Recent molecular phylogenetic studies have led to the erection of the phylum Amoebozoa, uniting naked and testate lobose amoebae, the mycetozoan slime moulds and amitochondriate amoeboïde protists (Archamoebae). Molecular data together with ultrastructural evidence have suggested a close relationship between Mycetozoa and Archamoebae, classified together in the Conosea, which was named after the cone of microtubules that, when present, is characteristic of their kinetids. However, the relationships of conoseans to other amoebozoans remain unclear. Here, we obtained the complete small-subunit (SSU) rRNA gene sequence (2746 bp) of the enigmatic, multiflagellated protist

*Multicilia marina*, which has formerly been classified either in a distinct phylum, Multiflagellata, or among lobose amoebae. Our study clearly shows that *Multicilia marina* belongs to the Amoebozoa. Phylogenetic analyses including 60 amoebozoan SSU rRNA gene sequences revealed that *Multicilia marina* branches at the base of the Conosea, together with another flagellated amoebozoan, *Phalansterium solitarium*, as well as with *Gephyramoeba* sp., *Filamoeba nolandi* and two unidentified amoebae. This is the first report showing strong support for a clade containing all flagellated amoebozoans and we discuss the position of the root of the phylum Amoebozoa in the light of this result.

**INTRODUCTION**

*Multicilia marina* is a multiflagellated amoeboïde protist that was first isolated in 1880 by L. Cienkowski (Cienkowski, 1881) at the Solovetskaya station (Onega Bay, White Sea). Its cells are usually spherical, about 30–40 μm in diameter, although some of them can have an oblong or irregular form. Typically, each cell has approximately 20–30 flagella. The basal apparatus of each flagellum is represented by a single kinetosome surrounded by a conical microtubular sheath. Submembrane interkinetosomal fibres connect each kinetosome to a neighbouring one and coordinate the movement of the flagella. No microtubular connectives between the basal apparatus and the nucleus have been observed (Mikrjukov & Mylnikov, 1996, 1998). The mitochondrial cristae are tubular. *Multicilia marina* normally feeds by phagocytosis, using lobopodia for the capture of large prey (gymnamoebae).

Cienkowski (1881) described *Multicilia* as an intermediate stage between flagellates and heliozoans. Kudo (1954) considered it to be a member of the order Rhizomastigina Batschli, together with the family Mastigamoebidae and two groups of heliozoans, the actinophryids and the dimorphids. Later ultrastructural studies revealed that the unusually weak and oscillating movements of the flagella of *Multicilia marina* (resulting in their superficial similarity to heliozoan axopodia), together with the poor coordination in their activity and a lack of anterior and posterior ends, make *Multicilia marina* distinct from all other known multiflagellated/ciliated protistan taxa, such as Caryoblastea, Pseudociliata, Hemimastigophora, Ciliophora, Opalinata and Parabasalida. A new phylum, Multiflagellata, of
uncertain affinities was proposed to include the genus *Multicilia* (Mikrjukov & Mylnikov, 1996, 1998). In contrast, Cavalier-Smith (1998) proposed that *Multicilia* belongs in the phylum Amoebozoa, uniting lobose amoebae, the myctozoan slime moulds and the amitochondrion Entamoebids and pelobionts (Archamoebae), and that the genus is either related to Archamoebae (Cavalier-Smith, 2000) or to Vannellidae (Cavalier-Smith; Smith et al., 2004). In the second edition of the Illustrated Guide to the Protozoa, *Multicilia* was placed among ‘residual heterotrophic flagellates’ (Patterson et al., 2000).

In spite of the rapid accumulation of sequences from amoebozoan organisms during the past few years (Amaral Zettler et al., 2000; Bolivar et al., 2001; Fahrni et al., 2003; Peglar et al., 2003; Cavalier-Smith et al., 2004; Nikolaev et al., 2004, 2005), to date no molecular data have been reported for *Multicilia*. Previous molecular phylogenetic studies, based on actin and small-subunit (SSU) rRNA gene sequences, indicated the monophyly of Amoebozoa, although with very weak support (Bolivar et al., 2001; Fahrni et al., 2003; Cavalier-Smith et al., 2004). Furthermore, several higher-level taxonomic lineages have been distinguished within Amoebozoa (e.g. Smirnov et al., 2005). However, the relationships between and within these lineages remain unclear. It has been proposed that the ancestral amoebozoan may have been a flagellated organism similar to *Phalans-terium* or *Mastigamoeba* and that the lobose amoebae evolved by multiple flagella losses (Cavalier-Smith et al., 2004).

