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Phylogenetic position of *Protoopalina intestinalis* based on SSU rRNA gene sequence

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Abstract

A robust recognition of phylogenetic affinities of Opalinidae—the peculiar multinucleated intestine commensals of frogs—is hindered by the absence of reliable molecular data. Up to now all attempts to sequence opalinid genes failed, as the obtained sequences labeled as *Protoopalina intestinalis*, *Cepedea virguloidea*, and *Opalina ranarum* in GenBank apparently originate from a zygomycete contamination. In this paper, we present the first molecular data for the family Opalinidae—SSU rRNA gene of *P. intestinalis*. Our phylogenetic analyses undoubtedly show opalinids as a sister group to *Proteromonas* within the Stramenopila clade, confirming the monophyly of Patterson's order Slopalinida. The enigmatic genus *Blastocystis* is resolved with high statistical support as a sister group to Slopalinida. The information contained in the SSU rRNA gene proved insufficient to uncover broader affinities of this group to other groups of Stramenopila. Nevertheless, our analyses clearly demonstrate that Cavalier-Smith's phylum Bigyra, which comprises Oomycetes and their relatives together with Slopalinida and *Blastocystis*, is not monophyletic.

Keywords: *Protoopalina*; Opalinidae; Stramenopila; Phylogeny; 18S rRNA gene

1. Introduction

Opalinids, first observed by Leeuwenhoek in 1683 (Dobell, 1932), are large (up to 2.8 mm), multinucleated, multiciliated protozoa with unusual morphology and ultrastructure. They are very common in the cloacae of frogs and toads. They are also the representatives of last few higher eukaryotic taxa that were not studied with molecular phylogenetic methods because of lack of DNA sequence data. Four genera of family Opalinidae are separated into two subfamilies (Metcalf, 1923). Subfamily Protoopalininae comprises binucleated genera *Protoopalina* (with cylindrical cells) and *Zelleriella* (with flattened cells). The second subfamily—Opalininae—comprises multinucleated genera *Cepedea* (cylindrical cells) and *Opalina* (flattened cells).

Opalinids resemble ciliates in having multiple flagella and were for a long time considered to be related to them (e.g., Stein, 1860—after Delvinquier and Patterson, 1993; Metcalf, 1923). In the 1950s, the hypothesis of opalinid–ciliate affinity was abandoned and opalinids were deemed to be either an isolated taxon in the phylum Zooflagellata or were treated as a separate phylum: Opalinata (e.g., Corliss, 1955; Grassé, 1952).

The first hypothesis on opalinid affinity based on reliable morphological data was formulated after a detailed ultrastructural study by Patterson (1985). The pattern of subpelicular microtubules and the structure of basal bodies and flagellar transition zone of opalinids strongly resembled those of *Karotomorpha bufonis* (Brugerolle and Joyon, 1975). *Karotomorpha*, the commensal in the frog intestine, is a uninucleated flagellate with four flagella. Genus *Karotomorpha* and related genus *Proteromonas* belong to the family Proteromonadidae. Patterson established a new order Slopalinida

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comprising families Proteromonadidae and Opalinidae. He also suggested a relationship between Slopalinida and heterokont algae due to the similarities in their basal body structures and the proposed homology between the somatonemes of *Proteromonas* and the mastigonemes of heterokont organisms.

The phylogenetic analysis of Silberman et al. (1996) confirmed the assumption that *Proteromonas lacertae* belongs to the Stramenopila. Quite surprisingly, the study also revealed that the hitherto enigmatic genus *Blastocystis*, the aciliated multinucleated gut parasite of both vertebrates and invertebrates with a spherical cell and a large central vacuole, is the sister group of *Proteromonas*.

These results and similarities in the structure of basal bodies and flagellar transition zone led Cavalier-Smith to postulate a new phylum Bigyra in the kingdom Chromista. The phylum Bigyra comprises Slopalinida, *Blastocystis*, Oomycetes, Hyphochytrea, and *Developayella* (Cavalier-Smith, 1997, 1998).

Although there are quite strong morphological indications of opalinid affinities to Stramenopila, we are still unable to confirm the hypotheses of Patterson and Cavalier-Smith on the basis of sequence data. The inclusion of opalinids in Stramenopila is based merely on their similarities in the structure of basal bodies and on the proposed relationship between opalinids and the family Proteromonadidae. The information contained in the SSU rDNA sequence of opalinids would not only clarify the phylogenetic position of the group, but could also elucidate the evolutionary history of the group Stramenopila.

Here we report the sequence of the SSU rRNA gene of *Protoopalina intestinalis*. We also demonstrate the phylogenetic affinities of opalinids and we examine the proposed monophyly of the order Slopalinida and the class Bigyra.

2. Materials and methods

2.1. DNA isolation, SSU rDNA amplification, and sequencing

Opalinids of the species *P. intestinalis* were isolated with a Pasteur pipette from the cloaca of a fire-bellied toad (*Bombina bombina*). The isolate contained numerous specimens of *Protopalina*, diplomonads, bacteria, and a few ciliates. *Protoopalina* cells were then separated from most of the other eukaryotes and bacteria by filtration through sterile filter paper (pore size ~10 μm). The filter was rinsed with 10 ml of physiological solution and the material on the upper side of the filter was washed into a sterile tube. *Protoopalina* and ciliates were the only eukaryotes observed after the filtration. The genomic DNA was isolated from these cleared and concentrated *Protoopalina* cells using High pure PCR template preparation kit (Roche).

Eukaryote-specific primers MedlinA (CGT GTT GAT CCT GCC AG) and MedlinB (TGA TCC TTC TGC AGG TTC ACC TAC) were used to amplify SSU rDNA (Medlin et al., 1988). The PCR products were then purified using QIAquick PCR purification kit (Qiagen) and used as a template for secondary amplification. Three different primer pairs were used for secondary amplification (Fig. 1). Because of the proposed close affinity of opalinids to Proteromonadidae, primers BA (CCA TGG CAG TAA GGG GTA ACG AA) and BB1 (GRA CAT CTA AGG GCA TCA CAG ACC) were designed on the basis of SSU rDNA sequences of *Protoopalina* and *Blastocystis* as specific primers for the *Blastocystis* + Slopalinida group. The PCR product amplified using the BA–BB1 primers was then cloned into pGEM-T Easy vector (Promega) and sequenced using 3100-Avant genetic analyser. Approximately 50% of the obtained sequences were very similar to sequences of ciliates (Blast *E* value for Ciliophora, Trichostomatia $< 10^{-149}$). Clones of the second type containing sequences with closest match to *Pr. lacertae* (Blast *E* value 10^{-107}) were ascribed to *Pr. intestinalis*. Three clones of *Protoopalina* were wholy sequenced. Primers PK (CAC ACC AGA TAT GGG TTA TGC) and PKR (GCC CTC CAA TKG ATT CG) were designed according to the obtained sequence as *Protoopalina*-specific primers and were used for the secondary amplification in tandem with primers MedlinB and MedlinA, respectively. The PCR product obtained using PK and MedlinB primers was cloned and two clones were sequenced. The PCR product obtained using primers PKR and MedlinA was sequenced directly using the two external and two internal primers—BA and B430R (TYC GCG CCT GCT GCC T). GenBank accession numbers of the three sequences are AY576544–AY5765446.

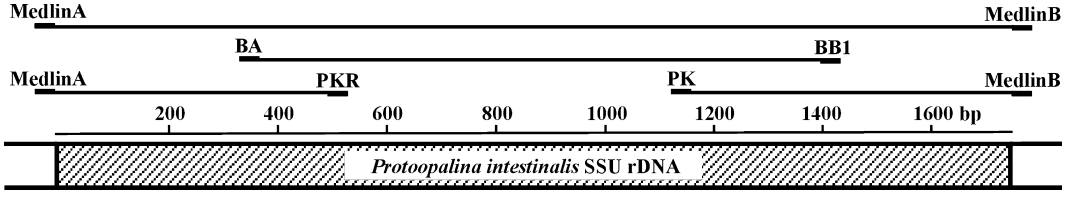

Fig. 1. The graphic representation of the SSU rDNA gene of *Protoopalina intestinalis*. Locations of the used primers and the lengths of the corresponding PCR products including their overlapping parts are indicated.

2.2. Phylogenetic analyses

A data set for study of the phylogenetic affinities of *P. intestinalis* consisted of 44 SSU rDNA sequences representing all major eukaryotic lineages. The sequences were aligned using the program ClustalX 1.18 (Thompson et al., 1997). The resulting alignment was manually edited using the program Bioedit (Hall, 1999). For phylogenetic analyses, 1244 unambiguously aligned positions were used. Alignment is available from corresponding author upon request (e-mail address: mkostka@centrum.cz).

To elucidate the phylogenetic affinities among the main groups of the stramenopiles, we constructed another alignment, containing 39 taxa and 1393 positions, with broad sampling of stramenopiles and five alveolates and two haptophytes as outgroups.

Maximum likelihood (ML) phylogenetic trees were constructed using PHYML (Guindon and Gascuel, 2003) employing the Tamura–Nei model + Γ + I chosen with Modeltest 3.06 (Posada and Crandall, 1998). Support for topological elements was assessed by tree reconstructions of 100 bootstrap-resampled replicates in which all characters were used. Another method to construct the maximum likelihood trees was conducting bayesian analysis using MrBayes 3.0 (Huelsenbeck, 2000) with four simultaneous Markov chains Monte Carlo, temperature 0.2, 2×10^6 generations, and burnin 10,000 trees.

Both maximum parsimony (MP) and Fitch–Margoliash method with log det distances (LogDet) were performed with PAUP 4.0 (Swofford, 1999). MP analysis was conducted with 1000 repeated tree searches in which the starting tree was constructed by random taxa addition and swapped by the TBR algorithm. The support for the topology of MP and LogDet trees was estimated by the use of 1000 bootstrap-replicates (only 10 repeated searches started with tree constructed by random taxa addition for each bootstrap replicate when bootstrapping MP tree).

3. Results and discussion

We determined 1751 bp of *P. intestinalis* SSU rRNA gene. The G-C content is quite low: 34.6%. The sequence contains many unique A-T rich regions especially in those positions that are known to vary among different eukaryotic taxa.

Analysis of the data set containing main eukaryotic groups showed that *P. intestinalis* belonged to the Stramenopila group (Fig. 2). All methods supported this result with high bootstrap values (ML 93%, MP 87%, and LogDet 98%). The relatively low posterior probability (0.70) in the bayesian analysis for the Stramenopila group was caused by frequent artificial grouping of the parabasalid clade (*Trichomonas* + *Tritrichomonas*) with *Wobblia* among trees generated by bayesian analysis. This ambiguous placement of the parabasalid clade and several other long branches was observed in three independent runs of MrBayes.

The monophyly of Slopalinida was supported by very high bootstrap values (ML100%, MP 99%, and LogDet 100%) as well as by posterior probability 1.00. Similarly, the sister-group status of *Blastocystis* to Slopalinida was well supported (ML 100%, MP 97%, and LogDet 100%, posterior probability 1.00). Preliminary results of analyses including 536 bp of SSU rDNA sequence of *Opalina ranarum* isolated from the common frog (*Rana temporaria*) and of *K. bufonis* isolated from the northern leopard frog (*Rana pipiens*) also suggest that the family Opalinidae and the order Slopalinida are probably monophyletic (data not shown).

Two sequences labeled as *O. ranarum* and *Cepedea virguloidea* (Accession Nos. AF141969 and AF141970) in the GenBank database were also included in the analysis. The GenBank database contains another sequence labeled as *P. intestinalis* (AF142474) which is incomplete and closely resembles the two aforementioned sequences. These sequences were used in several other works (e.g., Guillou et al., 1999; Karpov et al., 2001) showing them to be a sister group to Stramenopila. However, our analysis clearly demonstrated that the two sequences were closely related to *Mucor polycephalum* (ZygOpalina and ZygCepedea in Fig. 2) with high bootsrap values (ML 100%, MP 100%, and LogDet 100%) and posterior probability 1.00. Clearly, these sequences do not represent opalinids but zygomycetes that were associated with opalinids in their habitat or contaminated the sample. The notion of their sister position to Stramenopila in the previous studies was caused by wrong interpretation of incorrectly rooted trees.

Topology of our best ML tree and the shortest MP tree was identical within the stramenopila, alveolata, and opisthokonta clades. The best LogDet tree differed from these in the position of Labyrinthulea, which were resolved as the sister group of Oomycetes + Hyphochytriomycetes + *Developayella* + autotrophic stramenopiles. The resolved interrelationships among the groups of stramenopiles were weakly statistically supported by all three methods. The interrelationships among major eukaryotic groups (i.e., Stramenopila, Opisthokonta, Viridiplantae, Cryptophyta, Cercozoa, etc.) were resolved differently by each method.

