Protistology 2 (1), 34–47 (2001)
April, 2001

Protistology

Rootlet homology, taxonomy, and phylogeny of bicosoecids based on 18S rRNA gene sequences

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Summary

The cytoskeleton of *Pseudobodo tremulans* was investigated by transmission electron microscopy to resolve homologies among flagellar rootlets within the bicosoecids. The rootlet arrangement in *P. tremulans* is typical for bicosoecids. The basal body of anterior flagellum (bb2) has 2 rootlets: a dorsal rootlet of 2 microtubules and broad ventral rootlet (8+3 microtubules); the basal body of posterior flagellum (bb1) has two rootlets of 1 and of 2 microtubules respectively. There is a fibrillar rootlet that descends to the nucleus, and a set of fibrillar connectives around and between the basal bodies. The ventral rootlet is shown to originate from basal body 2, revealing that it is a homologue of rootlet 2 (not rootlet 3 as was accepted earlier for bicosoecids) of heteroconts. The fibrillar rootlets are not necessarily homologous in this order, as they may originate from either or both basal bodies. In general, all the flagellar microtubular rootlets of bicosoecids are homologues of those described from other stramenopiles. The bicosoecid rootlet 2 is much more developed than in colourless chrysophyceans. Rootlet 3 is often absent in bicosoecids while it is prominent in chrysophyceans as the ventral rootlet associated with feeding. Nuclear small subunit rRNA gene sequence (ssu rDNA) for 6 species of bicosoecids belonging to 2 families (Cafeteriidae and Siluaniidae) are partly congruent with the morphological character-state data. In agreement with morphological data, the molecular trees robustly support a clade that includes *Cafeteria* and *Symbiomonas* (Cafeteriidae) and a clade composed of *Adriamona*s plus *Siluania* (Siluaniidae). The ssu rDNA trees also depict a paraphyletic Cafeteriidae (*Pseudobodo* and *Cafeteria* are not sister taxa) and a paraphyletic Siluaniidae (*Adriamonas* and *Caecitellus* are not sister taxa). The further investigation of the representatives of 2 other families (Pseudodendromonadidae and Bicosoecidae) by electron microscopy and molecular methods is required to clarify the phylogeny of bicosoecids.

Key words: *Pseudobodo tremulans*, heterotrophic flagellate, ultrastructure, ssu rRNA, phylogeny, evolution, stramenopiles, heterokonts, bicosoecids

Introduction

Bicosoecids are composed of a small number of species of heterotrophic flagellates which live in marine and freshwater habitats. During the last 10 years several new bicosoecid genera and species have been established and re-described (Fenchel and Patterson, 1988; Patterson et al., 1993; Teal et al., 1998; Karpov et al., 1998; O'Kelly and Nerad, 1998; Guillou et al., 1999). Two revisions of this order have been made during this period (Moestrup, 1995; Karpov, 2000b), and the last classification delineated 4 families and 10 genera with approximately 60 species.

Ultrastructural and molecular studies established the affinity of this group within the stramenopiles (heterokonts) and that it is one of its earliest diverging groups (Leipe et al., 1994, 1996; Silberman et al., 1996). The molecular phylogenies are consistent with the hypothesis that chloroplast acquisition is a derived character within this lineage. Understanding the early events of evolution within the stramenopiles may help elucidate the processes that have made this entire evolutionary lineage so successful, pro-

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ducing ecologically important and speciose groups such as diatoms and brown algae, as well as a wide range of adaptive forms inclusive of taxa with fungal morphology (oomycetes), ciliate morphology (opalines) and heliozoa (actinophryids).

Recent studies have expanded the diversity of organisms encompassed within the order Bicosoecida (Karpov et al., 1998; Karpov, 2000b), and this assemblage has become a significant ecological component of the marine heterotrophic flagellate community. The bicosoecids are heterotrophic stramenopiles without plastids, having one or two flagella, with or without a lorica. The underlying fine structure of the flagellar apparatus unites these organisms into a coherent group. The cytostomal rootlet has a characteristic L-shaped cross section and represents 8+3 microtubular pattern. It always passes towards, and supports the cytostome region, which may be presented by a lip or a true cytostome with cytopharynx. A mitochondrion with vesicular or tubular cristae is usually associated with the middle part of this rootlet. A transitional spiral fibre, if present, is located either above or under the transverse plate. Bicosoecids may be sedentary or planktonic, freshwater and marine. We regard the bi-flagellated taxa with tubular hairs on the anterior flagellum as the ancestral form.

However, the plasticity of morphological characters makes it difficult to confidentially establish the validity of particular characters or even the specific relationships among the described bicosoecids. This study aims to clarify flagellar rootlet homologies and phylogenetic relationships among the bicosoecids.

The general ultrastructure of a typical aloricate marine bicosoecid, *Pseudobodo tremulans*, has been recently described (Karpov, 2000b). Here we present data on the flagellar rootlet composition of *P. tremulans* in comparison with rootlets of other bicosoecids and chrysophyceans, and detailed phylogenetic analyses based on ssu rDNA sequence comparison.

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 a brackish water sample (salinity 1.0–1.2%) collected in a littoral region of the White Sea near the Marine Biological Station of Zoological Institute, Russian Academy of Sciences (Kartesh), in August, 1986. Cultures were grown in artificial seawater and periodically supplied with suspensions of a single bacterial strain of *Klebsiella aerogenes.*

Electron microscopy

For sections, 1 ml of cells was mixed with 1 ml of a solution containing 4% glutaraldehyde, 0.1M cacodylate buffer and 0.48M sucrose. After fixation for 2 hours 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 hour 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.

For 3-dimensional reconstruction of the cytoskeleton, serial sections of 6 cells were examined. To exclude potential mistakes with rootlet interpretation, we did not analyse micrographs of predivisional stages, when additional basal bodies appear (e.g., just prior to mitosis, additional rootlets appear in conjunction with the replication of basal bodies).

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

DNA isolation, cloning, and sequencing

Genomic DNA was isolated from a cell pellet of *P. tremulans* with the PureGene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. The ssu rRNA gene was *in vitro* amplified using eukaryotic specific primers (Medlin et al., 1988). The single amplicon was gel purified (Prep-a-Gene, BioRad Laboratories, Hercules, CA) and cloned into the pCR2.1-TOPO T/A vector (Invitrogen Inc., Carlsbad, CA). To assess possible microheterogeneity in rRNA genes and to compensate for possible Taq polymerase errors during PCR amplification, plasmid DNA from 10 independent recombinant clones, isolated by a modified rapid boiling protocol (Holmes and Quigley, 1981), were pooled prior to DNA sequencing on a LICOR 4200 apparatus. Sequence data for the entire *P. tremulans* ssu rDNA was obtained from both strands and has been deposited in GenBank (accession No. AF315604).