To ascertain the amoebozoan affiliation of *Multicilia* and to investigate the relationships between flagellated and non-flagellated members of the phylum, we obtained the complete SSU rRNA gene of *Multicilia marina* and performed phylogenetic analyses, including an exhaustive sampling of eukaryotes and all available sequences of Amoebozoa. We also constructed a predicted secondary structure of the SSU rRNA molecule for *Multicilia marina* and compared it with those of other eukaryotes. Our study confirmed that *Multicilia* is an amoebozoan and allowed us to propose a new hypothesis about the phylogenetic relationships within the phylum Amoebozoa.

**METHODS**

**Cell cultures and DNA extraction, amplification, cloning and sequencing.** The culture of *Multicilia marina* used was obtained from the culture collection of the Institute of Biology of Inland Waters, Russian Academy of Sciences (Borok, Russia). It was isolated from samples of brown algae collected from the Black Sea at a depth of 1 m and a salinity of 18%, between the settlement Novyi Svet and the village Vesolye (Sudak district of Crimea).

DNA was extracted using a DNeasy Plant Minikit (Qiagen). The complete SSU rRNA gene sequence of *Multicilia marina* was amplified using the universal primers sA (5’-ACCTGTTTGTATCTGCAGCT-GAT-3’) and b (5’-TGATCCTTGCAAGTCTTACCTA-3’). PCR was performed in a total volume of 30 μl with an amplification profile consisting of 40 cycles of 30 s at 94 °C, 30 s at 50 °C and 2 min at 72 °C, followed by 5 min at 72 °C for the final extension. The PCR products were purified using a High Pure PCR Purification kit (Roche), then ligated into the pGEM-T vector system (Promega), transformed in XL-2 ultracompetent cells (Stratagene), sequenced using an ABI PRISM BigDye Terminator cycle sequencing kit and analysed using an ABI-377 DNA sequencer (Perkin Elmer), all according to the manufacturers’ instructions.

In addition, we obtained the SSU rRNA gene sequence (as described above) of an unidentified amoeba (unidentified eukaryote clone Borok), which was a contaminant in a culture of the centric diatom *Chlathrila elegans* isolated from waste treatment plants in Borok.

**SSU rRNA gene phylogenetic analyses and secondary-structure modelling.** Complete SSU rRNA gene sequences of *Multicilia marina* and the unidentified eukaryote clone Borok were aligned manually with sequences from diverse eukaryotes using the Genetic Data Environment software (Larsen et al., 1993), following the secondary-structure model proposed by Wuys et al. (2001). A first dataset, including the two sequences obtained in this study and sequences of 23 lobose amoebae, 25 opisthokonts and 50 bikonts, was used to infer the position of *Multicilia marina* among eukaryotes (1421 unambiguously aligned positions). More-detailed phylogenetic relationships within the Amoebozoa were then assessed using an alignment of 64 sequences, including the two sequences obtained in this study and 58 sequences of other amoebozoans (including the more divergent myctozoans, pelobionts and entamoebids) and four opisthokonts as an outgroup (1260 unambiguously aligned positions). GenBank/EMBL/DDBJ accession numbers for all sequences used in our analyses are indicated in Figs 1 and 2.

For both datasets, a maximum-likelihood (ML) tree was obtained with PAUP* (Swofford, 1998), using the general time-reversible (GTR) model of evolution (Lanave et al., 1984; Rodriguez et al., 1990), taking into account a proportion of invariable sites and a gamma distribution of the rates of substitution for the variable positions, with eight rate categories. All parameters were estimated from the dataset. Bayesian analyses were performed on the two datasets with MrBayes (Huelsenbeck & Ronquist, 2001), using the same model. Two runs starting from different random trees were performed for 1 000 000 generations and sampled every 100 generations, with four simultaneous chains. The trees sampled before the chains reached stationarity were discarded as a burn-in (2000). In addition, a ML bootstrap analysis was performed for the second dataset with PhyML (Guindon & Gascuel, 2003). The predicted secondary structure of the SSU ribosomal molecule was modelled with Mfold (Zuker & Gascuel, 1999) and visualized with RnaViz (De Rijk & De Wachter, 1997).