Analysis of the second data set containing broad sampling of stramenopiles resolved the main groups of stramenopiles well (Placididea, Labyrinthulea, Bicosoecida, Slopalinida + *Blastocystis*, Hyphochytrea, Oomycetes, and autotrophic stramenopiles). However, the interrelationships among them remained unclear as each of the used methods provided a different weakly statistically supported topology (trees not shown). Our

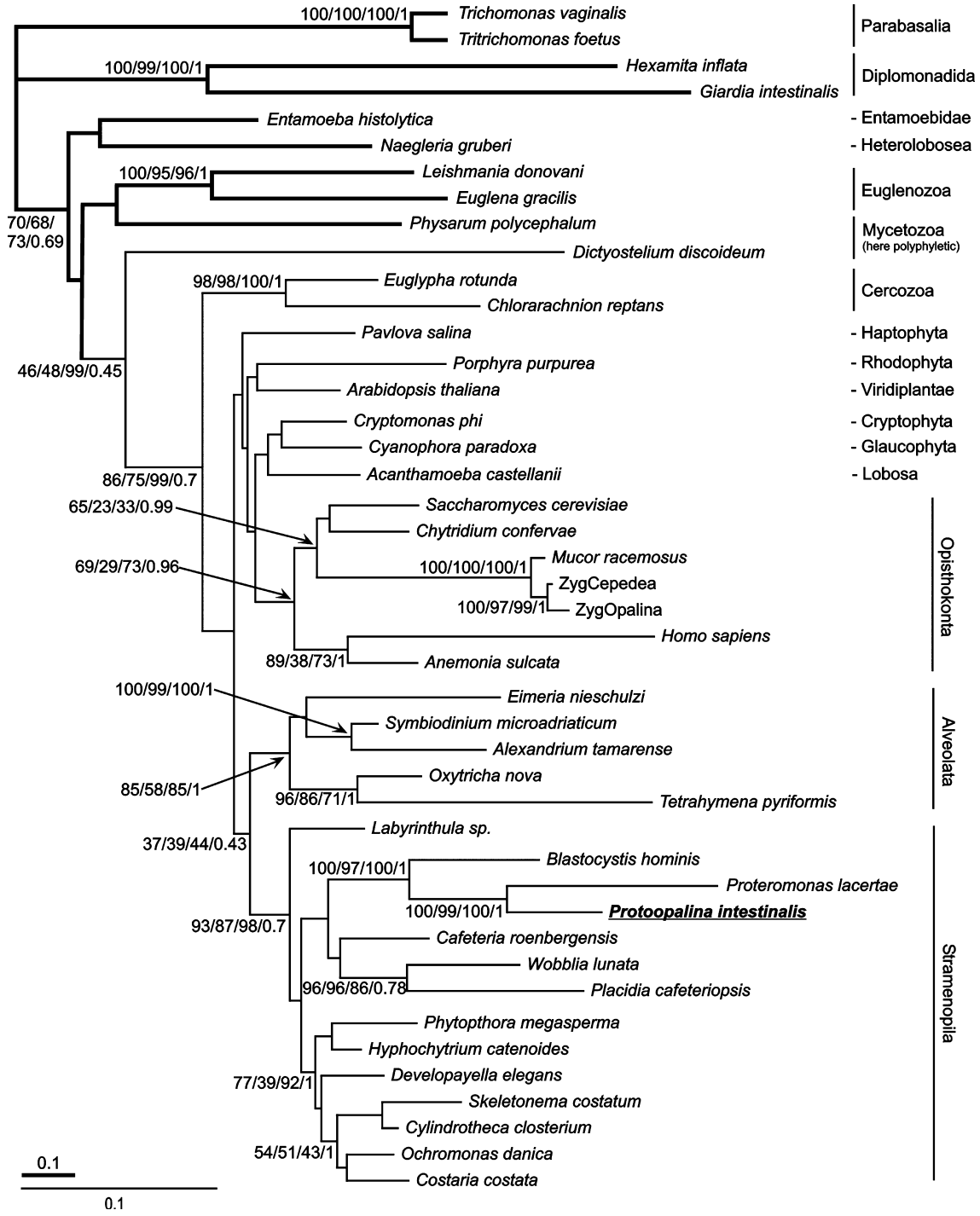

Fig. 2. Maximum likelihood tree showing relationships among 44 eukaryotic taxa. Bootstrap values from maximum likelihood (100 replicates), maximum parsimony (1000 replicates), LogDet (1000 replicates), and bayesian posterior probabilities, respectively, are shown at the nodes. Bootstrap values are not shown at some non-essential nodes and at little supported inner nodes of Stramenopila (where the topology is uncertain, see the discussion). For basal taxa (i.e., *Trichomonas*, *Tritrichomonas*, *Hexamita*, *Giardia*, *Entamoeba*, *Naegleria*, *Leishmania*, *Euglena*, and *Physarum*), a different scale is used to shorten the length of their branches in the figure (solid lines). Note the position of ZygCepedea and ZygOpalina—sequences labeled as *Cepedea virguloidea* and *Opalina ranarum*, respectively, in the GenBank database.

extensive survey showed that the inner topology of the stramenopila clade is inconsistent in the literature as well (compare, e.g., Dawson and Pace, 2002; Moreira and López-García, 2002; Moriya et al., 2000). The only group reconstructed by all methods was the group of Oomycetes + Hyphochytriomycetes + *Developayella* + *Pirsonia* + autotrophic stramenopiles (ML 96%, MP 94%, LogDet 100%, and posterior probability 1.00). It is,

therefore, highly probable that Cavalier-Smith's group Bigyra is not monophyletic.

The results of our analyses are in agreement with Patterson's hypotheses based on morphological data—our results support the affiliation of opalinids to Stramenopila and the monophyly of Slopalinida. They also confirm the sister position of *Blastocystis* to Slopalinida. It seems, however, that the information contained in SSU rDNA sequences is insufficient to reconstruct the phylogeny within Stramenopila and we need more sequences of other genes to understand the history of this interesting and ecologically important group.

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Short communication

Phylogenetic position of *Karotomorpha* and paraphyly of Proteromonadidae

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1. Introduction

The taxon Slopalinida (Patterson, 1985) comprises two families of anaerobic protists living as commensals in the intestine of vertebrates. The proteromonadids are small flagellates (ca. 15 μm) with one nucleus, a single large mitochondrion with tubular cristae, Golgi apparatus and a fibrillar rhizoplast connecting the basal bodies and nucleus (Brugerolle and Mignot, 1989). The number of flagella differs between the two genera belonging to the family: *Proteromonas*, the commensal of urodelans, lizards, and rodents, has two flagella, whereas *Karotomorpha*, the commensal of frogs and other amphibians, has four flagella. The surface of the cell is folded, the folds are supported by single microtubules (*Proteromonas*) or by ribbons of several laterally interconnected microtubules (*Karotomorpha*). The transitional flagellar region contains double transitional helix. The posterior part of *Proteromonas* cell is covered with fine tubular hairs—the somatonemes (Brugerolle and Joyon, 1975).

The representatives of the second family—Opalinidae—are quite different from the proteromonadid flagellates. They are multinucleated and multiciliated, often large (up to several mm). They are common commensals of frogs, some can inhabit the intestine of urodelans or fish. The family comprises three binucleated genera (*Protoopalina*, *Protozelleriella*, *Zelleriella*) and two genera with up to hundreds of nuclei (*Cepedea*, *Opalina*). Besides nuclei the cell contains a large number of mitochondria with tubular cristae, Golgi complexes and small digestive vacuoles (Delvinquier and Patterson, 1993). The cell surface is heavily folded, the folds are supported by ribbons of microtubules in a very similar way as in *Karotomorpha*. The ultrastructure of flagellar transitional region is alike that of proteromonadids as well, double transitional helix is present. These similarities led Patterson (1985) to unite the two families in the order Slopalinida and to postulate the paraphyly of the family Proteromonadidae (*Karotomorpha* being closer to the opalinids). The ultrastructure of flagellar transition region and proposed homology between the somatonemes of *Proteromonas* and mastigonemes of heterokont flagellates led him further to conclude that the slopalinids are relatives of the heterokont algae, in other words that they belong among stramenopiles. Phylogenetic analysis of Silberman et al. (1996) not only confirmed that *Proteromonas* is a stramenopile, but also showed that its sister group is the genus *Blastocystis*, the strange intestinal parasite of both vertebrates and invertebrates with multinuclear spherical cells and no flagella (Stenzel and Boreham, 1996). The morphological diversity within the slopalinida + *Blastocystis* group is thus tremendous, ranging from flagellates to multinucleated nonflagellated human parasites or ciliate-like opalinids. The monophyly of slopalinids was confirmed by phylogenetic analyses of SSU rDNA later on (Kostka et al., 2004; Nishi et al., 2005), yet none of these analyses included any molecular data for *Karotomorpha* and thus could not answer the problem of the paraphyly of the family Proteromonadidae.

In this study, we report the SSU rDNA gene of two *Karotomorpha* isolates, we examine the phylogenetic position of *Karotomorpha* within slopalinids and question the monospecifity of the genus.

2. Materials and methods

2.1. DNA isolation, SSU rDNA amplification and sequencing

Two *Karotomorpha* isolates were isolated with a glass Pasteur pipette from cloacae of two frog hosts—northern

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leopard frog (*Rana pipiens*, imported to the Czech Republic from North America; isolate RAPI1) and common toad (*Bufo bufo*, captured from wild in the Czech Republic; isolate ROP8). Three genera of protists were observed in the RAPI1 isolate: *Karotomorpha*, an enteromonad *Trimitus* sp., and a parabasalid *Trichomitus* sp. *Karotomorpha* and an unidentified parabasalian were observed in the ROP8 isolate. Neither of the two *Karotomorpha* isolates was cultured.

The genomic DNA was immediately isolated using High pure PCR template preparation kit (Roche). Eukaryotic-specific primers MedlinA (CGT GTT GAT CCT GCC AG) and MedlinB (TGA TCC TTC TGC AGG TTC ACC TAC) (Medlin et al., 1988) were used to amplify SSU rDNA of the RAPI1 isolate. The resulting product was cloned into pGEM-T Easy vector (Promega) and sequenced using 3100-Avant genetic analyser. Among 5 examined clones, two belonged to *Trichomitus* (BlastN E-value 10^{-108}), two were identical to those of *Trimitus* (Kolisko et al., 2005) and one had the closest match to *Protoopalina intestinalis* (BlastN E-value 10^{-108}). This sequence was ascribed to *Karotomorpha* sp. Partial SSU rDNA sequence of the other isolate (ROP8) was amplified with primers F2 (GAA GAA TTY GGG TTY GAT TT) and R1 (CCT TCC TCT AAA TRR TAA GA) designed on the basis of SSU rDNA sequences of *Proteromonas* and *Blastocystis* as specific primers for the *Blastocystis* + Slopalinida group. The resulting PCR product was cloned and sequenced. All five examined clones belonged to *Karotomorpha* (showed 95% similarity to *Karotomorpha* from the RAPI1 isolate). GenBank accession numbers of the two sequences are DQ431242 and DQ431243.

2.2. Phylogenetic analyses

The dataset prepared to study the phylogenetic position of *Karotomorpha* consisted of a total of 1440 unambiguously aligned positions of 43 SSU rDNA sequences including the two *Karotomorpha* isolates, 34 other stramenopiles and seven outgroups (alveolates and haptophytes). All available sequences of slopalinids were included (but only one representative was chosen for those which were identical—AB105337–AB105339 and AB105341–AB105343). Sequences with GenBank Accession Nos. AF141969, AF141970 and AF142474 were not included because they are zygomycete contaminations, see Kostka et al., 2004. The sequences were aligned using the program ClustalX 1.18 (Thompson et al., 1997). Resulting alignment was manually edited using the program BioEdit (Hall, 1999).

Maximum likelihood (ML) phylogenetic trees were constructed using PAUP 4.0β10 (Swofford, 2002) employing the Tamura-Nei model+Γ+I chosen with Modeltest 3.06 (Posada and Crandall, 1998). Maximum parsimony (MP), Fitch-Margoliash method with LogDet distances (LogDet) and maximum likelihood distances (MLDist) were also performed with PAUP 4.0β10. All heuristic tree searches were conducted with 10 replicates with the starting tree constructed by random taxa addition and swapped by the TBR algorithm. The support for topology was estimated by the use of 100 (ML) or 1000 (MP, LogDet, MLDist) bootstrap-replicates. Bayesian analysis (BA) was conducted using MrBayes 3.1.1 (Huelsenbeck and Ronquist, 2001) with 4 simultaneous Markov chains Monte Carlo, temperature 0.2, 2.5×10^6 generations (until average standard deviation of split frequencies was lower than 0.01) with the sampling frequency 100 and burn-in 6250 trees.

3. Results and discussion

We determined 1424 bp of *Karotomorpha* sp. (ROP8 isolate) and 1858 bp of *Karotomorpha* sp. (RAPI1 isolate) SSU rRNA gene. The sequences differed in 4.7% positions, ca. 65% of the differences were concentrated in three variable regions of the total length of 91 bp. These regions correspond to the varible region V4, helix 43 and a region between helices 45 and 46, as numbered by Wuyts et al. (2000). Opalinid SSU rDNA contains opalinid specific insert (Nishi et al., 2005) in the last mentioned region. Inserts of both *Karotomorpha* isolates in this region differ from opalinid insert in being rather GC rich. The difference between the two *Karotomorpha* isolates (4.7%) is comparable to or greater than that of well-defined species of other parasitic flagellates, e.g., *Trichomonas vaginalis*/*T. tenax* (2.1%), or even genera of other stramenopiles, e.g., chrysophytes *Ochromonas tuberculata*/*Chromulina chionophila* (4.2%). Alverson and Kolnick (2005) shown that there may be some intragenomic polymorphism in SSU rDNA genes—up to nearly 2% in some *Skeletonema* species. However, the distribution of polymorphic sites is different in their case—they never cluster in variable regions, but are scattered along the SSU rDNA sequence. We therefore assume that intragenomic polymorphism (sequencing different paralogs) is not responsible for majority of observed differences between SSU rRNA genes of the two *Karotomorpha* isolates. Grassé (1926) redescribed the species *Karotomorpha bufonis* from European amphibians and described a new species *Karotomorpha swezei* from American amphibians. However, Kulda (1961) showed that the latter *Karotomorpha* species was described on the basis of misinterpretation of morphological data. Nevertheless, according to our findings, we can assume that the genus *Karotomorpha* might contain more species, one of them European, another one American. These species may be morphologically undistinguishable (on our Giemsa-stained preparations we were not able to distinguish between ROP8 and RAPI1 isolates).