Comparative molecular analysis

Two separate ssu rDNA data sets were analysed to investigate the phylogenetic affinity of *P. tremulans* to heterokont protists, and the bicosoecids in particular. DNA sequences were manually aligned using conserved primary and secondary structures. Only unambiguously aligned positions were considered in phylogenetic analyses. A 32 taxon data set including autotrophic and heterotrophic stramenopiles, and alveolate ssu rRNA gene sequences as an outgroup allowed for 1537 positions to be used in phylogenetic analyses. The choice of outgroup was based on previous ssu rDNA analyses suggesting a close relationship between stramenopiles and alveolates (Van de Peer and De Wachter, 1997). Fine scale assessment of relationships among the bicosoecids was conducted on a 10 taxon

data set that included all available bicosoecid sequences (6 taxa) and 2 closely related stramenopile lineages (4 taxa); thus increasing the number of aligned positions to 1593. Nested likelihood ratio tests (ModelTest v.3.0, Posada and Crandall, 1998) determined that a general-time-reversible (GTR) model of nucleotide substitution incorporating an estimation of the fraction of invariable sites (I) and among-site rate variation (G) best described the data. Chi-square tests determined that the overall nucleotide frequencies were homogeneous for all data sets except when constant sites were removed from the broad-scale data set. Pair-wise base frequency comparison showed that of 32 taxa analysed, none of the bicosoecid sequences deviated significantly from the mean base frequency, but 3 other taxa did (*Proteromonas*, *Alexandrium*, and *Cyptosporidium*).

Molecular phylogenies were reconstructed with likelihood, distance, and parsimony methods. For distance and maximum likelihood methods, a GTR+G+I model was used to reconstruct phylogenetic trees. Both minimum evolution and least-square optimality criteria were used to calculate distance matrices. Negative branch lengths were handled differently for each objective function: branch lengths were constrained to be non-zero for least-squares distance calculations or were set to zero in minimum evolution calculations. Potential base-frequency affects were ameliorated by log determinant transformation of evolutionary distances calculated with an estimation of invariant sites; these trees were similar to the topology obtained in parsimony analyses. Heuristic searches were conducted on the 32-taxon data set while exhaustive searches were conducted on the smaller data set. Heuristic parsimony analyses were conducted with 100 random stepwise additions. Branching order and stability was assessed by analyses of 100 or more bootstrapped data sets (Felsenstein,1985) calculated heuristically on the 32-taxon data set and via branch and bound on the smaller data set. Kishino-Hasagawa tests (1989) were performed to determine if the likelihood of alternative tree topologies were significance different. All phylogenetic analyses were performed with the program PAUP* version 4.01b (Swofford, 1998).

Phylogenetic signal, taxon-variance ratios, and identification of taxa that could be a source of problems (i.e., possibly contributing to long-branch attraction artefact) in phylogenetic analyses were assessed using RASA version 2.4 (Lyons-Weiler et al., 1996; Lyons-Weiler and Hoelzer, 1999).

Results

Pseudobodo tremulans has two unequal flagella emerging from a small apical papilla, and a pear- or egg-shaped body with a broad posterior end. The overall body shape is approximately triangular (Fig. 1). The posterior flagellum emerges on the left side of cell (Fig. 1) and bends around the cell to attach to the substratum. Swimming cells are also observed, especially during exponential growth of the culture. Swimming cells move rather quickly in a straight line with the anterior flagellum directed forward and the posterior flagellum applied to the cell surface. There is a cytostomal region opposite to the flagellar insertion (Fig. 1), which is supported by a prominent band of microtubules.

Cytoskeleton of Pseudobodo tremulans

The microtubules surrounding the cytostome region originate from the basal body of anterior (hairy) flagellum (bb2 in accordance with accepted nomenclature – Andersen et al., 1991) (Figs 2, 3, 6–9). They form a rootlet consisting at its origin of 11 microtubules arranged in an 8+3 pattern (Figs 6–9). All of these microtubules comprise the ventral rootlet (r3 – according to O'Kelly and Patterson, 1996), which is L-shaped in cross-section (Figs 7–9), and connected to fibrillar material (Figs 2–3, 7). It splits rather early into two unequal parts. The narrow branch consists of 3 microtubules ("abc" – according to Moestrup and Thomsen, 1976; O'Kelly and Patterson, 1996) that turns slightly left. The broader branch of 8 microtubules increases in number up to 10 as it passes to the right side forming a loop supporting the cytostomal region. It then passes left and back to the vicinity of the 3-microtubular branch termini. Thus, the broad branch of r3 forms a ridge of the feeding "basket", which is very mobile and delineates the main part of the feeding apparatus of the cell. One more microtubule ("x"-fibre) joins to the outer side of this broad rootlet near its proximal end (compare Figs 7–8 with Fig. 9).

Another microtubular rootlet (r1), consisting of 2 microtubules, originates from the dorsal side of bb2 (Figs 10–12). It passes forward and slightly to the right, penetrating the papilla. The distal part of r1 initiates 2–3 sets of secondary microtubules which pass dorsally back towards the posterior end of the cell (Figs 10–13).