**RESULTS**

**Observations on the culture of *Multicilia marina***

The culture of *Multicilia marina* was maintained in artificial Shmaltz–Pratt medium (NaCl, 16·07%; KCl, 0·38%; MgCl2·6H2O, 3·15%; MgSO4·7H2O, 3·95%; CaCl2·H2O, 0·83%; KNO3, 0·06%; K2HPO4·3H2O, 0·006%; pH 6·5–7·5) and was fed with the lobose amoeba *Vannella* sp. All attempts to cultivate *Multicilia* using as food the bacterium *Pseudomonas fluorescens* (which grew after addition of rice or wheat grains to the culture), the alga *Chlorella*, the chrysomonads *Spumella* or *Prochlorothrix* *sorokinii*, or the chrysomonads *Spumella* or *Paraphysomonas*, failed. During light microscopic observation, it was established that, at the time of feeding, cells of *Multicilia marina* used their ventral side to cover the amoeba *Vannella*, and then stood motionless. The predator resumed its motion several seconds after the capture of its prey.
Fig. 1. SSU rRNA gene-based phylogeny of eukaryotes, showing the positions of *Multicilia marina* and the unidentified eukaryote clone Borok. Both organisms clearly belong to the Amoebozoa (grey box). The topology was inferred using the ML method with the GTR model, with a gamma distribution and a proportion of invariable sites. Numbers at nodes represent posterior probabilities resulting from a Bayesian analysis using the same model. Values under 0.5 have been omitted. All branches are drawn to scale; the bar represents 0.05 substitutions (corrected) per site. The tree is presented in an unrooted format, with a basal trifurcation.
Unusual length of the SSU rRNA gene sequence of *Multicilia marina*

The length of the complete, amplified SSU rRNA gene sequence of *Multicilia marina* was 2746 bp, which is the second-longest amoebozoan SSU rRNA gene sequence after that of *Pelomyxa palustris* (3502 bp). The length of the SSU rRNA gene sequence of *Multicilia marina* is similar to those of pelobionts and *Endolimax nana*. In other Amoebozoa, the SSU rRNA gene is generally longer than...
that in most eukaryotes, but does not exceed 2200 bp, with the exception of Acanthamoeba spp. (Table 1).

According to a predicted secondary structure (see Supplementary Fig. S1 in IJSEM Online), nine insertions are responsible for the extraordinary length of the SSU rRNA gene of Multicilia marina, which are located in the variable regions V2, V4, V5, V7 and V8 (see Table 1). One of the three insertions in region V2 corresponds to the additional helix E8_1, which is absent from most eukaryotes. The other two correspond to extensions in the terminal loops of helices 10 and 11. Two insertions are present in region V4 (extensions in the terminal loops of helices E23_12 and E23_14). The insertion in region V5 corresponds to an extension in the terminal loop of helix 29. The insertion in region V8 corresponds to the additional helix E45_1, which is absent from most eukaryotes, but present in all Archamoebae and some other amoebzoans. Remarkably, at 426 bp, variable region V7 (helices 43 and E43_1 to E43_4) of the SSU rRNA gene of Multicilia marina is among the longest of all eukaryotic sequences available to date, with longer insertions present to our knowledge only in the strepsipteran insects. The secondary structure proposed for this region in Supplementary Fig. S1 is tentative and other structures are possible.

**Phylogenetic position of Multicilia marina**

Fig. 1 shows the result of an ML analysis of 100 SSU rRNA gene sequences of eukaryotes, including Multicilia marina, the unidentified eukaryote clone Borok, 23 lobose amoebae and 75 other eukaryotes representing the most sequenced lineages of opisthokonts and bikonts. In the absence of a prokaryotic outgroup, the tree is presented in an unrooted format, with a basal trifurcation. The analysis clearly demonstrates that Multicilia marina belongs to the Amoebozoa, the monophyly of which was supported by a Bayesian posterior probability (PP) of 1.00. Opisthokonts also form a strongly supported clade (PP = 1.00). In contrast, bikonts form two clusters: Ancyromonas and apusomonads branch together next to the opisthokonts, although without Bayesian support, whereas the remaining bikont lineages form a weakly supported group (PP = 0.54) and resolution among them is typically poor.