Analyses of our data set resulted into a tree (Fig. 1) showing a monophyletic genus *Karotomorpha* as a member of well resolved order Slopalinida. Short fragments of SSU rDNA genes of some opalinids in alignment (only 159 positions, rest substituted with "N"s) obviously confuse the LogDet and MLDist analyses implemented in PAUP, causing low bootstrap support for the genus *Karotomorpha*. When these four sequences (*Protoopalina japonica* RN1,

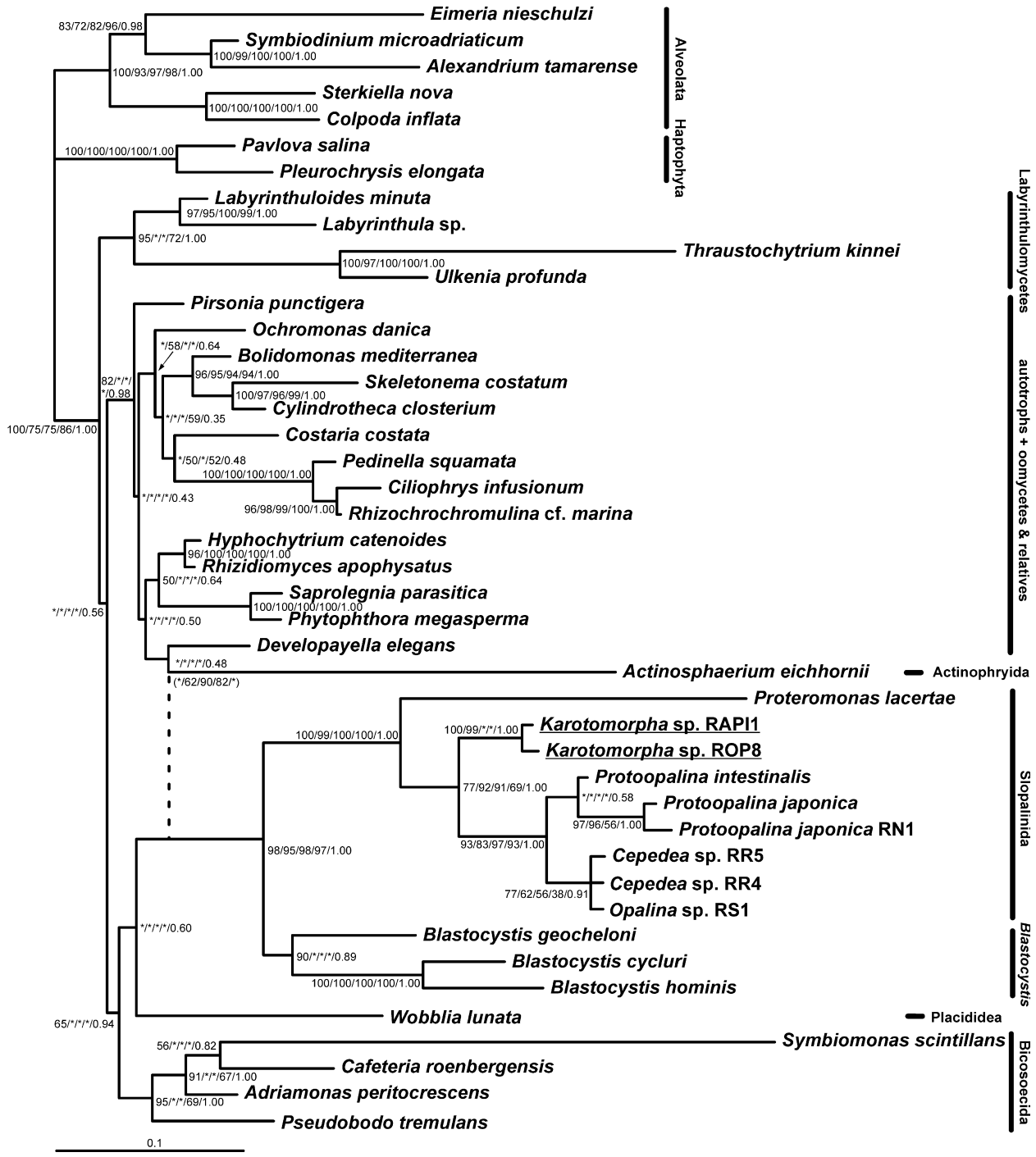

Fig. 1. Tree resulting from Bayesian analysis of SSU rDNA showing relationships among 36 stramenopile taxa + seven alveolate and haptophyte outgroups. Bootstrap values from maximum likelihood (100 replicates), maximum parsimony (1000 replicates), Fitch-Margoliash method with Log Det distances (1000 replicates), maximum likelihood distances (1000 replicates) and Bayesian posterior probabilities are shown at the nodes, respectively. Asterisk represents bootsrap value lower than 50%. *A. eichhornii* was shown by ML and BA to be the sister group to *Developayella*, but other methods resolved it as a sister group to Slopalinida + *Blastocystis*. Statistical support for both positions is shown in the picture.

Cepedea sp. RR4 and RR5, *Opalina* sp. RS1) are omitted from analyses, bootstrap support for monophyletic *Karotomorpha* grows to the values of 100 for both LogDet and MLDist. Similar effect has analysis of only those positions present in all taxa (LogDet 92%, MLDist 66%). The monophyly of the order Slopalinida was very well supported by bootstrap values 99% and more and Bayesian posterior probability 1.00. Within slopalinids, the family Proteromonadidae was shown to be paraphyletic as *Karotomorpha* was more closely related to opalines than to the genus *Proteromonas* (bootstrap support for this topology was ML 77%, MP 92%, LogDet 91%, MLDist 69%, posterior probability 1). *Blastocystis* was resolved as a sister group to the order Slopalidida with very good bootstrap support.

Slopalinids + *Blastocystis* were nested within stramenopiles, which was well supported, too. This result is in agreement with other studies based on SSU rRNA gene (Kostka et al., 2004; Nishi et al., 2005), but disagrees with alternative placement of opalinids among alveolates as based on tubulin genes in the latter study.

Other groups of stramenopiles were recovered: Labyrinthulomycetes, bicosoecids, autotrophic stramenopiles, Oomycetes + their relatives (Hyphochytriomycetes and *Developayella*). Interrelationships among the main groups of stramenopiles remained unresolved, only a grouping comprising autotrophs and oomycetes + relatives was recovered. Quite surprisingly, actinophriid heliozoan *Actinosphaerium* was shown by ML and Bayesian analysis to belong to this group with good statistical support (ML 82%, BA 0.98). However, the other methods used to reconstruct tree topology showed *Actinosphaerium* as a sister group of slopalinids + *Blastocystis* with reasonable bootstrap support (MP 62%, LogDet 90%, MLDist 82%), in agreement with the study of Cavalier-Smith and Chao (2006). In any case, none of our analyses showed *Actinosphaerium* to be sister group of *Ciliophrys* or other pedinellid. Based on morphological data, pedinellids were hypothesised to be close relatives of actinophryid heliozoans represented here by *Actinosphaerium* (see Mikrjukov and Patterson, 2001; Nikolaev et al., 2004).

The results of our analyses confirmed Patterson's hypothesis of a close relationship between *Karotomorpha* and opalinids. The family Proteromonadidae comprising both genera *Proteromonas* and *Karotomorpha* was shown to be paraphyletic. The addition of the two *Karotomorpha* SSU rDNA sequences further supports monophyly of Slopalinida and its position within Stramenopila.

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Short Communication

Non-monophyly of Retortamonadida and high genetic diversity of the genus *Chilomastix* suggested by analysis of SSU rDNA

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1. Introduction

Retortamonads (Retortamonadida) are a small group of protists comprising flagellates living mostly as intestinal commensals of both vertebrates and invertebrates (Kulda and Nohýnková, 1978), although free-living representatives have been also found (Bernard et al., 1997). Potential pathogenicity has been reported for some species from vertebrates. Although medically unimportant, retortamonads have attracted attention because of their evolutionary history. Their cells lack some typically eukaryotic organelles, the mitochondrion in particular, and retortamonads were once considered to be one of a series of eukaryotic lineages – 'Archezoa' – that had diverged before the acquisition of the mitochondrial organelle (Cavalier-Smith, 1983, 1987). However, this hypothesis has fallen into disfavor, as relict mitochondria have been found in most of the putative archezoan groups (see Simpson and Roger, 2004). Although retortamonads are one of the last eukaryotic groups for which no sign of a mitochondrial past has yet been found, it has been shown that they are closely related to diplomonads (Silberman et al., 2002; Hampl et al., 2008; Kolisko et al., in press), whose cells do possess a mitochondrial remnant, the 'mitosome' (Tovar et al., 2003). It is, therefore, generally assumed that retortamonads are also secondarily amitochondriate.

Although numerous species of retortamonads have been described they are assigned to just two genera, the biflagellated *Retortamonas* and the quadriflagellated *Chilomastix* (Kulda and Nohýnková, 1978). The characteristic features of retortamonads include four basal bodies arranged in two pairs, two or four flagella, one of them being directed posteriorly and associated with well-developed cytostome, which continues as a curving cytopharynx. There is also a microtubular corset underlying the cell surface (Brugerolle, 1973, 1977, 1991; Kulda and Nohýnková, 1978; Bernard et al., 1997). The morphological synapomorphies of Retortamonadida were defined by Simpson and Patterson (1999). Retortamonad cells also possess all features typical for "true excavates" (Simpson and Patterson, 1999; Simpson, 2003) and are currently classified into the eukaryotic supergroup Excavata (Cavalier-Smith, 2002; Simpson, 2003; Adl et al., 2005).

Retortamonadida was proposed as a holophyletic lineage. Until the present study, molecular data of a single retortamonadid genus, *Retortamonas*, have been available (Silberman et al., 2002; Hampl et al., 2008; Kolisko et al., in press) and *Chilomastix* was always assumed to be sister to *Retortamonas*. Molecular phylogenetic studies have shown clearly that retortamonads are closely related to diplomonads (Diplomonadida), *Carpediemonas* and *Dysnectes* (Silberman et al., 2002; Simpson et al., 2002; Kolisko et al., 2005; Yubuki et al., 2007), together forming the monophyletic group Fornicata (Simpson, 2003). Fornicate morphological synapomorphies have been defined recently (Adl et al., 2005; Yubuki et al., 2007).

Retortamonads have often been regarded to be closely related to diplomonads on the basis of the ultrastructure of the flagellar apparatus and the presence of cytostomes and cytopharynges (Brugerolle, 1977, 1991; Cavalier-Smith, 1993). Together, they were named Eopharyngia (Cavalier-Smith, 1993) though eopharyngian morphological synapomorphies have not been defined so far (see Simpson, 2003). The close relationship between the two groups has been further supported by molecular phylogenetics (Silberman et al., 2002; Hampl et al., 2008; Kolisko et al., in press). Diplomonads, the largest fornicate group, comprise both parasitic and free-living flagellates. The most remarkable difference

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between retortamonads and diplomonads is that while retortamonads are unizoic (their cells contain a single set of organelles), diplomonads are mostly diplozoic (their cells contain two axially symmetrical sets of organelles including doubled nucleus and cytoskeleton). On the basis of the presence or absence of cytostomes (Kulda and Nohýnková, 1978), use of canonical vs. non-canonical genetic code (Keeling and Doolittle, 1997) and molecular phylogenetic studies (Kolisko et al., 2005; Keeling and Brugerolle, 2006; Jørgensen and Sterud, 2007), diplomonads have been divided into two monophyletic groups, Hexamitinae and Giardiinae. Interestingly, unizoic enteromonads, which had been hypothesized as ancestors of diplozoic diplomonads (Siddall et al., 1992), branch within the Hexamitinae indicating that either diplomonads arose several times independently from unizoic cells or that unizoic enteromonads arose from diplozoic diplomonads (Kolisko et al., 2005, in press). It has been recently established that the symmetry of diplomonad cells is, at least in case of *Giardia intestinalis*, only superficial and that there is considerable asymmetry in the karyotypes and behavior of the two nuclei during the cell cycle (Tůmová et al., 2007). Moreover, mastigonts of a single *Giardia* cell exchange a flagellum during each cell cycle (Nohýnková et al., 2006).

Although phylogenetic analyses based on the SSU rDNA gene sequences strongly support monophyly of Eopharyngia, the exact relationship between retortamonads and diplomonads remains unclear. Phylogenetic studies analysing the SSU rDNA gene are undecided as to whether retortamonads are sister to the Giardiinae lineage, making diplomonads paraphyletic, or whether diplomonads are monophyletic and retortamonads form their sister branch. (Silberman et al., 2002; Simpson et al., 2002; Kolisko et al., 2005; Keeling and Brugerolle, 2006; Yubuki et al., 2007). On the other hand, analyses of HSP90 gene sequences support monophyly of diplomonads to the exclusion of retortamonads (Kolisko et al., in press) which corresponds with analyses based on ultrastructural data (Siddall et al., 1992; Simpson, 2003). Apart from Eopharyngia, two free-living excavate flagellate genera, *Carpediemonas* and *Dysnectes*, belong to Fornicata. However, both ultrastructural and molecular-phylogenetic approaches have not fully resolved phylogenetic relationships between the three fornicate lineages (Yubuki et al., 2007).

So far, hypotheses on retortamonad evolutionary history have been based solely on sequence data from the genus *Retortamonas*. Although *Chilomastix*, the second of the two retortamonad genera, has not been forgotten by protozoologists, the unavailability of isolates made most research impossible. We have cultured two different *Chilomastix* species, *C. mesnili* and *C. wenrichi*, have sequenced their SSU rDNA and performed phylogenetic analyses. The present paper represents the first phylogenetic study that includes sequences from the genus *Chilomastix*. Our data strongly suggest that Retortamonadida are not monophyletic, but that they are paraphyletic and that diplomonads branch inside them. We, therefore, propose a new scenario of evolution of Eopharyngia. Our study also reveals considerable genetic diversity within *Chilomastix*.

2. Materials and methods

2.1. Organisms

Chilomastix wenrichi isolate CAVIA2 was obtained from the large intestine of a Guinea pig (*Cavia porcellus*). *Chilomastix mesnili* isolate FAB was obtained from feces of a human patient suffering from diarrhea who had recently returned to the Czech Republic from South America. The isolates were xenically cultured with bacteria (FAB) or with bacteria and *Blastocystis* sp. (CAVIA2) in Dobell and Leidlaw's biphasic medium (Dobell and Leidlaw, 1926) at 37 °C and were maintained by serial transfer every 2–4 days. The isolates are deposited in the culture collection of the Department of Parasitology of Charles University in Prague, Czech Republic. To confirm species identity of the *Chilomastix* isolates, their morphology was examined on protargol-stained preparations. Moist films spread on coverslips were prepared from pelleted cultures obtained by centrifugation at 500 g for 8 min. The films were fixed in Bouin-Hollande's fluid for 15 h, were washed with 70% ethanol, and were stained with 1% protargol (Bayer, I. G. Farbenindustrie AG, Germany) following the Nie's (1950) protocol.