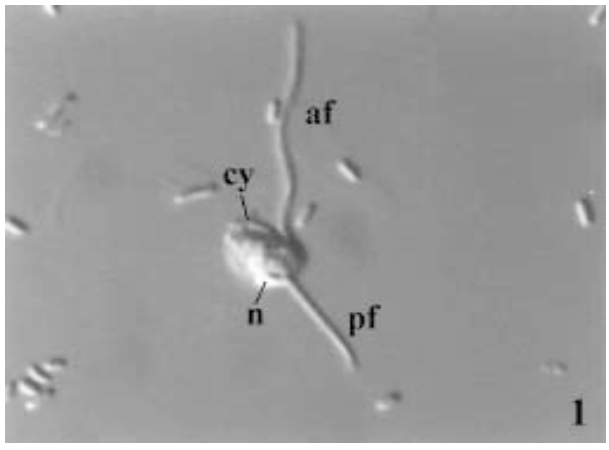
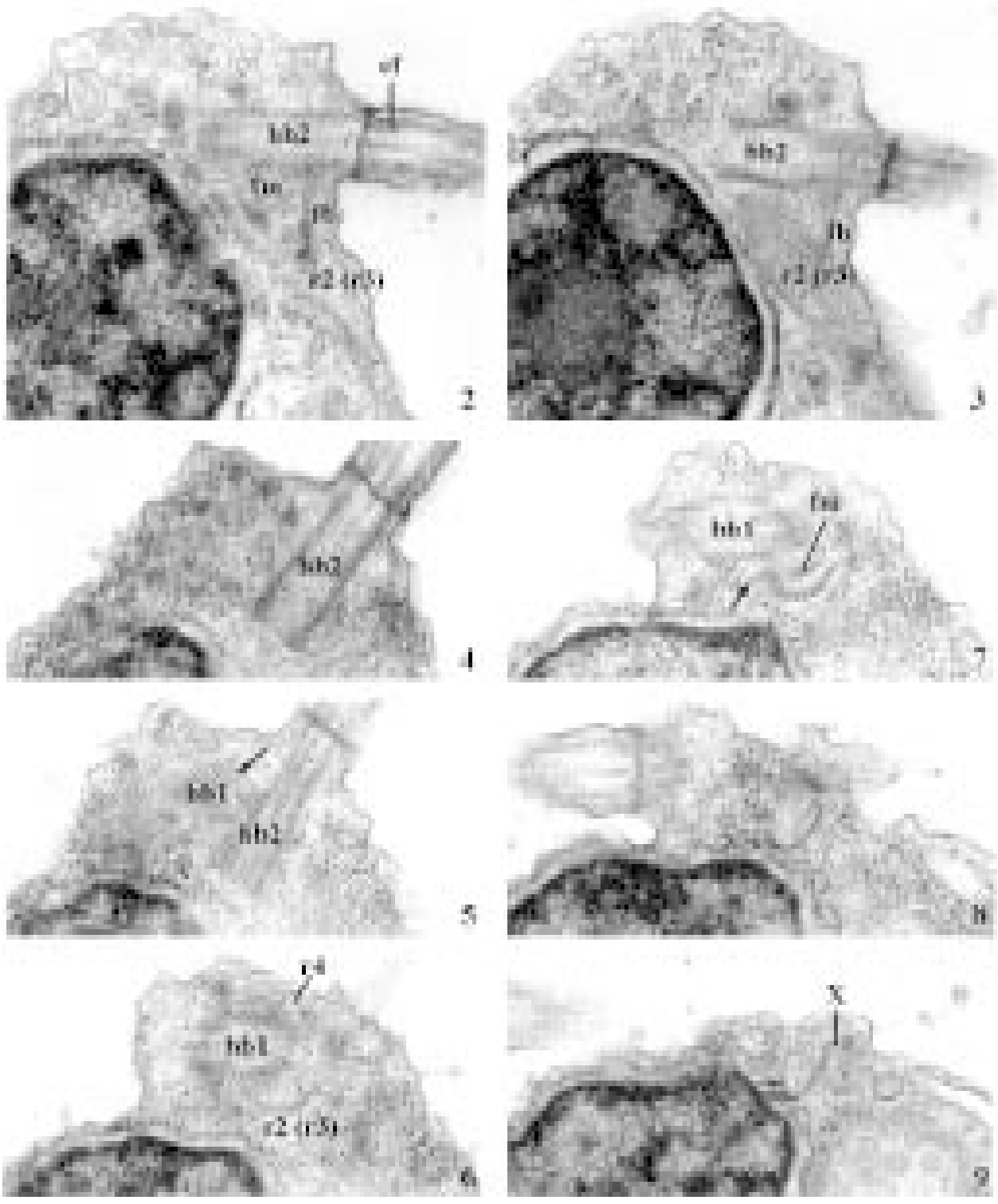
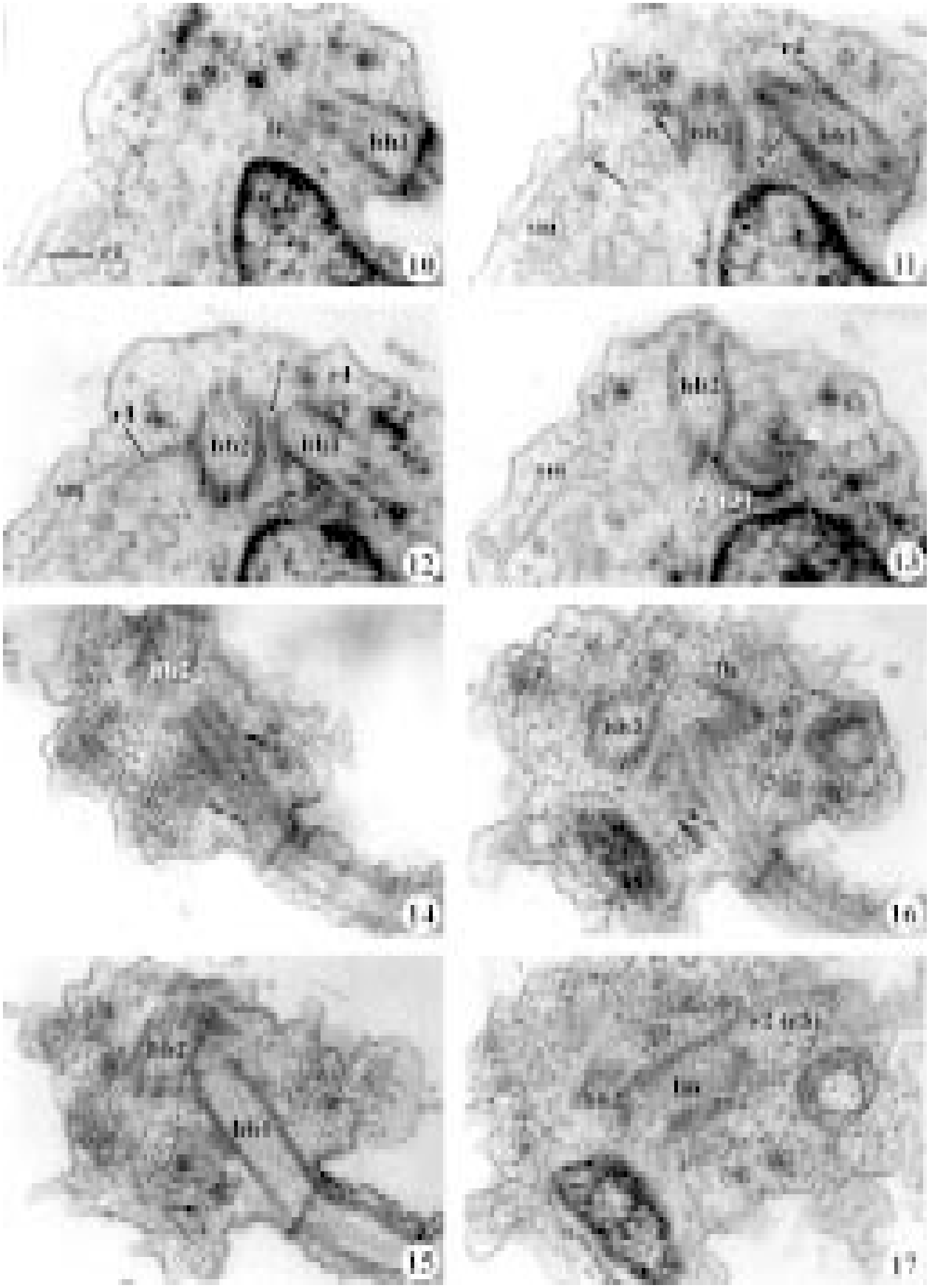


Fig. 1. LM view of *Pseudobodo tremulans.* Abbreviations: af – anterior flagellum, cy – cytostome, n – nucleus, pf – posterior flagellum. Magnification: 2,200x.