In the tree presented in Fig. 1, Multicilia marina branches next to Gephyamoeba sp. (PP = 0.84) and together they form a sister-group to a strongly supported clade (PP = 0.97) comprising Filamoeba nolandi, the unidentified eukaryote clone Borok and an environmental clone (RT5ii44) that was described by Amaral Zettler et al. (2002). The Multicilia + Filamoeba + Gephyamoeba clade is supported by a PP of 0.80. Phalansterium solitarium branches at the base of this clade, but with lower support (PP = 0.65).

**Phylogenetic relationships among Amoebozoa**

To further investigate the position of Multicilia marina among Amoebozoa, ML and Bayesian analyses were performed on a second dataset including the two sequences obtained in this study and those of 12 mycetozoans, 5 pelobionts, 6 entamoebids and 35 other amoebzoans, with 4 opisthokonts as an outgroup (Fig. 2). As in Fig. 1, Multicilia marina branches within a clade containing Phalansterium solitarium, Gephyamoeba sp., F. nolandi and the two unidentified clones Borok and RT5ii44. Analysis of the second dataset revealed that this clade, supported by a PP of 0.91 and a bootstrap value (BV) of 84 %, also includes Archamoebae and Mycetozoa.

Resolution within this clade was relatively good. As in Fig. 1, Multicilia marina branches as the sister taxon to Gephyamoeba sp. (PP = 1.00; BV = 91 %). F. nolandi and the two unidentified clones Borok and RT5ii44 form a strongly supported clade (PP = 1.00; BV = 93 %). Archamoebae (supported by a PP of 1.00 and a BV of 99 %) and Mycetozoa (PP = 1.00; BV = 99 %) branch together (PP = 0.81; BV = 68 %) as a sister group to the ‘Filamoeba clade’ (PP = 0.93; BV = 71). Finally, Phalansterium solitarium occupies the most basal position in the clade, with a PP of 0.99 and a BV of 80 %.

As in Fig. 1, other groupings within the Amoebozoa are congruent with a recent molecular phylogeny and classification of the phylum (Smirnov et al., 2005), and were named accordingly in Fig. 2. Three well-supported clades are recognized, the classes Tubulinea and Flabellinea and the order Acanthopodida. As shown previously (Fahrni et al., 2003; Cavalier-Smith et al., 2004; Smirnov et al., 2005), the relationships between these clades and the three independent lineages formed by Dermamoeba algensis, Mayorella sp. and ‘Platyamoeba’ stenopodia were very poorly resolved (the generic status of the latter species needs revision; see, for example, Smirnov et al., 2005). In the tree shown in Fig. 2, the Tubulinea occupy a basal position within Amoebozoa, with a PP of 0.56 and a BV of 81 %.

**DISCUSSION**

**Multicilia marina is an amoebozoan**

Phylogenetic analysis of SSU rRNA gene sequences clearly showed that, despite its numerous flagella, Multicilia marina is more closely related to lobose amoebae than to any other group of eukaryotes. Furthermore, the monophyly of the Amoebozoa including Multicilia marina was strongly supported in our analyses (Fig. 1), in spite of the inclusion of several bikont lineages of unknown affinities, such as apusomonads, centrohelids, Ancyromonas, Diphylleia, Trimastix and Breviata anathema [after this paper had been accepted, ‘Mastigamoebae’ inverts were redescribed as Breviata anathema (Walker et al., 2006)], some of which have been shown to group near or with lobose amoebae in previous large-scale phylogenies of eukaryotes (e.g. Brugerolle et al., 2002; Cavalier-Smith & Chao, 2003; Cavalier-Smith, 2004). This result contradicts the initial idea that Multicilia is an intermediate stage between flagellates and heliozoans, as
Table 1. Sequence length and unusual secondary-structure features of the SSU rRNA gene of *Multicilia marina* and other Amoebozoa

The size of helices 6, 10, 11, 16, E23_12, E23_14, 26, 29, 37, 44 and 46 is conserved in most eukaryotes; 'h' indicates that, in the taxon concerned, the terminal loop of the helix extends into a hairpin that is usually absent from other eukaryotes. Helices E8_1, E43_4 and E45_1 are absent from most eukaryotes; + and − indicate the presence or absence of these helices in the different amoebozoan taxa, respectively. In some *Acanthamoeba* species the helix E10_1 branches into two terminal hairpins, whereas in *Entamoeba* spp. two helices instead of one are found between helices 10 and 11. Parentheses indicate that the structure is present only in a subset of the members of the taxon.

