basis, a new composition for the order Bicosoecida (=Bicosoecales), with representatives of the order Pseudodendromonadida Hibberd, 1985, forming a family within it has been proposed (Karpov, 2000). Here the genus *Symbiomonas* is added to the family Cafeteriidae.

According to the IZCN the **order Bicosoecida** (Grassé) Karpov, 1998 includes 4 families:

Bicosoecidae Stein, 1878: lorica present, cytopharynx and body scales absent.
Bicosoeca Stein, 1878.
Siluaniidae Karpov, 1998: cytopharynx present, lorica absent.
Siluania Karpov, 1998,
Adriamonas Verhagen et al., 1994,
Caecitellus Patterson et al., 1993.
Cafeteriidae Moestrup, 1995: lorica and cytopharynx absent.
Cafeteria Fenchel & Patterson, 1988,
Pseudobodo Griessmann, 1913,
Symbiomonas Guillou et Chrétiennot-Dinet, 1999,
Acronema Teal *et al*., 1998,
Discocelis Vørs, 1988.
Pseudodendromonadidae Karpov, 2000: lorica absent, body scales and cytopharynx present.
Pseudodendromonas Bourrelly, 1953,
Cyathobodo Petersen et Hansen, 1961.

Molecular phylogeny

To date, the 2 most complete molecular analyses in regard to bicosoecids include only three representative species: *Cafeteria roenbergensis, Siluania monomastiga* and *Symbiomonas scintillans* (Guillou et al., 1999). Their broad scale analysis shows the bicosoecids as an early di-

verging stramenopile lineage and *Symbiomonas* more closely related to *Cafeteria* than to *Siluania* within this group. Phylogenetic analysis of a data set restricted to the heterotrophic stramenopiles places *Symbiomonas* as a sister taxon to a clade composed of *Cafeteria/Siluania*. Both molecular trees show clear separation of *Symbiomonas* from 2 other genera, but one cannot make any definitive conclusions regarding bicosoecid classification from these restricted data used in molecular analyses. Further insights into the relationship among the bicosoecids await molecular phylogenetic analyses that include representatives of all 4 proposed families.

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