Figs. 2–9. Cytoskeleton structure of *Pseudobodo tremulans*.**2–3** – two consecutive sections through bb2 and cytostomal rootlet – r2 (former r3); **4–9** – selected serial sections, showing the origin and structure of cytostomal rootlet (arrows on fig. 5 shows r4, on fig. 7 – s-tubule; three bars on figs 8–9 indicate "abc" microtubules of r2 – former r3). Abbreviations: bb1 – basal body of posterior flagellum, bb2 – basal body of anterior flagellum, cf – coiled fiber, fb – fibrillar bridge between bb2 and cytostomal rootlet, fm – fibrillar material associated with cytostomal rootlet, fr – fibrillar rootlet, r1–r4 – microtubular rootlets, s – s-tubule, sm – secondary microtubules, other abbreviations as in fig. 1. Magnification: 50,000x for all figures.



Two microtubular rootlets originate from bb1. One (r4) consists of 2 microtubules and passes to the left (Figs 11, 12, 14). Another rootlet consists just of 1 microtubule that passes towards the nucleus (Figs 11, 12, 16).

There is one more microtubule associated with the basal bodies (Figs 7–9, 12, 15) which we interpret to be the S-tubule. It passes parallel to r3 on its inner side, traversing between the two basal bodies (Fig. 12).

There are short fibrillar structures connecting bb1 with bb2 and also connecting both basal bodies to the ventral rootlet (Figs 2, 3, 7, 16). We do not interpret these fibrillar connectives to be independent rootlets. A short, broad, inconspicuous fibrillar rootlet passes from the middle of bb1 to the nucleus (Figs 10–12). The general scheme of the microtubular rootlet system is presented in figure 18 c.

The two basal bodies are composed of microtubule triplets and normally lie at an oblique angle to each other in different planes, but their arrangement may vary to a nearly anti-parallel position in cells preparing for division. Two additional basal bodies appear adjacent to the old ones prior to mitosis.

Molecular data

The 1807 base pair ssu rRNA gene of *P. tremulans* is characteristic of most eukaryotes, with a G+C content of 45.4% (average G+C content for the entire data set = 45.3%). The test statistic tRASA (Lyons-Weiler et al., 1996; Lyons-Weiler and Hoelzer, 1999) demonstrated that both the 32- and 10-taxon data sets possess a hierarchical character state pattern characteristic of true phylogenetic signal, although *Proteromonas* and *Symbiomonas* had high, though non-significant taxon-variance ratios (data not shown). The latter two species contributed to branch length heterogeneity in phylogenetic trees. The *Proteromonas* branch did not affect the resolution within or among the bicosoecid lineage, but the *Symbiomonas* branch did (to be discussed below).

Ssu rRNA gene sequences from a wide representation of stramenopile lineages, inclusive of all the available bicosoecid sequences, were analysed to assess the phylogenetic relationship of *P. tremulans* within this assemblage. After an initial phylogenetic analysis with a broad array of eukaryotic taxa demonstrated robust support for the monophyly of the major lineages (e.g., animals + fungi, stramenopiles, alveolates, haptophytes, chlorophytes, rhodophytes, cryptophytes, etc., data not shown), six alveolate ssu rDNA sequences were chosen to root the stramenopiles. Maximum likelihood, distance (minimum evolution and least-squares optimality criteria), and parsimony methods all show that *P. tremulans* branches within a strongly supported monophyletic stramenopile clade as the most basal bicosoecid (Figs 19, 20). The earliest diverging stramenopiles were all heterotrophic organisms, while the more derived stramenopiles were predominantly photosynthetic. The opalinids diverged first followed a polytomy composed of the labyrinthulids, *Blastocystis* plus *Proteromonas*, and the bicosoecids. As has been seen in other analyses (Leipe et al., 1996) the Oomycetes, *Hyphochytrium*, and *Developayella* form a clade that is sister to a clade composed of predominantly photosynthetic stramenopiles.

All methods of phylogenetic reconstruction robustly support the monophyly of bicosoecids (Figs 19, 20). In our data sets, *Pseudobodo* was the most basal member of this lineage. A sister relationship between *Siluania* and *Adriamonas* was also strongly supported with high bootstrap values. The relationship among *Caecitellus*, *Symbiomonas*, and *Cafeteria* was not very well resolved in the broad-scale phylogenetic analyses (Fig. 19). *Symbiomonas* branched alternatively with either *Caecitellus* or adjacent to the *Siluania* plus *Adriamonas* clade in distance and maximum likelihood analyses, respectively. When *Symbiomonas* was removed from analyses, *Adriamonas* plus *Siluania* and *Caecitellus* plus *Cafeteria* form separate, well supported clades while preserving the basal position of *Pseudobodo* within this lineage (data not shown).

A smaller data set (Fig. 20) composed of all the available bicosoecid ssu rDNA sequences (6) with *Hyphochytrium*, *Developayella*, and two labyrinthulids serving as outgroup taxa allowed the unambiguous alignment of 1593 characters and a more detailed examination of the phylogenetic relationship among bicosoecid species. Although RASA analyses once again showed that *Symbiomonas* (and a labyrinthulid *Ulkenia profunda*) contributed to branch-length heterogeneity (though the taxon variance ratio was not statistically significant), their long-branches did not attract one another artificially. Thus long-branch attraction artefact did not seem to be problematic in this data set.

All phylogenetic methods recovered the same optimal tree topology with each node supported by high bootstrap values (Fig. 20). Limiting the number of diverse taxa while increasing the number of characters analysed alleviated the instability in branching pattern seen in the broad-scale data set. Consistent with the above analyses, *P. tremulans* diverged earliest amongst the bicosoecids analysed, followed by a clade composed of *Siluania* plus *Adriamonas*, and a clade composed of *Cafeteria*, *Symbiomonas*, and *Caecitellus*. These analyses robustly demonstrate a sister relationship between *Symbiomonas* and *Caecitellus*.

Figs. 10–17. Cytoskeleton structure of *Pseudobodo tremulans*.**10–13** – series of consecutive sections through basal bodies (arrows on fig. 11 show r1, on fig. 12 – r3; **14–17** – series of consecutive sections through basal bodies of another cell (arrows on fig. 14 show r4, on figs 15–16 – r3). Abbreviations as in figs 1–9. Magnification: 50,000x for all figures.