<table>
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<tr>
<th>Taxon</th>
<th>Sequence length (bp)</th>
<th>6</th>
<th>E8_1</th>
<th>10 E10_1</th>
<th>11</th>
<th>16 E23_1</th>
<th>E23_4</th>
<th>E23_12</th>
<th>E23_14</th>
<th>26</th>
<th>29</th>
<th>37</th>
<th>E43_1</th>
<th>E43_4</th>
<th>44</th>
<th>E45_1</th>
<th>46</th>
</tr>
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<td></td>
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<td>1</td>
<td>1</td>
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<tr>
<td>Flabellinea, Dactylopodida</td>
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<td>(+)</td>
<td>1</td>
<td>1–3</td>
<td>(h)</td>
<td>1</td>
<td>(+)</td>
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<td>1–3</td>
<td>h</td>
<td>0</td>
<td>−</td>
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<tr>
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<td>−</td>
<td>2</td>
<td>3</td>
<td></td>
<td>1–3</td>
<td>(h)</td>
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<td></td>
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<td>h</td>
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<td>(h)</td>
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<td>(h)</td>
<td>h</td>
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<td>2746</td>
<td>+</td>
<td>h</td>
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<td>2</td>
<td>h</td>
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<td>*</td>
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<td>h</td>
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<td>h</td>
<td>*</td>
<td>h</td>
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<td>h</td>
<td>+</td>
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<td>*</td>
<td>h</td>
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<td>h</td>
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†According to Wuyts *et al.* (2001), up to two helices arise from helix E23_1 (E23_2 and E23_3), up to three helices arise from helix E23_4 (E23_5 to E23_7) and up to two helices arise from helix E43_1 (E43_2 and E43_3); numbers indicate how many helices arise from these helices in the different amoebozoan taxa.

‡An asterisk indicates the presence of an important insertion of as yet unknown secondary structure.
proposed by Cienkowski (1881) on the basis of the similarity of its body form, mode of locomotion and the presence of multiple flagella. It is also in disagreement with the more recent placement of Multicilia in the phylum Multiflagellata of uncertain affinities (Mikrjukov & Mylnikov, 1996, 1998) or among heterotrophic flagellates of uncertain affinities (Patterson et al., 2000).

The results of our study confirm the hypothesis that Multicilia belongs in the phylum Amoebozoa, as first suggested by Cavalier-Smith (1998). However, the position of Multicilia among Amoebozoa in our SSU rRNA gene trees differs from the classification of this genus in the class Discosea, together with Vannellididae, Vexilliferidae and Paramoebidae (Cavalier-Smith, 2003; Cavalier-Smith et al., 2004). This classification was based on observations of cell surface glycostyles in Multicilia (Mikrjukov & Mylnikov, 1996), possibly similar to the glycostyles present in vannellids (Bovee, 1965). However, it cannot be excluded that the glycostyles observed in Multicilia originate from its prey, Vannella. According to our analyses, the closest sequenced relative of Multicilia is Gephyramoeba sp. ATCC 50654, and both organisms branch far from Vannellididae and Vexilliferidae. Although well-supported in our analyses, the relationship between Multicilia and Gephyramoeba is difficult to assess morphologically, because the ATCC strain of Gephyramoeba sequenced by Amaral Zettler et al. (2000) was not described in detail and was possibly misidentified (A. Smirnov, personal communication).

**Monophyly of all flagellated amoebozoans**

Our SSU rRNA gene analyses, including the first sequence of Multicilia to be obtained and an exhaustive sampling of available complete sequences of Archamoebae and Mycetozoa, allow us to recover for the first time a monophyly of all flagellated amoebozoans (pelobionts, myxogastrids, Multicilia and Phalansterium), which branch together with a few non-flagellated lineages (entamoebids, dictyostelids, Filamoeba and Gephyramoeba) within a strongly supported clade (PP of 0.91 and BV of 84%). As this clade contains all flagellated amoebozoans examined to date, we propose to name it Conosea (after Cavalier-Smith, 1998), in agreement with the new system of Amoebozoa proposed recently by Smirnov et al. (2005).