2.2. DNA isolation, amplification, cloning and sequencing

Genomic DNA was isolated using the High pure PCR template preparation kit (Roche Applied Science). Eukaryote-specific primers MedlinA (CGTGTTGATCCTGCCAG) and MedlinB (TGATCCTTC TGCAGGTTCACCTAC) (Medlin et al., 1988) were used to amplify SSU rDNA with an annealing temperature of 45 °C. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and were cloned into the pGEM®-T EASY vector using the pGEM®-T EASY VECTOR SYSTEM I (Promega). Eleven clones from three independent PCRs of the isolate CAVIA2 were partially sequenced using the primer MedlinA. Two of the obtained sequences were SSU rDNA of *Blastocystis* sp. The other nine sequences, which were almost identical, were ascribed to *Chilomastix wenrichi* and were further sequenced. Four clones of the *C. mesnili* isolate FAB originating from two independent PCRs were sequenced. All clones were sequenced bidirectionally by primer walking. Sequence data reported in this paper are available in GenBank under accession numbers EF450168 and EU009463–EU009466.

2.3. Phylogenetic analyses

Four data sets containing sequences of SSU rDNA were created. The first data set contained 9 sequences of fornicates including two *Chilomastix* species and 33 sequences representing a broad range of other eukaryotic taxa. The second data set contained 16 sequences of fornicates and 6 sequences of other excavates. The third data set contained only sequences of fornicate taxa. The fourth data set contained the same sequences as the second data set, plus fragments of SSU rDNAs obtained from fin whale (*Balaenoptera physalus*) feces (GenBank accession numbers AY392799, AY392812, AY392815, and AY392816) by Jarman et al. (2004). Sequences from each data set were aligned using the T-Coffee method (Notredame et al., 2000) with the help of the T-Coffee@igs server http://www.igs.cnrs-mrs.fr/Tcoffee/ (Poirot et al., 2003). To fit the requirements of the server, sequences of *Retortamonas* spp., *Chilomastix mesnili* and *Euglena gracilis*, which were longer than 2000 nucleotides, were shortened by deleting the most divergent parts of their long insertions not aligned with any other taxon by ClustalX 1.81 (Thompson et al., 1997). The resulting alignments were manually edited using BioEdit 7.0.4.1 (Hall, 1999). The fourth data set was then trimmed: the sites for which sequences obtained from whale feces had only gaps were omitted. The resulting alignments contained 1044, 1067, 1094, and 177 characters, respectively. The alignments are available from the corresponding author upon request.

Phylogenetic analyses were conducted using the maximum parsimony (MP), Fitch-Margoliash with Logdet (LD) distances, Fitch-Margoliash with maximum likelihood distances (MLdist), and maximum likelihood (ML) methods implemented in PAUP* 4.0b10 (Swofford, 2002), and by the Bayesian method implemented in MrBayes 3.1.1 (Ronquist and Huelsenbeck, 2003). The models of nucleotide substitution for the ML and MLdist analyses were chosen by hierarchical nested likelihood ratio tests implemented in Modeltest 3.06 (Posada and Crandall, 1998). The models were selected as follows: TrNef + I + Γ for the first and second data sets, TrN + I + Γ for the third data set, and TrNef + Γ for the fourth

data set. The proportion of invariable sites for LD analysis was estimated from a neighbor-joining tree. MP, LD, MLdist, and ML trees were constructed by ten replicates of a heuristic search in which the starting tree was obtained by the stepwise addition procedure with a random order of taxa addition and swapped using the tree bisection and reconnection (TBR) algorithm. The trees were bootstrapped with 1000 (300 for ML in case of the first data set) replicates, each with ten replicates of random taxon addition with TBR branch swapping. For the Bayesian analyses, base frequencies, rates for the six different types of substitution, the proportion of invariable sites, and the shape parameter of the gamma correction for the rate heterogeneity (approximated by four discrete categories) were allowed to vary. A covarion model was used to allow rate heterogeneity along the tree. The number of generations of Markov chain Monte Carlo was 10^6 for the second and fourth data set, 2×10^6 for the third data set, and 3×10^6 for the first data set (until average standard deviation of split frequencies was lower than 0.01) and the trees were sampled every 100 generations. First 2500 (second and fourth data set), 5000 (third data set) or 7500 (first data set) trees were discarded as burn-in.

Alternative positions of the genus *Chilomastix* were tested using AU tests implemented in consel 0.1i (Shimodaira and Hasegawa, 2001). The trees of highest likelihood whose topologies corresponded to the tested hypotheses were constructed by ten replicates of a heuristic search with TBR branch swapping under constraints defined by particular hypotheses. The trees were tested against the 500 trees of highest likelihood found during the heuristic search for the best tree. Site likelihoods were calculated using PAUP*.

3. Results

The SSU rDNA sequences of the two *Chilomastix* species were rather different in length and base composition. The first BlastN hit for *C. wenrichi* was *Octomitus intestinalis* with an *E* value of 10^{-82}; the first BlastN hit for *C. mesnili* was an uncultured eukaryote (GenBank accession number AY392816) with an *E* value 10^{-103}. The sequences differed markedly in length. Whereas *Chilomastix wenrichi* had a short SSU rDNA sequence (1488 bp with primers; GC content 63%), the corresponding sequence from *C. mesnili* was rather long (ca 2500 bp; GC content 56%). There were considerable differences among particular *C. mesnili* SSU rDNA clones (uncorrected *p*-distance up to 1.6%), including up to 14 bp long indels, suggesting that several different paralogs of the SSU rRNA gene exist in the *C. mesnili* genome. Only minor differences were found between *C. wenrichi* SSU rDNA clones (up to 0.5%; no indels) and were probably due to Taq-polymerase errors.

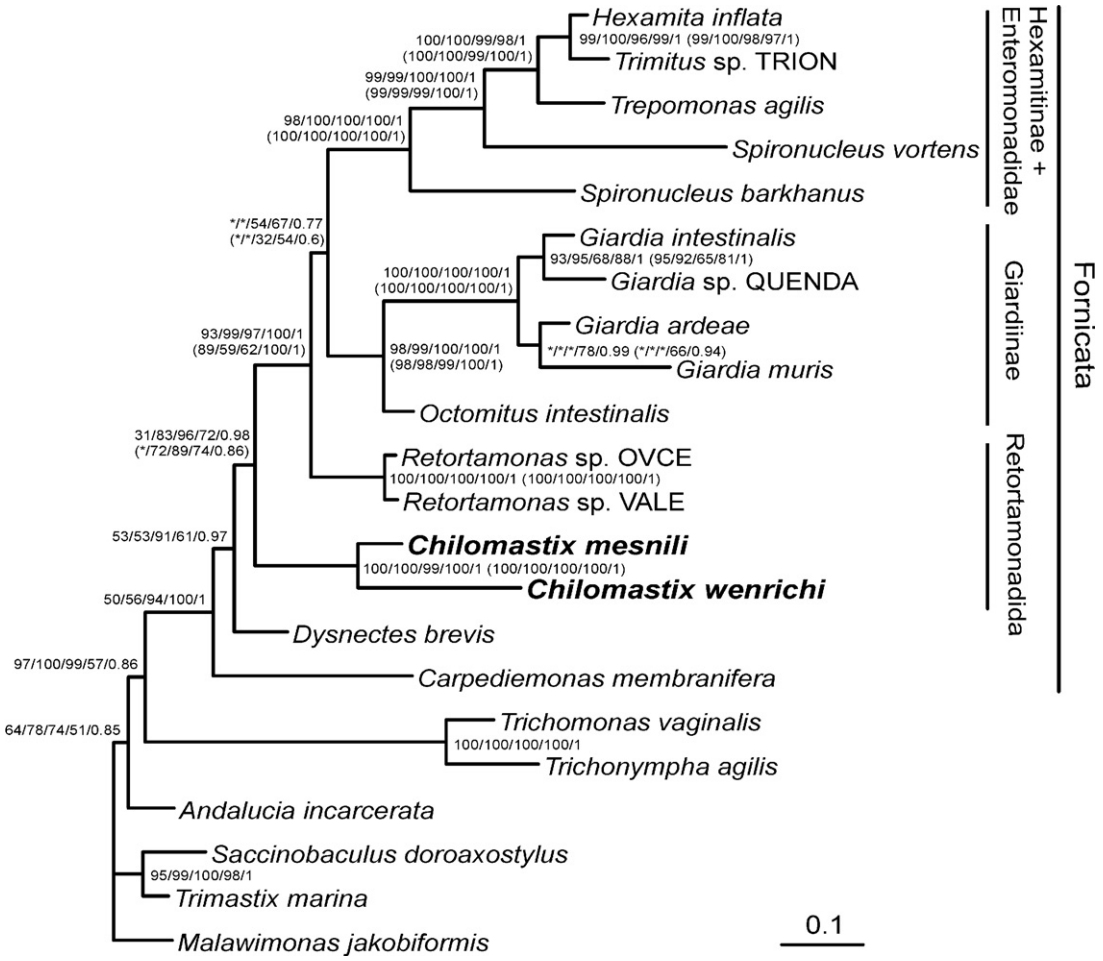

Fig. 1. Phylogenetic tree of Fornicata, rooted by other excavates, based on the SSU rRNA gene sequences. The tree was constructed from the second data set by the maximum likelihood method under TrNef + I + Γ model. Bootstrap values from maximum parsimony, Fitch-Margoliash method with Log Det distances, maximum likelihood distances, maximum likelihood, and Bayesian posterior probabilities are shown at the nodes, respectively. The corresponding values obtained by analyses of the third data set are shown in parentheses at the important nodes. Asterisks indicate nodes with a different topology resolved by the respective method.

Phylogenetic analysis of the first data set with a broad sampling of eukaryotic diversity showed monophyletic Fornicata (including *Chilomastix*) with low to high statistical support (bootstrap values for MP, LD, MLdist and ML were 51, 65, 88, and 100, respectively; Bayesian posterior probability 1; not shown), always with *Carpediemonas* forming basal branch. Therefore, detailed analysis of Fornicata was performed. A maximum likelihood tree based on the second data set is given in Fig. 1. Monophyletic Fornicata were recovered by all methods and were statistically strongly supported by MLdist and ML (bootstrap values 91 and 100, respectively) and the Bayesian analysis (posterior probability 1), but only weakly supported by the MP and LD analyses (bootstrap values 50 and 56, respectively). *Carpediemonas membranifera* (rather than *Dysnectes brevis*) formed the basal branch of Fornicata, though only the MLdist analysis supported the position well (bootstrap value 91), the support from the other methods being weak (bootstrap values 53–61, Bayesian posterior probability 0.97). A monophyletic Eopharyngia grouping was recovered by all methods and was well-supported by some of them (bootstrap values for MP, LD, MLdist, and ML were 31, 83, 96, and 72, respectively; Bayesian posterior probability 0.98).

Within Eopharyngia, the genus *Chilomastix* formed a clade that was strongly supported by all methods (bootstrap values 99–100, Bayesian posterior probability 1). Surprisingly, *Chilomastix* fell in a basal position within Eopharyngia, and support for this placement was strong with all methods (bootstrap values 93–100, Bayesian posterior probability 1). The rest of Eopharyngia formed three robust clades (bootstrap values always 98–100, Bayesian posterior probability 1), (i) *Retortamonas*, (ii) a Hexamitinae clade including enteromonads and (iii) Giardiinae. The relationships between the three clades, however, remained unclear and were not well supported by any method. In MP and LD analyses, *Retortamonas* and Giardiinae were sister groups (bootstrap values 42 and 80, respectively). By contrast, Giardiinae and Hexamitinae formed a common branch in MLdist, ML and Bayesian analyses (bootstrap values 54 and 67, respectively; Bayesian posterior probability 0.77).

Phylogenetic trees obtained from analyses of the third data set had the same topology as the trees from the second data set, except for the MP analysis, where a monophyletic Eopharyngia were not recovered (*Chilomastix* branched with *Dysnectes* instead of other Eopharyngia). In the LD, MLdist, ML and Bayesian analyses Eopharyngia were recovered with similar support (bootstrap values 72, 89 and 74, respectively; Bayesian posterior probability 0.86) as in the analyses of the first data set. The monophyly of the genus *Chilomastix* was again highly supported (bootstrap values 100, Bayesian posterior probability 1), and *Chilomastix* always formed the deepest branch within Eopharyngia (bootstrap values for the clade of *Retortamonas* and diplomonads were for MP, LD, MLdist and ML, 89, 59, 62 and 100, respectively; Bayesian posterior probability 1). Giardiinae, Hexamitinae and *Retortamonas* were always recovered as clades and were well supported (bootstrap values 98–100, Bayesian posterior probabilities 1). As in the case of the second data set, *Retortamonas* and Giardiinae clades formed a common branch in the MP and LD analyses (bootstrap values 55 and 35, respectively), while Giardiinae and Hexamitinae formed a common branch in the MLdist, ML and Bayesian analyses (bootstrap values 32 and 54, respectively; Bayesian posterior probability 0.6).

In a likelihood framework, three alternative hypotheses concerning the placement of the genus *Chilomastix* were evaluated using AU tests (the first hypothesis was tested using both second and third data set, while the remaining two hypotheses were tested using only the second data set). The first hypothesis was that Retortamonadida is monophyletic, i.e. *Retortamonas* and *Chilomastix* are clade. This hypothesis was rejected at the 1% confidence level for both data sets (p-values 0.004 and 2×10^{-7}, respectively). The second hypothesis was that *Chilomastix* forms the basal branch of Fornicata. This hypothesis could not be rejected ($p = 0.738$). The third hypothesis was that *Chilomastix* branches more basally than Parabasala which are sister to remaining fornicates. This hypothesis was rejected at the 5% confidence level ($p = 0.02$).