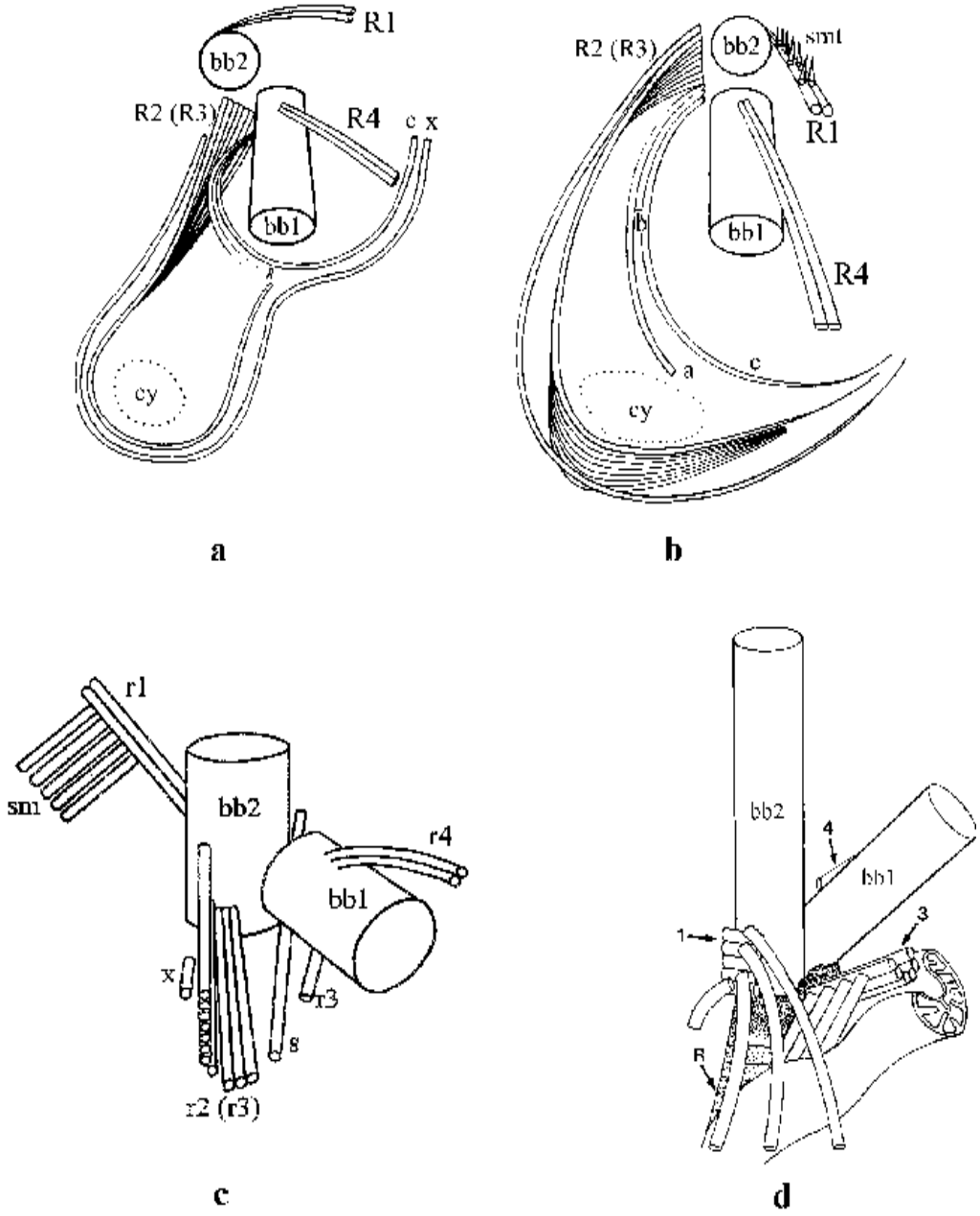


Fig. 18. General schemes of various bicosoecid microtubule cytoskeletons: *Caecitellus parvulus* (**a**), *Cafeteria roenbergensis* (**b**), *Pseudobodo tremulans* (**c**), with a comparison to the chrysophyceae *Epipyxis pulchra* (**d**) showing rootlet homologies. Abbreviations: a,b,c – "abc" microtubules of r2, bb1, bb2 – basal bodies 1 and 2 respectively, cy – cytostome region, located ventrally, r – fibrillar rootlet, r1, r2, r3, r4, 1, 3, 4 – rootlet numbers accordingly to Andersen et al., 1991, s – s-tubule, smt – secondary microtubules, x – x-tubule (**a**– after: O'Kelly and Nerad, 1998, **b**– after: O'Kelly and Patterson, 1996, **c** – present paper, **d** – after: Andersen and Wetherbee, 1992).

Discussion

The features of bicosoecid flagellar apparatus are presented in Table 1. This broad list reflects the increasing number of described genera belonging to the order Bicosoecida sensu Karpov (2000a,b), plus *Wobblia lunata* (Moriya et al., 2000), which shares some characters with bicosoecids. Before discussing specific characters traits, taxonomic considerations of two organisms need to be addressed.

Taxonomic considerations

Acronema sippewissettensis and *Cafeteria roenbergensis* are almost identical at the ultrastructure level (Table 1), differing only in features that have not been extensively studied in *Acronema*. The main character that distinguishes *Acronema* from *Cafeteria*, as proposed by Teal et al. (1998), is the presence of acronematic flagellum in *Acronema*. This feature (presence/absence of acronema) is rather variable among flagellates (Moestrup, 1982; Melkonian, 1984; Zhukov and Karpov, 1985), and