A common origin for Archamoebae and Mycetozoa was proposed previously by Cavalier-Smith (1998). In a multigene analysis of expressed sequence tag data from Mastigamoeba, Entamoeba and Dictyostelium, Baptiste et al. (2002) later claimed to have confirmed the monophyly of an Archamoebae + Mycetozoa clade, but the lack of any lobose amoebae in their study made this conclusion irrelevant. On the other hand, analyses of actin and SSU rRNA genes from a broad taxonomic sampling of Amoebozoa provided opposite results, showing Archamoebae and Mycetozoa as separate lineages (Fahrni et al., 2003). Finally, these two taxa did not group together, even with the inclusion of Phalansterium (Cavalier-Smith et al., 2004). It appears that the addition of the sequences of Multicilia and one unidentified amoeba was important to recover the monophyly of all flagellated amoebae. The monophyly of Conosea is indeed sensitive to taxon sampling, as Phalansterium does not usually branch with Filamoeba, Gephyramoeba, Archamoebae or Mycetozoa in the absence of Multicilia and the two unidentified clones Borok and RT5iin44 (data not shown). Comparison of the present results with those of previous analyses also suggests that the use of a larger sampling of Archamoebae and Mycetozoa was important to stabilize the relationships within Conosea (data not shown). In particular, the addition of two recently published sequences of Trichiales (Fiore-Donno et al., 2005) allows the breaking of the very long stem branch leading to myxogastrids. This apparently increases the bootstrap support for the close relationship between Multicilia and Gephyramoeba, as well as for the monophyly of Mycetozoa and their sister-group relationship with Archamoebae.

Apart from Multicilia, the flagellated amoebae include pelobionts, myxogastrids and Phalansterium, a genus that was previously considered as a zooflagellate (Ekelund, 2002) and was only recently transferred to the Amoebozoa, based on an analysis of its SSU rRNA gene sequence (Cavalier-Smith et al., 2004). These flagellated Amoebozoa are all characterized by a unikont flagellar apparatus, with a kinetid composed of one centriole and one flagellum, with a microtubular cone (Cavalier-Smith, 1998). Ultrastructural studies of Multicilia marina (Mikrjukov & Mylnikov, 1996, 1998) revealed that the basal apparatus of each flagellum is also represented by a single centriole and possesses a microtubular cone. However, in contrast with other flagellated amoebozoans, which possess a kinetid with the broad end of the conus of microtubules orientated towards the nucleus (Cavalier-Smith, 1998), in Multicilia the kinetid broad end is orientated towards the cell surface (Mikrjukov & Mylnikov, 1996). This might be an ancestral state or a consequence of the multiciliarity, where the flagellar apparatus does not lean on the nucleus but on the surface skeletal elements.

Some of the features observed in the primary and secondary structures of the SSU rRNA gene of Multicilia suggest its possible close relationship to Archamoebae (Table 1). The extraordinary length of the SSU rRNA gene in Multicilia is shared with the majority of Archamoebae (Hinkle et al., 1994; Milyutina et al., 2001), although this is not strong evidence for a common origin, because even closely related amoebae species may differ widely in their SSU rRNA gene length (in the Acanthopodida, for example, it varies from 1972 bp in Balamuthia mandrillaris to greater than 2500 bp in some Acanthamoeba spp.). Multicilia and all Archamoebae, except Entamoeba, possess an insertion in variable region V4, in helix E23_14 (data not shown). This insertion corresponds in Multicilia to an additional hairpin in the loop of helix E23_14. Unfortunately, because the secondary structure of region V4 has not yet been fully determined in Archamoebae, we do not know whether this region also corresponds to an additional hairpin in the loop of helix...
E23_14 in this taxon. *Multicilia* also shares with all Archamoebae an insertion in region V8 (additional helix E45_1). The presence of helix E45_1 is a characteristic feature of Archamoebae, although it is present in some other Amoebozoa (*Acanthamoeba* spp., *Clydonella* sp. and *Vexillifera minutissima*).

Finally, there are also some ultrastructural features of the flagellar root system that may suggest a relationship between *Multicilia* and Archamoebae (Cavalier-Smith, 2000). These include the presence of the microtubular cone and of a short flagellar band around the flagellum, as well as a single microtubular band (Brugerolle, 1991; Simpson *et al.*, 1997; Mikrjukov & Mylnikov, 1998; Brugerolle & Müller, 2000; Walker *et al.*, 2001). The loss of some components of the axoneme of the flagellum (Brugerolle & Patterson, 2000), such as the outer dynein arms, and the weak motion of the flagella in some Archamoebae (*Mastigina*, *Pelomyxa*) are also shared by *Multicilia*. Perhaps the ancestor of Archamoebae lived, as *Multicilia*, in an aerobic environment, possessed tubulocristate mitochondria and was multi-flagellated. If this hypothesis is correct, then *Pelomyxa*, with its multiple flagella (Griffin, 1988; Goodkov & Seravin, 1991), may have maintained the ancestral state of the group and, because of a sedentary lifestyle, its flagella have been preserved in a reduced form only.