Topologies of trees constructed from the fourth data set were ill-resolved due to a low amount of data (the alignment consisted of 177 characters) and differed according to the particular method. However, *Chilomastix mesnili* always formed a robust clade (bootstrap values 98–100, Bayesian posterior probability 1) with the eukaryotes obtained from fin whale feces (not shown).

4. Discussion

The evolution of Fornicata, one of recently recognized major eukaryotic groups (Simpson et al., 2002; Simpson, 2003; Adl et al., 2005), is poorly understood and the relationships among fornicate taxa are still poorly resolved. Previous hypotheses concerning the evolution of retortamonads and diplomonads have been based solely on ultrastructural data (Brugerolle, 1973, 1977, 1991; Kulda and Nohýnková, 1978; Simpson and Patterson, 1999) or, when including molecular data, consider only a single retortamonad genus, *Retortamonas* (Silberman et al., 2002; Kolisko et al., 2005; Keeling and Brugerolle, 2006; Yubuki et al., 2007). These studies have always assumed (or recovered) the monophyly of Retortamonadida on the basis of the strikingly similar cell structure of *Retortamonas* and *Chilomastix*. Simpson and Patterson (1999) defined exclusive retortamonadid morphological synapomorphies not shared with other eukaryotes including, most importantly, diplomonads. Results of the present study are, however, in considerable disagreement with the anticipated models of evolution of Fornicata.

In our analyses, Fornicata is split into four branches: *Carpediemonas membranifera*, *Dysnectes brevis*, *Chilomastix* spp., and *Retortamonas* spp. + Diplomonadida. The close relationship of *Retortamonas* and diplomonads is consistent with previous studies, as is the weak resolution of the position of *Retortamonas* within this clade (Silberman et al., 2002; Simpson et al., 2002; Kolisko et al., 2005; Keeling and Brugerolle, 2006; Yubuki et al., 2007). The hypothesis of a monophyletic Diplomonadida forming the sister branch to *Retortamonas* is favored by morphological data and by analyses of HSP90 gene (Kolisko et al., in press). Interestingly, *Chilomastix* forms a sister branch to the clade of *Retortamonas* + diplomonads instead of branching with *Retortamonas* only. The basal position of the genus *Chilomastix* in Eopharyngia was robustly supported by all examined methods of tree reconstruction, and a monophyletic Retortamonadida was rejected by AU tests. Although we could not rule out the possibility that *Chilomastix* branches even more basally than *Carpediemonas* and *Dysnectes*, we support the hypothesis of a monophyletic Eopharyngia as it was preferred by most phylogenetic methods (only the maximum parsimony analysis of one of two full-length datasets recovered an alternative optimal tree) and conforms to previous hypotheses. However, morphological synapomorphies of Eopharyngia have not been defined so far (see Simpson, 2003). As independent evolution of the distinctive retortamonadid morphology in ancestors of *Retortamonas* and *Chilomastix* would seem rather improbable, we interpret our topology as suggesting that Retortamonadida are paraphyletic rather than polyphyletic.

Our results allow us to propose a new scenario of the evolution of Eopharyngia. If Diplomonadida truly form an internal branch of Retortamonadida, they must have once possessed complete set of retortamonadid (and also excavate) features. These characters would have been lost during the early evolution of Diplomonadida, and diplozoic cells appeared. The groove-like cytostomes of Retortamonadida changed to tube-shaped ones in some

phagotrophic Hexamitinae or have been lost altogether in the pinocytotic Giardiinae. Two microtubular fibres surrounding the nuclei, the infra- and supranuclear fibres, were formed either *de novo* or by modification of existing retortamonadid microtubular structures, possibly in connection to the loss of the microtubular corset. The supranuclear fibre was putatively homologized by Simpson (2003) with the R4 (or anterior) root of some excavate taxa, including the fornicates *Carpediemonas* and *Dysnectes*. Interestingly, neither *Retortamonas* nor *Chilomastix* possesses an R4. We therefore assume that the supranuclear fibre could be either a novel structure or a remnant of the subpelicular corset supporting the dorsal side of each nucleus, rather than a homolog of R4.

Our new hypothesis on the phylogeny and evolution of Eopharyngia is based on single-gene analyses. To rule out the possibility that the paraphyly of Retortamonadida is an artifact, perhaps caused by the divergent nature of their SSU rDNA sequences, analyses of more genes must be performed in the future. However, only SSU rDNA sequences have been published so far for *Dysnectes* and *Chilomastix* (Yubuki et al., 2007; this paper). The taxon sampling of the molecular-phylogenetic analyses is also still poor, in particular the sampling of Retortamonadida. Sequences for only two apparently closely related *Retortamonas* species are available to date. The situation is further complicated by the fact that previous TEM studies were performed on *Retortamonas* species from insects (Brugerolle, 1977, 2006) while molecular studies have examined only *Retortamonas* spp. from vertebrates (Silberman et al., 2002). According to our TEM studies (Kulda et al., unpublished), *Retortamonas* spp. from vertebrates differ considerably from those from insects by the absence of the subpellicular microtubular corset (an extended version of the dorsal fan of other typical excavates), which we regard as a very important structure in evolution of Eopharyngia. The possibility that they represent, in fact, two different evolutionary lineages should be investigated and new phylogenetic studies based both on morphological and phylogenetic data should be performed. A convincing reconstruction of eopharyngian phylogeny can be obtained only by a multi-gene study. It is clear, however, that before such a study can be conducted, it will be of crucial importance to improve the taxonomic sampling of this still enigmatic taxon.

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Introduction of *Mayorella gemmifera* Schaeffer, 1926 into phylogenetic studies of Amoebozoa

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Summary. Marine amoeba *Mayorella gemmifera* Schaeffer, 1926 that was isolated by F. C. Page and deposited in the Culture Collection of Algae and Protozoa (now UK National Culture Collection) in 1981 as strain CCAP 1547/8 was re-examined using light and electron microscopy. Its morphology, consistent with species original description and the newly obtained SSU rDNA sequence qualifies this strain for phylogenetic studies on the Amoebozoa. Since *Mayorella bigemma* Schaeffer, 1926, the type species of *Mayorella* Schaeffer, 1926, is not available in culture collections, the CCAP strain of *M. gemmifera* can substitute the latter and supplement the only *Mayorella* strain (JJP-2003) representing thus far the genus in phylogenetic studies. Comparison of *M. gemmifera* with JJP-2003 strain revealed some differences in primary and secondary structures of their SSU rDNA sequences; nevertheless, our phylogenetic analyses clearly demonstrated that sequences of both *Mayorella* strains cluster together and their position within Amoebozoa is well supported.

Key words: *Mayorella gemmifera*, morphology, phylogeny, Amoebozoa

INTRODUCTION

The search for objective criteria distinguishing genera and species of amoeboid protists is currently focused mainly on molecular markers, which already illustrated their power in taxonomy of diverse amoeboid organisms. Phylogenetic analyses based on sequences of SSU rDNA discovered relationships congruent with morphology of individual groups of Amoebozoa as well as incongruencies unexpected at the level of species within the same genus. The critical importance of careful morphological identification of organisms, sequences of which are used for phylogenetic studies, has been stressed several times, most emphatically by Smirnov et al. (2008). The latter authors demonstrated serious misidentifications that influenced interpretation of phylogenetic trees, and fixed standard rules

for reliable identification of organisms and their subsequent re-examination, if necessary. Since the number of type strains maintained in culture collections is limited, as is the number of strains representing some specific groups of the Amoebozoa, the current demand to exploit well described strains from culture collections and obtain their molecular characterisation grows.

The primary and secondary structure of SSU rDNA and data on phylogeny of *Mayorella gemmifera* are presented as a contribution to the recent effort to learn more about and to verify phylogenetic relationships recognised within the Amoebozoa.

MATERIAL AND METHODS

Mayorella gemmifera strain CCAP 1547/8 was obtained from the UK National Culture Collection (UKNCC) in liquid medium S75S (Sigma Cereal Leaf-75% Seawater) containing bacteria, ciliates (*Cyclidium* sp.), flagellates and rice grains. A slightly modified medium was used at the beginning of subculturing carried out in our laboratory. Cerophyl (no more supplied by Sigma) was substituted with dried wheat leaves. Due to the recurrent decline of subcultures we changed culture conditions several times, trying to enhance multiplication of trophozoites. The strain was maintained in the culture for over two years without a substantial progress in its growth, although certain degree of culture refinement was attained by the balance established among amoebae, bacteria, flagellates and ciliates. An improvement of the growth of *Mayorella* trophozoites was achieved when a fish cell culture (FHM line), routinely used in our laboratory to feed histophagous ciliates, was used also in culturing the CCAP 1547/8 strain. Good parent cultures were subcultured weekly by transferring approximately half of amoeba cells suspended in fresh MY75S (Malt &Yeast Extract-75% seawater) medium into disposable (Falcon) flasks with almost complete monolayer of FHM cells. Cell culture medium was removed from each culture flask

immediately before suspension of amoebae was pipetted into it. *Mayorella* trophozoites, attached firmly to the bottom of culture flasks, were harvested for DNA extraction using disposable plastic scraper. Material for transmission electron microscopy was fixed with 3% glutaraldehyde *in situ*, peletted, postfixed in 1% osmium tetroxide and embedded in Spurr resin. Ultrathin sections were double stained with uranyl acetate in 50% methanol, post-stained with Reynold's citrate and examined with a JEOL JEM 1010 electron microscope operating at 80 kV.

DNA was extracted from cell pellets using the DNeasy™ Tissue Kit (Qiagen GmbH, Germany) according to the manufacturer's protocol. A set of universal eukaryotic primers (5'-ACCTGGTTGATCCTGCCAG-3' and 5'-CTTCCGCAGGTTCACCTACGG-3') (Barta et al. 1997) were used for amplification of the SSU rRNA gene. PCR was carried out in 25µl reaction volume using 10 pmol of each primer, 250 µM of each dNTP, and 2.5 µl 10 x PCR Buffer (Takara, Japan) and 1 Unit of TaqDNA polymerase (Takara, Japan). The reactions were run on a T3 Thermocycler (Biometra). The thermal cycling pattern was 95°C, 5 min; 94°C, 1 min; 44ºC, 1.5 min; 72°C, 2 min (×5); 94°C, 1 min; 48°C, 1.5 min; 72°C, 2 min (× 25); 72°C, 10 min. Amplification products were gel-isolated and cloned into pCR® 2.1 TOPO Cloning vector using the TOPO-TA Cloning Kit (Invitrogen) and sequenced on an automatic sequencer ABI 3130×1 using ABI Prism BigDye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) according to the manufacturer's protocol. The complete SSU rDNA sequence was obtained stepwise using a combination of flanking and internal primers and two *Mayorella* specific primers (Table 1).

For the comparison of *M. gemmifera* with *Mayorella* JJP-2003 strain sequences of these two organisms were aligned with Clustal V method implemented in MegAlign, part of DNA STAR package (Lasergene). The sequences of the whole dataset were aligned using

Clustal_X 1.18 (Thompson et al. 1997). Insertions and variable regions that were not aligned unambiguously were deleted from the alignment (1389 bp retained).

Maximum likelihood (ML), maximum parsimony (MP) and analysis employing Fitch-Margoliash method with LogDet distances (LD) were performed with PAUP 4.0β10 (Swofford 2001). ML analyses employed the general time reversible model + Γ+ I chosen with MrModeltest (Nylander 2004). All heuristic tree searches were conducted with 10 replicates. The starting tree constructed by random taxa addition was swapped by the TBR algorithm. In total 100 (ML) or 1000 (MP, LD) bootstrap replicates were used to estimate the support of topology. Bayesian analysis (BA) was conducted using MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003) with four simultaneous Markov chains Monte Carlo, 2×10^6 generations and the sampling frequency 100, temperature 0.2 and burn-in 5000 trees. The –lnL was plotted against the generation to check that burn-in was sufficient.

RESULTS

Light-microscopical observations made on living trophozoites of the CCAP 1547/8 strain in hanging drop preparations (Fig. 1) revealed all morphological characters summarised in generic diagnosis of *Mayorella* Schaeffer, 1926 by Page (1983). They corresponded to the description given by Page (1983) for the strain of *M. gemmifera* Schaeffer, 1926 that was of the same local origin as that one deposited as CCAP1547/8 in the UKNCC. Trophozoites with the greatest dimension fitting within the range 30–90 μm were oblong (L:B mostly 1.8–2.0) and formed relatively short blunt and conical sub-pseudopodia from the narrow zone of hyaloplasm. In the granuloplasm there were large vacuoles and also numerous vesicles of different diameter. The average diameter of nucleus was 6–9 μm. Floating forms (Fig. 1) resembled those documented in low magnification by Page (1983), in having long pseudopodia often broad at their bases.

The ultrastructural examination revealed other morphological features, of which the cell surface is of taxonomic value (Figs 2–6). Trophozoites had a thick cuticle (280–380 nm) stratified in two fibrilar layers (Figs 5 and 6). The inner layer, parallel to plasma membrane, was denser; the outer layer, less homogeneous, was composed of hair-like projections. In the cytoplasm, which contained numerous rounded mitochondria with tubular, branching cristae (Fig. 3), predominated small but plentiful vesicles and vacuoles containing amorphous material. The nucleus observed in ultrathin sections was mostly elongated. Golgi bodies were not abundant and some appeared to consist entirely of vesicles.