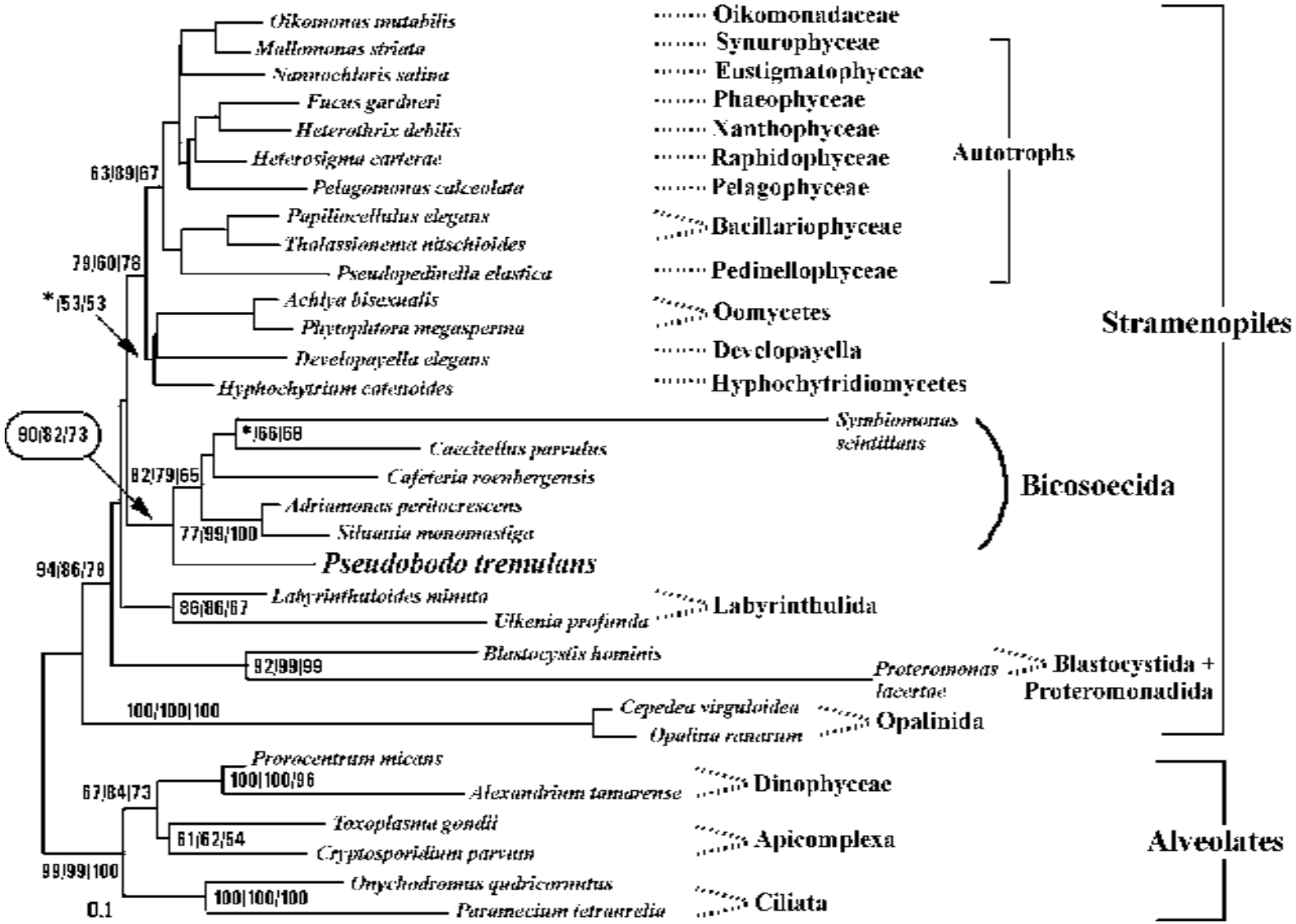


Fig. 19. Molecular tree based on SSU rRNA gene sequences. A maximum likelihood distance tree based on 1537 aligned positions (GTR + G +I model, a=0.51, I=0.299) is shown with bootstrap values over 50 for maximum likelihood, distance, and parsimony, respectively, indicated above the nodes. An asterisk (*) denotes bootstrap values less than 50. The stramenopile lineage is rooted with alveolate ssu sequences. The scale bar represents evolutionary distance for the number of changes per site.

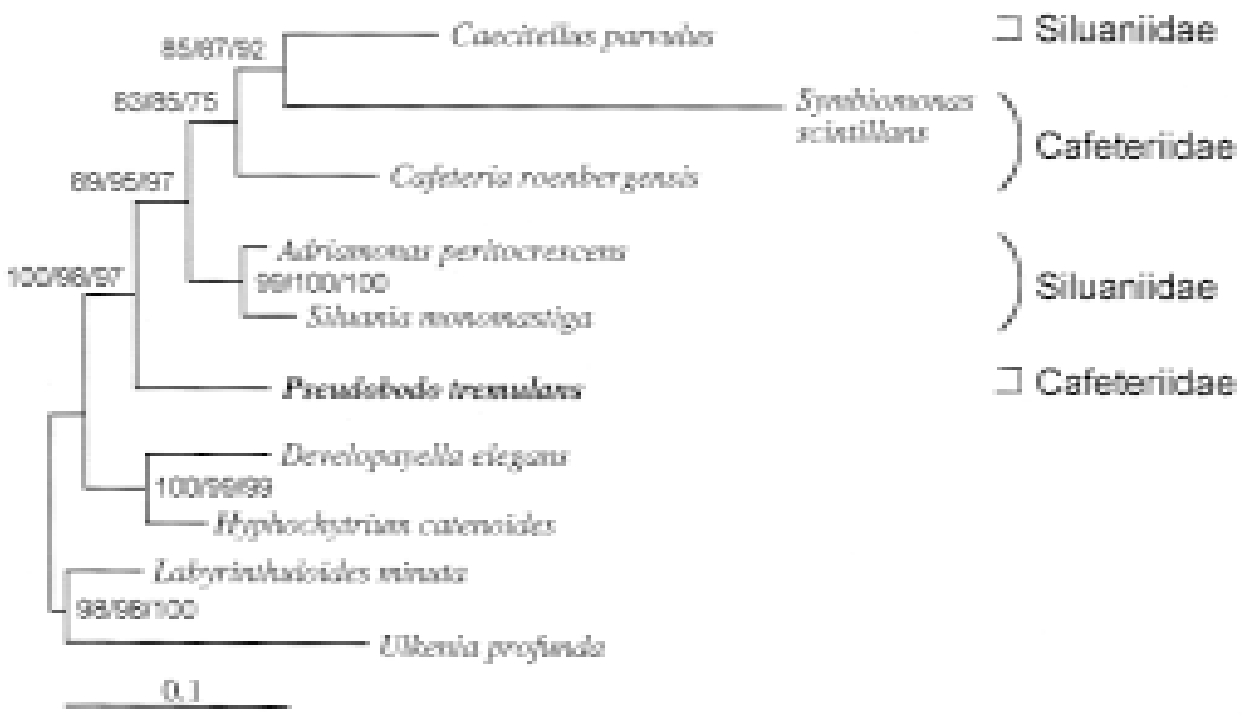


Fig. 20. Fine scale molecular phylogeny of bicosoecids using SSU rRNA gene sequences. Shown is a maximum likelihood tree (GTR +G + I model, a=0.565, I=0.325) generated from analysing 1593 aligned nucleotide positions. Bootstrap values for maximum likelihood, distance, and parsimony, respectively, are shown above the nodes. The bicosoecids were rooted with representatives of two independent heterotrophic stramenopile groups; bicosoecid families are indicated. The scale bar represents evolutionary distance for the number of changes per site.

thus not a valid character to distinguish among genera or even species. On the bases of light microscopic observations (D.J. Patterson, pers. comm.) and ultrastructural evidence we consider the name *Acronema sippewissettensis* to be a junior synonym of *Cafeteria roenbergensis.*

In addition, *Wobblia lunata* (Moriya et al., 2000) and *Pendulomonas adriperis* Tong, 1997 (Tong, 1997a,b; Lee and Patterson, 2000) have the same morphology and mode of movement. Both of these characters are distinctive for this organism, and cannot be confused with any other flagellates. Thus, *Wobblia lunata* is probably a junior synonym of *Pendulomonas adriperis*.

Cytoskeletal structure of bicosoecids

The ultrastructure of bicosoecids (sensu Karpov, 2000b) has been examined for several species from the genus *Bicosoeca* (fam. Bicosoecidae): *B. planctonica* (Belcher, 1975), *B. kepneri* and *B. lacustris* (Mignot, 1974b), *B. maris* (Moestrup and Thomsen, 1976), *B. socialis* and *B. petiolata* (Mylnikov, 1995), in aloricate bicosoecids without cytopharynx (fam. Cafeteriidae): *Cafeteria roenbergensis* (Fenchel and Patterson, 1988, O'Kelly and Patterson, 1996, Teal et al., 1998), *Discocelis saleuta* (Vørs, 1988), *Symbiomonas scintillans* (Guillou et al., 1999), *Pseudobodo tremulans* (Karpov, 2000b), in aloricate bicosoecids with cytopharynx (fam. Siluaniidae): *Caecitellus parvulus* (O'Kelly and Nerad, 1998), *Siluania monomastiga* (Karpov et al., 1998), and *Adriamonas peritocrescens* (Verhagen et al., 1994; O'Kelly et al., in press), and in scaly bicosoecids with cytopharynx (fam. Pseudodendromonadidae): *Cyathobodo* (Hibberd, 1976, 1985; Strüder-Kypke and Hausmann, 1998) and *Pseudodendromonas* (Mignot, 1974a; Hibberd, 1976, 1985). Here we describe the cytoskeletal elements that we regard as homologous among bicosoecids (Fig. 18, table 1), highlighting those characters that appear to be variable and those that are conservative among different species. Unfortunately, many cytockeletal characters of *Discocelis saleuta, Cyathobodo* and *Pseudodendromonas* cannot be included in comparative analyses because they have not been investigated in detail.