**Where is the root of Amoebozoa?**

The monophyly of flagellated amoebae depends not only on the sequences included in the analyses, but also on the position of the root of Amoebozoa. Because the radiation of Amoebozoa is not well resolved, its rooting varies depending on the gene, type of analysis, taxonomic sampling and selected outgroup. In the present study, the root of Amoebozoa is either placed between Tubulinea + Mayorella + ‘Platyamoeba’ stenopodia + Acanthopodida and Flabellinea + *Dermamoeba algensis* + Conosea (Fig. 1) or between Tubulinea and all other amoebozoans (Fig. 2). The latter result was also recovered in actin phylogenies published by Fahrni *et al.* (2003) and Nikolaev *et al.* (2005) and in the SSU rRNA gene phylogeny published by Smirnov *et al.* (2005). In other SSU rRNA gene trees, the root was placed between Tubulinea + Archamoebae and Mycetozoa + other lobose amoebae (Fahrni *et al.*, 2003) or between Acanthopodida and other Amoebozoa (Cavalier-Smith, 2002). Cavalier-Smith *et al.* (2004) used *Breviata anathema* as an outgroup, but this species is not related to Amoebozoa (Fig. 1; see also Edgcomb *et al.*, 2002) and did not increase the stability of the relationships within Amoebozoa. Finally, in other protein trees, the taxonomic sampling is too small to make any assumption about the position of the root.

Alternatively, the position of the root might be deduced from such features as the presence of flagella. Assuming that the ancestor of Amoebozoa was flagellated, as proposed by Cavalier-Smith (1998, 2000), the flagellum was lost several times during the evolution of amoebae. For example, a flagella loss occurred at least once within Archamoebae (in the Entamoebidae), or twice if *Entamoeba* and *Endolimax* are not monophyletic, as suggested by SSU rRNA gene analyses (see Fig. 2). In this particular case, the loss of flagella may be related to the parasitic mode of life of members of these genera. In the whole Amoebozoa, up to ten flagella losses have been proposed to match the observed tree topology (Cavalier-Smith *et al.*, 2004). However, the number of flagella losses depends on the phylogenetic position of the flagellated species within Amoebozoa and the position of the root in the group. According to the topologies shown in Figs 1 and 2, which place *Multicilia* and *Phalansterium* in the same clade, the number of flagella losses can be reduced to eight. If the rooting possibilities suggested by our analyses are not correct and the root actually lies between Conosea and all other amoebozoans, as observed in some ML trees (data not shown), this number would be reduced to six (Fig. 3). In this most-parsimonious case, the flagellum would have been lost once in the common ancestor of Tubulinea and Flabellinea, once in *Gephyromoea* sp., once in the common ancestor of the *Filamoeba* clade, once in the common ancestor of dictyostelids and once or twice in the parasitic Archamoebae.

The rooting would be much more complex if Amoebozoa are paraphyletic. This possibility cannot be excluded entirely because of (i) the as yet rather weak evidence for their monophony, and (ii) the as yet not well-established position of Amoebozoa between opisthokonts and bikonts. According to a recent hypothesis on the early divergence of eukaryotes (Stechmann & Cavalier-Smith, 2003; Richards & Cavalier-Smith, 2005), the Amoebozoa are close to the eukaryotic root. This root has been placed between the...
ancestrally uniflagellate opisthokonts and Amoebozoa on the one hand and the ancestrally biflagellate bikonts on the other. However, it cannot be excluded that the Amoebozoa might be paraphyletic. If this was the case, it would be obvious to search for early eukaryotes among flagellated amoebae. One of them, *Phalansterium*, has already been proposed as a candidate for the closest relative to the ancestral eukaryotes (Cavalier-Smith et al., 2004). Further multigene studies of this and other flagellated amoebozoans, including *Multicilia*, may possibly bring answers to some of the questions concerning early eukaryote evolution.

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