The SSU rDNA sequence prepared for *M. gemmifera* (strain CCAP 1547/8) has been deposited in GenBank under Acc. No. EU719190.The length of this sequence is 2934 bp. The comparison of the latter with the unique SSU rDNA sequence of *Mayorella* sp. published thus far (AY294143) revealed remarkable difference in the length and base composition. Comparable segments of corresponding sequences are 2863 bp long in the CCAP strain of *M. gemmifera* and 2131 bp in the JJP-2003 strain of *Mayorella* sp., respectively (the sequence of *M. gemmifera* slightly overlaps the other one at both ends). Alignment of the two sequences revealed that part of the V2 region, the segment homologous with helix E10_1, (Van de Peer et al. 2000), is that one most responsible for the length difference of the two compared sequences. Helix E10_1 in *M. gemmifera* is much longer (926 bp) than in JJP-2003 strain of *Mayorella* sp. (277 bp). The sequence dissimilarity between strains under study has been assessed to 35.3%. After expanded segment E10_1 was deleted from alignment, the percentage of sequence dissimilarity decreased to 24.2%.

Phylogenetic analyses (Fig. 7) clearly demonstrated that the CCAP strain of *M. gemmifera* and the other *Mayorella* strain, to date the only representatives of the genus in tree reconstructions, form a cluster well supported with bootstraps (ML 97%, LD 99%, MP 93%, MB posterior probability 1). Phylogenetic position of these two representatives of the genus

Mayorella within the analysed dataset of Amoebozoa is also well supported (ML 98%, LD 99%, MP 96%, MB 1). Of other groups, tubulinea represented by *Leptomyxa reticulata* and *Amoeba proteus*, and acanthamoebids represented by *Acanthamoeba castellani* and *Protacanthamoeba bohemica* are also well resolved. The relationships of the other groups of amoebae included in our analyses remained unresolved. Different tree-reconstructing methods resulted in different topologies resolved with low statistical support.

DISCUSSION

Based on light microscopical characters mentioned in the literature, re-identification of the CCAP 1547/8 strain with *Mayorella gemmifera* Schaeffer, 1926 was easily accomplished. Of the ultrastructural features it was only the cell surface and the narrow peripheral layer of cytoplasm of *M. gemmifera* documented by Page (1983) that was available for comparison. The difference noted in the fine structure of cell cuticle can be explained thanks to repeated ultrastructural studies of the CCAP strain. In certain percentage of trophozoites we observed a more compact periphery of the outer layer of cuticle similar to that documented by Page (1983). This structure of cuticle was characteristic of trophozoites that (judged by density of their cytoplasm) were not in a good shape when fixed. It is known that the cuticle may appear somewhat different depending on fixation procedures, as mentioned by Page (1988) with respect to species and generic diagnoses of amoebae. The fine structure of cell coat (cuticle) will be of primary interest when generic affiliation is verified in the numerous "*Mayorella*" species, description of which was based on morphotype observed in light microscope. Although we repeated fixation of trophozoites for transmission electron microscopy several times during a long period of subculturing, the appearance of cytoplasm was always the same. Based on our experience with other amoebae that could be cultured in liquid as well as on agar media, we suppose that a main determinant of cytoplasmic density is the medium

applied.

Since the type species of the genus, *Mayorella bigemma* (Schaeffer, 1918) Schaeffer, 1926 is not available, the CCAP strain of *M. gemmifera* might serve as a well-defined marine representative of the genus. Relevant ultrastructural and molecular studies of both freshwater and marine *Mayorella* strains maintained in culture collections are needed to expand the dataset and recognise the importance of fine structure of the cell coat for taxonomic status of these strains. The occurrence of a more complex structure of the cell coat than that found in *M. gemmifera* points to this necessity. The arrangement of the cell coat in three layers composed of fibrils arranged parallel and perpendicular to the plasma membrane was documented by Cann (1981) in the freshwater *Mayorella viridis* (Leidy, 1874).

The data obtained on secondary structure of SSU rDNA of *M. gemmifera* are in accordance with the observations by Fahrni et al. (2003), who analysed the sequences of representatives of lobose amoebae. While in other amoebae they found size variations mostly in regions V4, V7 and V8, for *Mayorella* sp. they observed expansion of the length in variable region V2. In other eukaryotes, the length of segment homologous with helix E10_1 is usually much shorter, e.g., 50 bp in *Saccharomyces* (Van de Peer et al.2000).

Since our intention was not to study a high level phylogeny of amoeboid organisms but to analyse the position of *M. gemmifera* within the Amoebozoa, the limited set of sequences was analysed. Low statistical support of topologies of some groups of the Amoebozoa seen in our phylogenetic reconstructions is well known also from other studies (Tekle et al. 2008, Smirnov et al. 2008).

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Table 1. Primers used in the study in addition to universal eukaryotic ones, given in 5'–3' direction.

Primer	Sequence (5'→ 3')
M13F	GTAAAACGACGGCCAGT
M13R	GAAACAGCTATGACCATG
460F	CAGCAGGCGCGCAAATTA
1200F	GATCAGATACCGTCGTAGTC
1200R	GACTACGACGGTATCTGATC
1350R	CCGTCAATTCCTTTAAGTTTC
1700R	GGCATCACAGACCTGTTAT
Mayor680R	CCCATGCAGGGATTTGTATTGT
Mayor1F	ATTTTCTCACTTGGCTCATTCCAC

Figure legend

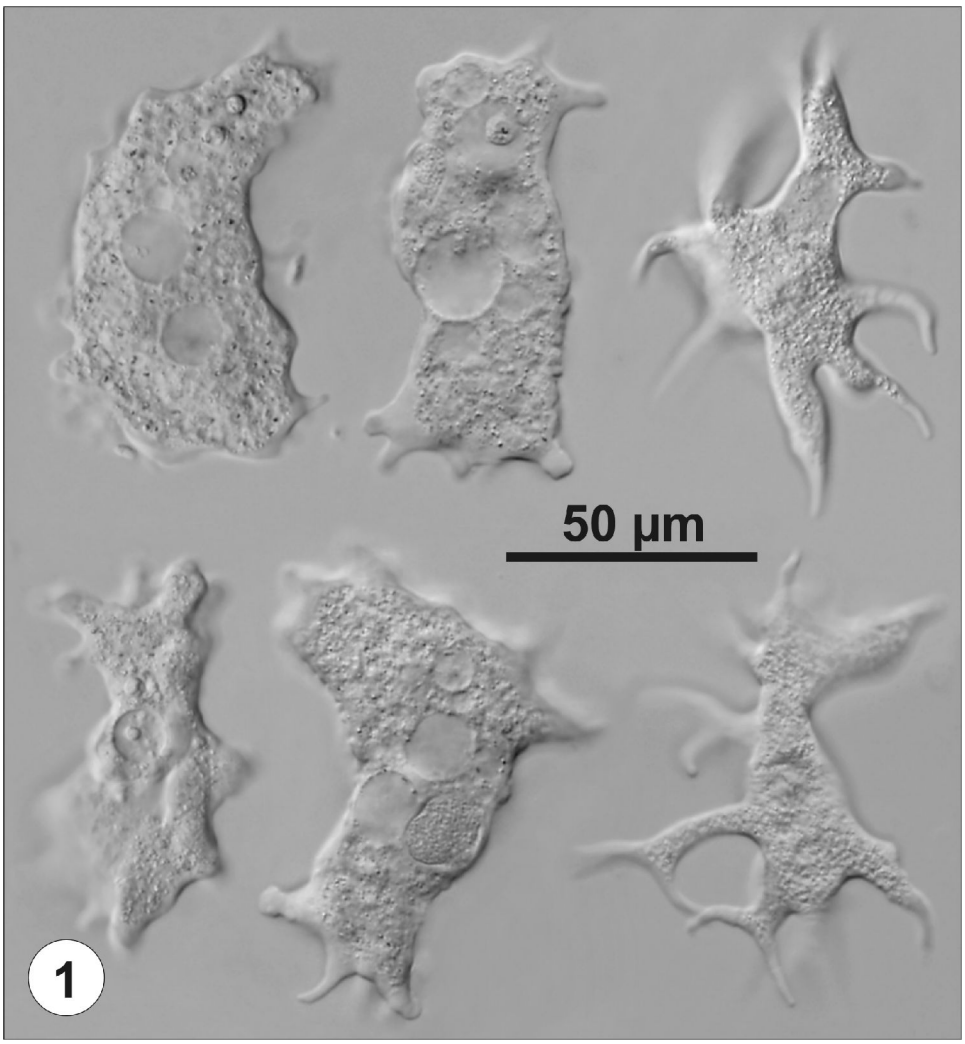
Fig. 1. Four attached (left) and two floating trophozoites (right top and bottom) of *Mayorella gemmifera* (strain CCAP 1547/8) as seen in hanging-drop preparation. Nomarski differencial interference contrast.

Figs 2–6. Details of ultrastructure of *Mayorella gemmifera* (strain CCAP 1547/8). Fig. 2. Trophozoite with the complete surface coat adjacent to the plasma membrane. The section shows mainly the granuloplasm that contains numerous mitochondria, vacuoles and vesicles;

a small part of hyaloplasm is seen on extreme left. Fig. 3. Mitochondria with tubular branching cristae. Fig. 4. Nucleus with a dense nucleolus. Figs 5, 6. Arrangement of the fibrilar cell coat as seen under different magnification.

Fig. 7. Maximum likelihood tree of SSU rDNA sequences of 13 amoebozoans and two opisthokonts as outgroup. The novel sequence of *Mayorella gemmifera* is in bold. The numbers at the nodes are bootstraps (ML, LD and MP) and bayesian posterior probabilities, respectively. Statistical support is shown for nodes with bootstraps higher than 50% and posterior probability higher than 0.5. Asterisk (*) indicate a bootstrap support lower than 50% for the respective method. All branches are drawn to the scale given at the bottom that indicates substitutions per site.

Figures:



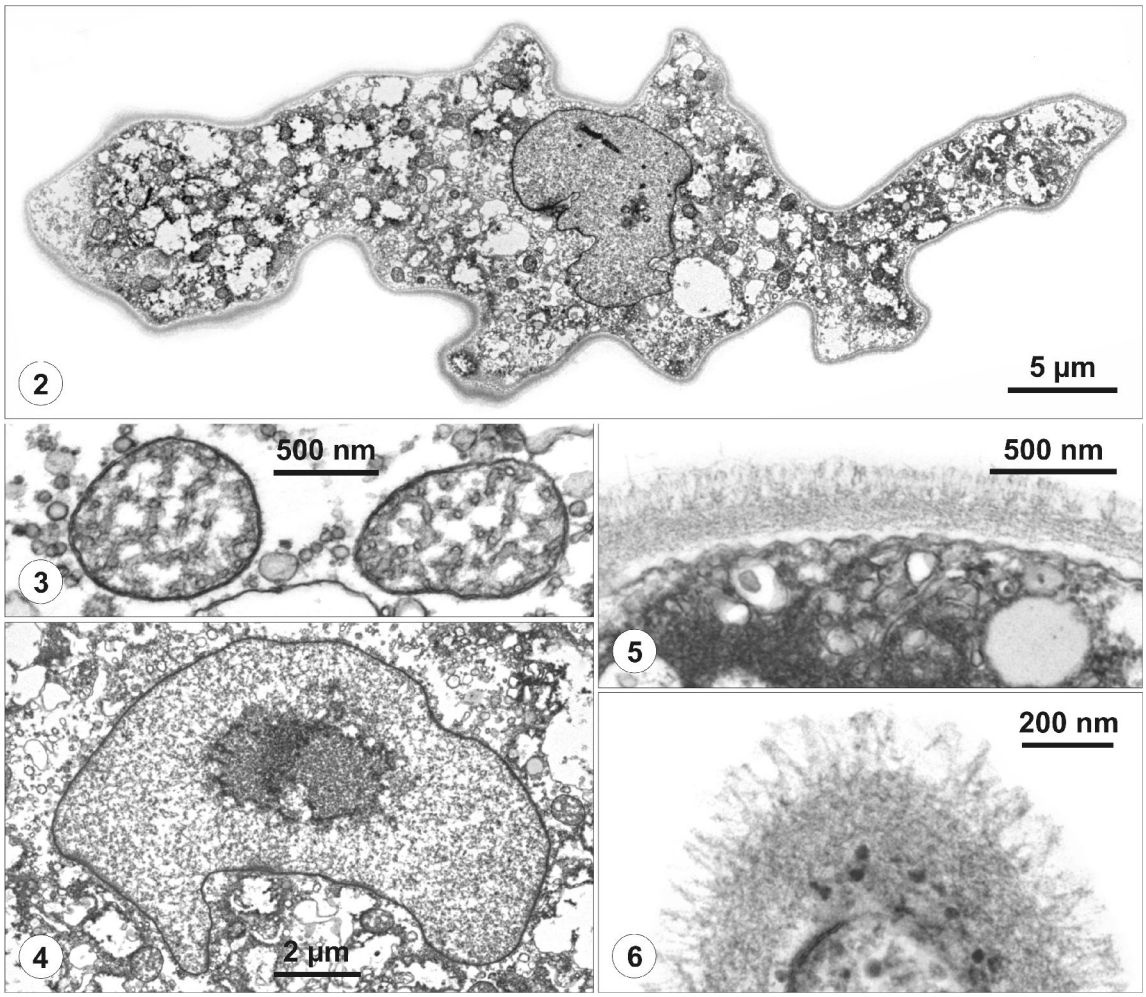
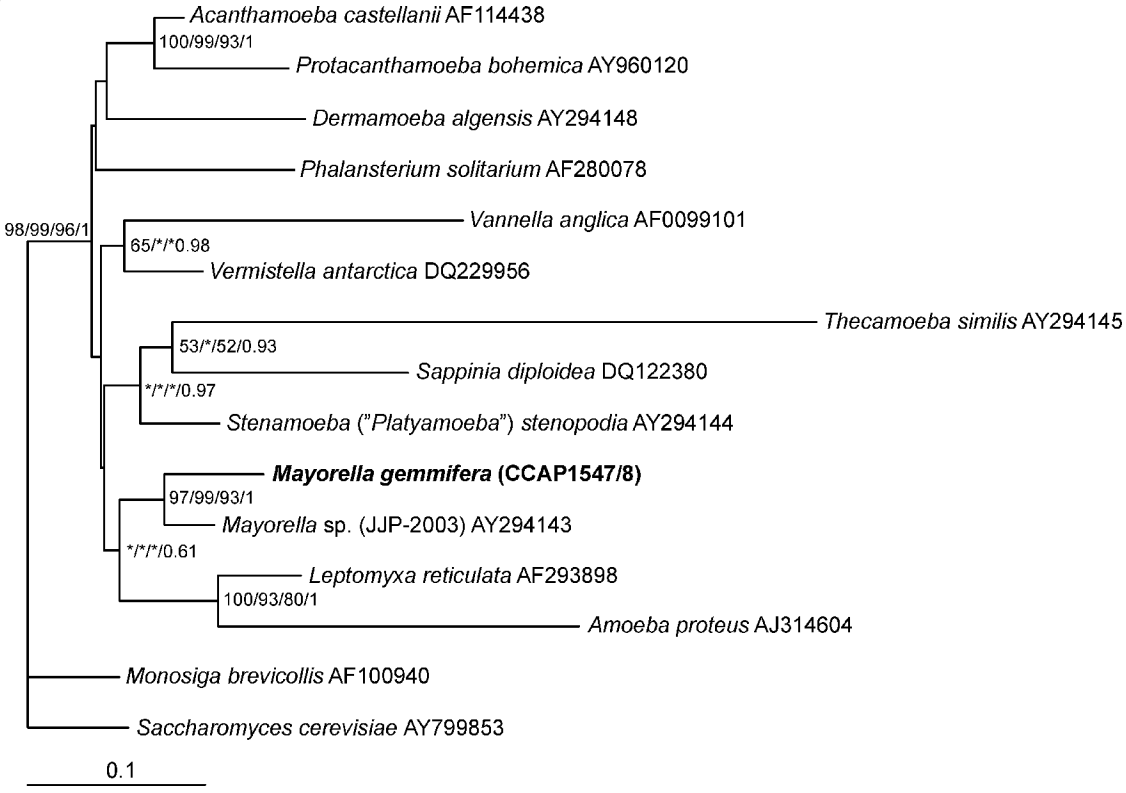