A number of characters relating to the flagellar apparatus in bicosoecids can be variable; e.g., the presence/absence of mastigonemes, electron dense core in the basal bodies, spiral fibre in the transition zone, and the paraxial rod. Mastigonemes are absent in 4 genera: *Caecitellus*, *Adriamonas*, *Cyathobodo* and *Pseudodendromonas*. A paraxial rod is present in the proximal part of posterior flagellum of *Bicosoeca kepneri* and *B. lacustris* (Mignot, 1974b), in posterior flagellum of *B. socialis* and in the anterior flagellum of *B. petiolata* (Mylnikov, 1995), but has not been described in other bicosoecids. A spiral fibre (not a transitional helix – see: Andersen et al., 1991; Karpov

and Fokin, 1995) has been found in 4 genera: *Adriamonas*, *Bicosoeca*, *Pseudobodo* and *Siluania* (Table 1). It was not reported in the original text for *Adriamonas peritocrescens*, but 2 strands of it are clearly visible under the transversal plate in figures 33 and 37 of the one paper on this taxon (Verhagen et al., 1994).

The orientation of basal bodies is the same in all investigated species within the order Bicosoecida. The two basal bodies lie at an oblique angle to one another; with bb1 oriented to the left (Fig. 18, table 1).

In the majority of investigated bicosoecids the flagellar rootlet 1 is composed of 2 microtubules (Fig. 18), although it is composed of 3 microtubules in *Cyathobodo* (Table 1). In those bicosoecids that have been studied, this rootlet originates from the dorsal side of bb2 passing in the same direction (dorsally); it often has secondary microtubules. Thus, r1 is considered homologous in all bicosoecids.

The structure of the ventral flagellar rootlet is also very conservative in bicosoecids. It has a broad and a narrow part, with the microtubules generally in an 8+3 arrangement (Fig. 18), but the number of microtubules does vary among species (see table 1). This rootlet is typically curved or "L" shape in cross section at its proximal end, to which fibrillar material is often connected. This rootlet is associated with the mitochondrion in its middle part and then extends to support the cytostome region. In the past, the origin of this rootlet was not clear. Because both basal bodies, and the origin of the ventral rootlet and associated fibrillar connections are so close to one another, it can mistakenly appear as if the ventral rootlet is connected to both basal bodies. Figures 2, 6–7, 13, and 17 in the present paper show that the ventral rootlet is associated with bb2. Its proximal end terminates directly on the surface of bb2 (Figs 5, 6) with an associated fibrillar bridge (Figs 2, 3). Moestrup (Moestrup, 1995) also noticed that one end of the bicosoecid ventral rootlet is associated with the hairy flagellum basal body (bb2). Bb1 is located close to the proximal end of this rootlet, but is not associated with it. As opposed to the previously accepted heterokont nomenclature *, we consider the ventral rootlet to be r2 and not r3. Thus, there are two microtubular rootlets that originate from bb2 in bicosoecids: r1 (dorsal, often ribbed and composed of 2 microtubules) and r2 (the broadest rootlet, often 8+3, previously called r3).

A highly conserved character of the bicosoecid cytoskeleton is the so-called x-fibre. Normally it appears separate from the proximal end of r2 (former r3), sometimes looking like an additional microtubule parallel to the broad part of the ventral rootlet (Fig. 18). It passes even further then r2 (former r3), ending adjacent to the "abc" microtubules (O'Kelly and Nerad, 1998). Unlike the other additional microtubules in the cytostomal region, the x-fibre passes along the external side of the broad part of ventral rootlet, while the other microtubules in cytostomal region appear on the opposite side of this rootlet. The x-fibre is found in the majority of bicosoecids. The lack of an x-fibre in *Siluania* is perhaps due to its small cell size, which has led to a general reduction in flagellar cytoskeletal elements in this protist.

One or two microtubular rootlets arise from bb1 (Fig. 18). Most bicosoecids possess rootlet r4 (Table 1). This 2-microtubule rootlet always originates from the same side of bb1 in the bicosoecid where it has been observed; r4 faces the anterior end of the cell and passes ventrally and slightly to the left. There is little doubt that r4 is homologous in all bicosoecids. *Symbiomonas* lacks this rootlet because of the absence of bb1. *Siluania* perhaps has it, but it is not clear, as bb1 is not always present in this species.

It is not clear if the other microtubular rootlet arising from bb1 can be regarded as a 'typical' feature in bicosoecids. This rootlet is composed of 2 microtubules, and according to the accepted nomenclature for heterokonts (Andersen, 1991; Andersen et al., 1991), should be called r3. But this rootlet has only been clearly demonstrated in *Bicosoeca maris* (Moestrup and Thomsen, 1976) passing along the posterior flagellum, and in *P. tremulans* (Figs 12, 16 of present paper) passing towards the nucleus. It has either not been demonstrated in other bicosoecids, or the data are doubtful (Table 1).

The first clear demonstration of an S-tubule was in *Bicosoeca maris* (Moestrup and Thomsen, 1976). In some thin sections (Fig. 41 in that paper) the microtubule looks as though it passes between both basal bodies. A similar tubule is found in *Cafeteria* (referred to as r2 by O'Kelly and Patterson, 1996) and it is also seen in *P. tremulans*. We do not explicitly consider the microtubule a rootlet because it actually traverses between the two basal bodies (Figs 12, 18 of present paper), instead of emanating from a basal body.

Fibrillar rootlets are less conservative in bicosoecids and have not been found in some species (Table 1). They can be easily overlooked, as these rootlets are very short and delicate (except in pseudodendromonads were they are always plainly visible); additionally, some fixation methods are not suitable for revealing these structures (O'Kelly and Patterson, 1996). When visible, the fibrillar rootlets track from one (bb1 in *Pseudobodo*) or both basal bodies to the nucleus, and may be divided into two branches. The homology among these rootlets is not clear, as they originate from different basal bodies in different species.

In general, the flagellar apparati among stramenopiles share a number of common elements, as can be seen when comparing bicosoecids and chrysophyceans (Fig. 18). Rootlet 1 in Chrysophyceae and Bicosoecida normally nucleate secondary microtubules, but chrysophyceans have 5 microtubules in r1 instead of 2 in r1 of bicosoecids (Andersen and Wetherbee, 1992; Moriya et al., 2000; Table

* Recently, Moestrup proposed another numbering system for flagellar rootlets: r1 and r2 originate from bb1, and r3 and r4 originate from bb2 (Moestrup, 2000).

Table 1. Some peculiarities of bicosoecids and *Pendulomonas* (=*Wobblia*)

genera	habitat	lorica	cytopharynx	body scales	mastigonemes	number of bbs	bb1 orientation	dense core in bb	transition zone	paraxial rod	fibrillar rootlets	r1	r2 (former r3)	x-fibre	S-tubule	r3	r4	references
Bicosoeca	m, fr	–			+	2	left	+/-	sf	+/-	–/-	2	8–3	+	–	2	2	Mignot, 1974b; Belcher, 1975; Moestrup and Thomsen, 1976; Mylnikov, 1995
Siluania	fr	–	–	–	+	1	–	–	sf	–	–	2	3–1	–	?	–	1?	Karpov et al., 1998
Adriamonas	so	–		–	–	2	left	–	sf*	–		2	8–3	1	?	–	2	Verhagen et al., 1994; O'Kelly et al., in press
Cercitellus	m	–	–	–	–	2	left	+	–	–	–	2	8–3	+	?	–	2	O'Kelly and Nerad, 1998
Cafeteria	m	–	–	–	+	2	left	+	–	–	–	2	8–3	+	–	–	2	Fenchel and Patterson, 1988; O'Kelly and Patterson, 1996
Cafeteria (=Acronema)	m	–	–	–	+	2	left ?	+	–	–	–	2	8–3	+	–	–	2	Teal et al., 1998
Pseudobodo	m, br	–	–	–	+	2	left	–	sf	–	–	2	8–3	+	–	1	2	Karpov, 2000b; present paper
Symbiomonas	m				+	1		+				2	6–3	+				Guillou et al., 1999
Diaphanoeca	m	–	–	–	–	2	?	–	–	–	–	–	–	?	?	?	+	Vørs, 1988
Pseudodendromonas	m	–	–	+	–	2	?	–	–	–	–	?	6–1	?	?	?	?	Mignot, 1974a; Hibberd, 1976, 1985
Cyathobodo	fr	–/–	–	+	–	2	left	–	–	–	–	3	6 (7)	?	?	?	?	Hibberd, 1976, 1985; Stüder-Kypke and Hausmann, 1998
Pendulomonas (=Wobblia)	m	–	–	–	+	2	left	–	db	–	–*	5	7–3	–	–	1*	2	Moriya et al., 2000

Abbreviations: br – brackish water, db – double transitional helix, fr – freshwater, m – marine, sf – spiral fibre, so – soil, "+" – present, "–" – absent, "+/–" – present in not all species of the genus, "?" – data unknown, * – this character has not been noticed by the authors, but exists in published material. Digits in the last 6 columns show the number of microtubules in the rootlets.

1). Its origin and orientation in chrysophyceans and bicosoecids is identical, leaving no doubt that the r1 rootlets are generally homologous (Moestrup, 1995, 2000). As for the other flagellar rootlets: r2 in Chrysophyceae is often absent or looks vestigial (or reduced) while r2 in bicosoecids is well developed because it is involved with feeding. In contrast to bicosoecids, chrysophyceans use r3 for feeding , which accounts for its robust structure (comprising up to 6 microtubules), while r3 in bicosoecids is often absent, or may be composed of 1–2 microtubules. Chrysophycean r3 also has secondary microtubules that are never found on either r2 or r3 of bicosoecids. The rootlet 4 is most conservative. In both groups it consists of two microtubules which have the same origin and orientation. Thus, the rootlets (r1–r4) in Chrysophyceae and Bicosoecida reveal a general homology which is common for all stramenopiles, but at the same time essential differences in their development and structure exist.

Another difference concerns the flagellar transition zone. Some bicosoecids contain a spiral fibre, but there is no bicosoecids with transition helix, which is characteristic for the majority of stramenopiles.

Molecular data

Analyses of ssu rDNA included members of 2 of the 4 families as delineated by morphologic features presented in the recent reclassification of the order Bicosoecida (Karpov 2000b): Cafeteriidae (*Cafeteria, Symbiomonas* and *Pseudobodo*), and Siluaniidae (*Siluania*, *Adriamonas* and *Caecitellus*). Members from the families Bicosoecidae (*Bicosoeca*) and Pseudodendromonadidae (*Cyathobodo* and *Pseudodendromonas*) are missing from the data set. The molecular and morphology based hypotheses of relationships amongst these taxa partly conflict. On one hand, the ssu rDNA tree depicts a paraphyletic Cafeteriidae (e.g., *Pseudobodo* and *Cafeteria* are not sister taxa) and a paraphyletic Siluaniidae (e.g., *Adriamonas* and *Caecitellus* are not sister taxa). Contrary to our analysis of morphological characters, the molecular tree robustly supports a clade that includes *Cafeteria, Symbiomonas* (Cafeteriidae), plus *Caecitellus* (Siluaniidae) to the exclusion of *Pseudobodo* (Cafeteriidae). On the other hand, morphological and molecular hypotheses are congruent with *Adriamonas* and *Siluania* (Siluaniidae) forming a strongly supported clade, in addition to *Symbiomonas* and *Cafeteria* (Cafeteriidae) forming a robust clade (Figs 19, 20).

In all of our analyses, *Pseudobodo* is the most basal bicosoecid. Inclusion of molecular data from representatives of the families Bicosoecidae and Pseudodendromonadidae would greatly aid in mapping morphological characters on our trees. At present it is not possible to distinguish various, independent, gains or losses of characters within the bicosoecids such as the cytopharynx, mastigonemes, lorica or body scales. Nevertheless, convergence of characters can be inferred from our trees. The reduction of cytoskeletal elements in *Symbiomonas* and *Siluania* is most likely due to physical constraints imposed by the extremely small size of these protists. Likewise, the inferred loss of flagellar hairs is believed to have happened independently in *Caecitellus* and *Adriamonas*. Thus, there seems to be plasticity in many of the morphological characters upon which bicosoecids have been classified. The morphological diversity of this group of flagellates is reflected in their molecular diversity. Increasing the breadth of taxa examined at the molecular level is sure to uncover even more diversity and hopefully clarify molecular and morphological inconsistencies.

Acknowledgements

The bulk of the ultrastructural studies were performed in the lab facilities of the Botanical Institute of the University of Koeln. S.A. Karpov thanks M. Melkonian and his colleagues for this possibility and help. Michael E. Holder (University of Houston) provided invaluable computational assistance. A portion of this work was supported by NIH grant # GM32964 to MLS, the state programme "Russian Universities", grant No. 992619 to SAK, Natural Sciences and Engineering Research Council grant# 227085 to Andrew J. Roger (Dalhousie University). For the use of ssu rDNA sequence data from *Adriamonas peritocrescens* and *Caecitellus parvulus* prior to public release, we thank Charles J. O'Kelly (Bigelow Laboratory of Ocean Sciences) and Michael H. Atkins (Woods Hole Oceanographic Institution), respectively. IGPP publication No. 5564.

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