2
5 µm
500 nm
3
500 nm
5
4
2 µm
200 nm
6

Fig. 7:


Acanthamoeba castellanii AF114438
100/99/93/1
Protacanthamoeba bohemica AY960120
Dermamoeba algensis AY294148
Phalansterium solitarium AF280078
98/99/96/1
Vannella anglica AF0099101
65/*/*0.98
Vermistella antarctica DQ229956
Thecamoeba similis AY294145
53/*/52/0.93
Sappinia diploidea DQ122380
//*/0.97
Stenamoeba ("Platyamoeba") stenopodia AY294144
Mayorella gemmifera (CCAP1547/8)
97/99/93/1
Mayorella sp. (JJP-2003) AY294143
//*/0.61
Leptomyxa reticulata AF293898
100/93/80/1
Amoeba proteus AJ314604
Monosiga brevicollis AF100940
Saccharomyces cerevisiae AY799853
0.1

SSU rRNA-based phylogenetic position of the genus *Saccamoeba* Frenzel, 1892 (Amoebozoa)

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Page heading: Phylogeny of the genus *Saccamoeba*

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Summary. Light- and electron-microscopic data on a new strain of free-living amoeba isolated from dead aquarium fish are supplemented with its SSU rDNA sequence. Detailed comparison of general morphology and ultrastructure of this strain with data available on *Trichamoeba*, *Hydramoeba* and *Saccamoeba* spp. justified its assignment to the genus *Saccamoeba* Frenzel, 1892 and identified it with the species *S. limax* (Dujardin, 1841). In phylogenetic analyses, this new strain of *S. limax* (NTSHR) should be used as a representative of the genus instead of ATCC 30942 strain. The latter strain is considered as disqualified from its representative role in the genus *Saccamoeba* by the lack of ultrastructural data and by its close phylogenetic relationship with a previously described strain (LOS7N) whose mitochondria are substantially different from those typical of *Saccamoeba* species.

Key words: Amoebozoa, *Saccamoeba limax*, general morphology, ultrastructure, SSU rDNA based phylogeny

Introduction

As a result of current effort directed to accumulation of molecular data on amoebae and amoeboid organisms, considerable progress has been made in understanding evolution of these and related organisms. The majority of insights came from the small subunit rDNA sequences and their phylogenetic analyses. However, with a rising number of sequences, problems emerge regarding the congruence of the molecular phylogeny of amoebae and morphological features of source organisms and their respective sequences. Serious errors in the interpretation of phylogenetic trees can be caused by misidentification of amoebae, sequences of which are included in analyses, or by poor description of those amoebae that

based on the current level of knowledge cannot be assigned unambiguously to a genus. Relative to a great diversity of amoebae, a minimum of well-described type strains is available in culture collections. They should be exploited, neotype strains used in place of non-existent type strains and newly described amoebae should be deposited in culture collections.

This work is part of our continuing studies on free-living amoebae related to fish and their environment. This contribution is aimed at morphological and molecular characterisation of a newly isolated strain and search of its phylogenetic position within representatives of Lobosea (Tubulinea sensu Smirnov et al. 2008).

Material and Methods

Amoebae were isolated, together with flagellates and three different species of ciliates, from decomposing gills of aquarium fish brought to the laboratory in a small amount of water containing also detritus from aquarium where many fishes died. Purification of mixed primary isolate and establishment of homogeneous population of amoebae designated as NTSHR strain was accomplished in three weeks using 1.75% Non-Nutrient (Amoeba Saline) Agar (NN). Clonal culture was derived in following three weeks. Subculturing was done weekly. To avoid unwanted increase in accompanying bacteria, NN agar was used during the whole period when material for morphological, ultrastructural and molecular studies was collected.

Living amoebae were observed on agar plate cultures (*in situ*) and in hanging drops prepared using Page's amoeba saline. Olympus BX51 microscope equipped with Nomarski differential interference contrast and fitted with digital camera DP 70 was used for observation and documentation of amoebae.

One-week-old agar plate cultures selected for examination by electron microscopy were fixed *in situ* with 3% glutaraldehyde in cacodylate buffer. Pelleted trophozoites were postfixed in 1% osmium tetroxide in cacodylate buffer and embedded in Spurr resin. Ultrathin

sections were stained with uranyl acetate and Reynold's lead citrate, and examined in Jeol JEM 1010 electron microscope.

DNA was extracted from pelleted trophozoites of NTSHR strain using the DNeasy™ Tissue Kit (Qiagen GmbH, Germany) according to the manufacturer's protocol. Universal eukaryotic primers (5'-ACCTGGTTGATCCTGCCAG-3' and 5'-CTTCCGCAGGTTCACCTACGG-3') (Barta et al. 1997) were used for amplification of the SSU rRNA gene. PCR was carried out in 25 µl reaction volume using 10 pmol of each primer, each dNTP in concentration of 250 µM, 2.5 µl 10 x PCR Buffer (Top-Bio, Czech Republic) and 1 Unit of TaqDNA polymerase (Top-Bio, Czech Republic). The reactions were run on a Tpersonal Thermocycler (Biometra). The thermal cycling conditions consisted of initial denaturation at 95°C (5 min), 30 cycles of denaturation at 94°C (1 min), annealing at 48°C (1.5 min) and extension at 72°C (2 min) followed by a final extension at 72°C (10 min). Following visualisation of PCR products via gel electrophoresis, amplification products were extracted from the agarose using JETQUICK Gel Extraction Spin Kit (Genomed, Germany), then cloned into pCR® 2.1 TOPO Cloning vector using the TOPO-TA Cloning Kit (Invitrogen) and sequenced on an automatic sequencer CEQ™ 2000 using CEQ DTCS Dye Kit (Beckman Coulter) according to the manufacturer's protocol. The complete SSU rDNA sequence of NTSHR strain was obtained stepwise using a combination of flanking and internal primers as mentioned elsewhere (Dyková et al. submitted).

SSU rDNA sequences of 22 amoebozoans and of two opisthoconts (outgroup) were included in the phylogenetic study. The alignment of sequences for phylogenetic analyses was prepared in Clustal_X 2.0.6 (Larkin et al. 2007). Ambiguously aligned positions were deleted manually in BioEdit (Hall 1999). The resulting alignment contained 1439 positions. Maximum likelihood (ML) analyses were performed with the program RAxML 7.0.3 (Stamatakis 2006) with the use of GTR + Γ model and rapid bootstraping (100 replicates).

Program PAUP* 4.0b10 (Swofford 2002) was used to compute maximum parsimony (MP) analyses and analyses employing Fitch-Margoliash method with LogDet distances (LD). Heuristic searches were conducted with 10 repeated searches in which the starting tree was constructed by random taxa addition and swapped with the TBR algorithm. The number of bootstrap replicates was 1000 for both MP and LD. Bayesian analysis (BA) was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Four simultaneous MCMC chains were run for 10^6 generations with sampling frequency 100 generations and burn-in 2500.

Results

Light and electron microscopy of NTSHR strain

Trophozoites growing on the surface of agar displayed irregular shape similar to that of polypodial amoebae (Fig. 1). When subcultured via suspension in amoeba saline, they grew as isolated individuals even after one week. The aggregates of cells were found rather exceptionally. In trophozoites observed in translucent light through Petri dishes, i.e., at low magnification, the division of their cytoplasm into hyaloplasm and granuloplasm with numerous optically active inclusions could be seen.

In hanging drop preparations, morphology of trophozoites attached to coverslips differred. According to their locomotive activity they exhibited polypodial forms or transform in monopodial, "limax" forms (Figs. 1–3). Monopodial trophozoites, 55 (49–63) μm long and 12 (11–13) μm wide, were the most active, difficult to document due to the speed of their locomotion. They advanced by steady flow of cytoplasm in one direction and most of them had hyalopasmic anterior cap. One spherical nucleus, a contractile vacuole situated near the posterior end and a knob-like uroid could be observed (Fig.2). In addition to these smooth "limax" forms that were symmetrical along their long axis, slightly curved or laterally bulging monopodial forms were observed among most active trophozoites. In comparison with

monopodial forms, polypodial trophozoites projecting pseudopodia with hyaloplasmic caps in several directions were less active. In these trophozoites, the nucleus, contractile vacuole and uroid could not be observed regularly when hanging drop technique was used (Fig. 3). The inclusions resembling crystals were clearly seen in less active trophozoites (Fig. 3) and in these pelleted by centrifugation and observed in translucent light (Fig. 4). Due to the size of trophozoites, true floating forms were difficult to produce and observe. Trophozoites detached from support and shaken in amoeba saline resembled irregular spheres with bumps (without defined pseudopodia). They fell down very fast to the bottom of slide chamber, tended to adhere immediately and subsequently transformed into polypodial trophozoites. Cyst formation was not observed although several agar plate cultures of NTSHR strain stored at 20ºC were checked repeatedly for four months.

The cell surface was coated with very thin amorphous glycocalyx (Figs. 5, 8, 9). Based on series of ultrathin sections, the nucleus could be classified as vesicular with central nucleolus (Fig. 10). Rounded and oval sections of mitochondria showed their tubular cristae (Fig. 6). The arrangement of Golgi complex resembled dictyosomes (Fig. 7). The rough endoplasmic reticulum consisted mostly of short cisternae. The contractile vacuole was surrounded by spongiom of numerous small vesicles (Fig. 12). The cytoplasm of trophozoites contained multiple food vacuoles, vesicles with remnants of amorphous material, and dividing bacteria. The cytoplasmic structures that in light microscope resembled crystals, differed in ultrathin sections, in which predominated vesicles containing amorphous material (Fig. 11). Structures with suspected crystal organisation were found in thin sections only exceptionally (Fig. 13). In addition to cytoplasmic microtubules, at the border between granuloplasm and hyaloplasm were microfilaments, markedly aggregated between the uroid and main cell cytoplasmic mass (Fig. 15). The uroidal structure of trophozoites under study was of the villous type (Fig. 14).

Molecular characterisation of NTSH strain and its phylogeny

The sequence of SSU rDNA of NTSHR strain (2048 bp) was deposited in GenBank under Acc. No. EU869301. The results of phylogenetic analyses (Fig. 16) have shown the strain as a member of well-supported amoebozoa (bootstraps: ML 100%, MP 96%, LD 93% and BA posterior probability 1.00). Within amoebozoa, the strain belongs to Tubulinea as a member of (Amoebidae + (*Hartmannella cantabrigiensis* + (*Glaeseria mira* + (*Saccamoeba* sp. SC007 + NTSHR)))). This group and all its nodes are well supported by bootstrap values (99% and higher) and posterior probabilities (1.00). Although the partial SSU rDNA sequence available from *Saccamoeba* sp. strain SC007 was relatively short (882 bp), the corresponding segment of NTSHR sequence was almost identical (94% sequence similarity).

Taxonomic affinities of NTSHR strain

Light microscopical and ultrastructural characteristics of three genera, *Trichamoeba* Fromentel, 1874, *Hydramoeba* Reynolds et Looper, 1928 and *Saccamoeba* Frenzel, 1892 had to be taken into consideration to classify properly NTSHR strain. In all papers containing essential and well documented data for comparison, the mutual similarities of the three genera mentioned above are stressed and their distinctions are considered rather weak (Page and Robson 1983, Siemensma and Page 1986, Page 1988). The vesicular type of nucleus with centrally located nucleolus, floating form lacking pseudopodia, and thin cell surface coat, differentiate trophozoites of NTSHR strain from *Trichamoeba* and *Hydramoeba* and enable its assignment to *Saccamoeba*. Molecular support of this decision could only be inferred from the partial SSU rDNA sequence of *Saccamoeba* sp. (strain SC007) that in corresponding segment revealed 94% identity with NTSHR. Based on comparison of data available on named species of the genus, NTSHR strain was identified with *Saccamoeba limax* (Dujardin, 1841). The inclusion of SSU rDNA sequence of NTSHR strain into dataset analysed phylogenetically disqualified the ATCC 30942 strain from its role of the representative of the

genus *Saccamoeba*. The branching pattern of sequences and its bootsrap support revealed a close relationship of ATCC 30942 with LOS7N/I that differs substantially from *Saccamoeba* spp. in having unique arrangement of tubular cristae of mitochondria.

Discussion

To the best of our knowledge, in *Saccamoeba* spp., only monopodial locomotive forms were documented but possible bulging of "limax" forms was mentioned several times. The less active type of NTSHR trophozoites that by their shape and content of cytoplasm slightly resembled trophozoites of *Amoeba* Bory de St. Vincent, 1822, *Chaos* Linnaeus, 1767 and *Polychaos* Schaeffer, 1926, as documented by Page and Baldock (1980), Page and Kalinina (1984) and Page (1988), was described also in *Trichamoeba* spp. (Chakraborty and Old 1986, Siemensma and Page 1986) and *Hydramoeba hydroxena* (Entz, 1912). In the latter species, Page and Robson (1983) called such trophozoites "proteus" forms. The nuclear structure distinguishes our strain from *Amoeba* spp. that possess internal fibrous lamina supporting nuclear membrane as well as from amoebae of the other genera mentioned above that have nucleolar material either located on the periphery of nucleus or possess granular type of nucleus. Also cell surface of NTSHR trophozoites differed substantially from those representing the genera mentioned above. The results of our ultrastructural studies could be best compared with those of Page and Robson (1983), Page (1985) and Siemensma and Page (1986). In two *Saccamoeba* species, Page (1985) demonstrated tubular cristae of mitochondria that were very similar (in their twisting appearance and rare branching) to mitochondria of NTSHR strain. This feature, together with other similarities in the fine structure (Golgi bodies, cytoplasmic microfilaments, spongiom surrounding the contractile vacuole), supports the assignment of NTSHR strain to the genus *Saccamoeba*. Future studies should clarify whether differences described in the fine structure and in floating forms of *Saccamoeba*,

Trichamoeba and *Hydramoeba* are in congruence with differences in molecular markers and do fulfill criteria for independent genera. Cysts (absent in our strain) were described in *S. stagnicola* (strain CCAP 1527/3) when the strain was cultured in liquid medium (Page 1985). In general, Page did not considered formation of cysts a stable feature.

In the context of its structural characters, phylogenetic position of the newly obtained sequence of NTSHR strain is surprising in its distance from the only complete sequence representing the genus *Saccamoeba*, i.e., ATCC 30942 strain denominated *S. limax* (Amaral Zettler et al. 2000). Unfortunately, the sequence of *S. stagnicola* (CCAP1527/3 strain) stated to have been deposited (http://amoeba.ifmo.ru/species/), is actually missing in GenBank. The high level of similarity (94%) of corresponding segments of SSU rDNA sequences of NTSHR and *Saccamoeba* sp. strain SC007 (Hewet 2006) supports the generic assignment of NTSHR strain based on light and electron microscopical observations. The comparison of the same segments in other sequences of the same clade, e.g., NTSHR and *Glaeseria mira* or in sequences of sister clade (*Amoeba proteus* and *A. leningradensis*) reveals lower values, i.e., 78% and 91%, respectively.

The fact that the AF293902 sequence of ATCC strain denominated *Saccamoeba limax* clustered with the sequence of our strain LOS7N/I should be stressed because of high level of similarity (99.58%) calculated for this couple of sequences and also because of the unusual pattern of mitochondrial structure documented for LOS7N/I by Dyková et al. (2002). No ultrastructural data are available for ATCC 30942 strain of "*S. limax*" but one can hardly believe that one taxon (genus *Saccamoeba*) could exhibit two different types of mitochondrial cristae. Phylogenetic relationship of both strains recognized in this study should be supplemented with their comparative ultrastructural study. In connection with sister branching of these two strains it might be noted that unusual type of arrangement of tubular cristae was found also in *Cashia limacoides* (Page, 1967) and characterised by Page (1985) as helical.

Based on results of this study we recommend the substitution of the sequence of ATCC 30942 strain with that of NTSHR if a representative of *Saccamoeba* is required for a dataset. The sequence of ATCC 30942 strain should be suppressed until relationships between the fine structure and molecular characters are more firmly established. Hopefully, the arrangement of mitochondrial cristae found in the phylogenetically closely related strain (LOS7M/I) would stimulate such comparative studies. Basic morphology of mitochondrial cristae and changeover (from discoid to tubular) has long been recognised to be of great phylogenetic value but phylogenetic importance of more delicate changes in organisation of tubular cristae has not been studied yet.

Although originally aimed at identification of newly isolated amphizoic amoeba, this study pointed out, once again, the lack of data, which makes the interpretation of distantly clustering sequences of allegedly congeneric amoebae quite difficult.

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Figure legends

Figs. 1–4. Trophozoites of NTSHR strain of *Saccamoeba limax*. Fig. 1. Agar plate culture. Trophozoites on the surface of agar (seen through inverted Petri dish × 150). Fig. 2. Monopodial locomotive forms. Uroids are marked with arrows. Fig. 3. Less active trophozoites resembling polypodial amoebae and transient forms with lateral bulges of hyaloplasm on the way to transform in monopodial locomotive forms. Fig. 4. Crystals in the

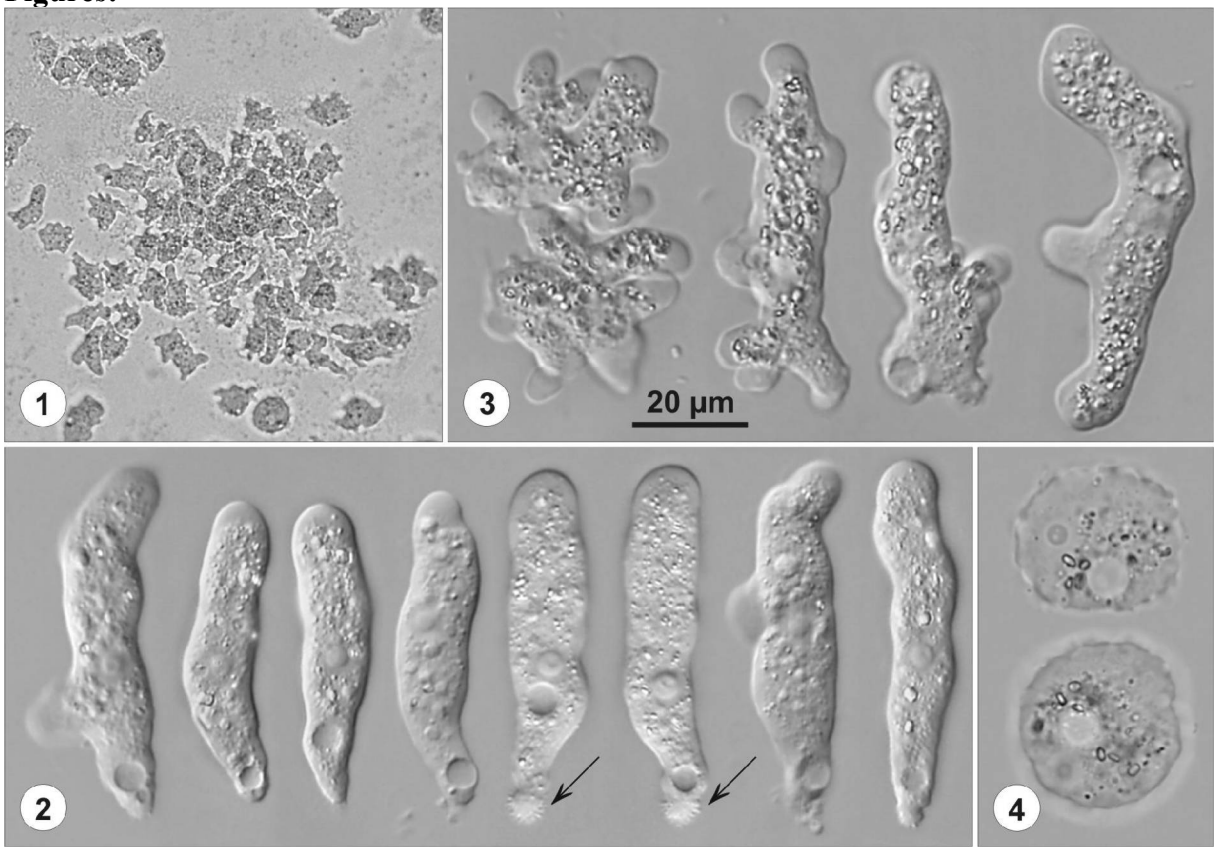
cytoplasm of trophozoites pelleted by centrigfugation, observed in translucent light, × 650. Figs. 2, 3 = same scale.

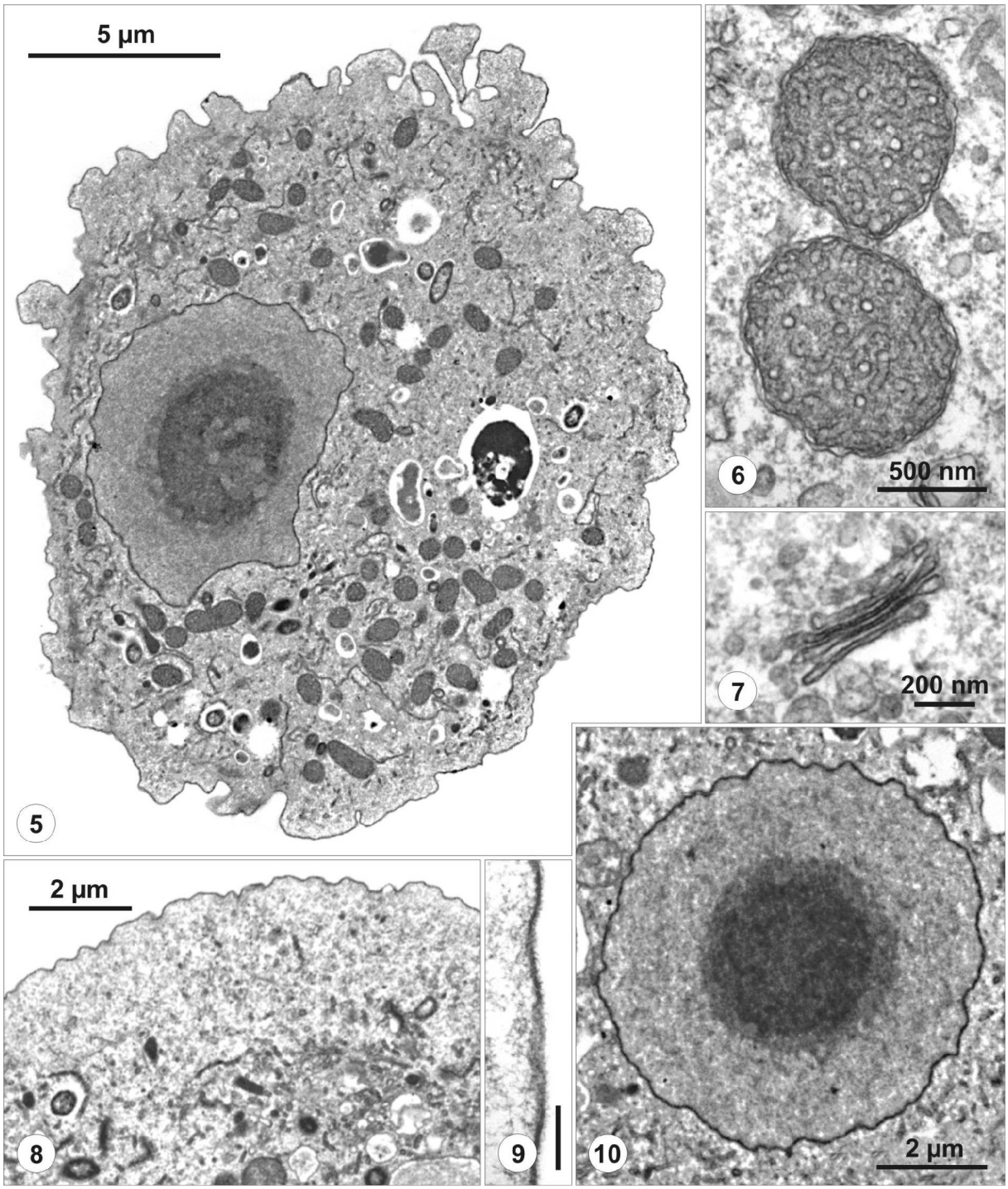
Figs. 5–9. Fine structure of trophozoites of NTSHR strain of *Saccamoeba* sp. Fig. 5. Overview section of trophozoite shows prominent vesicular nucleus, numerous mitochondria, vacuoles with remnants of food, bacteria and vesicles of unknown origin. Fig. 6. Mitochondria with twisted tubular cristae. Fig. 7. Golgi complex resembling dictyosome. Figs. 8, 9. Cell periphery and cell surface covered with very thin amorphous glycocalyx. Fig. 10. Nucleus of vesicular type with centrally located nucleolar material. Scale bar for Fig. 9 = 200 nm.

Figs. 11–15. Fine structure of trophozoites of NTSHR strain of *Saccamoeba* sp. Fig. 11. Layer of hyaloplasm on the periphery of trophozoite and part of granuloplasm containg suspected crystal, remnants of food in vacuoles, vesicles of unknown origin and bacteria. Fig. 12. Contractile vacuole surrounded by spongiom. Fig. 13. Crystal-like structure from the cytoplasm of trophozoite. Fig. 14. Transverse section through villous uroid. Fig. 15. Aggregates of microfilaments observed between hyaloplasm of uroid (arranged in villi) and main cell cytoplasmic mass.

Fig. 16. Maximum likelihood tree based on SSU rDNA sequences of 22 amoebozoans and two opisthokonts (outgroup). The newly introduced sequence (NTSHR) is in bold. Bootstrap values for ML, MP, LD and Bayesian posterior probabilities are given at nodes, respectively. Asterisks represent bootstraps lower than 50%. Nodes with black dots obtained bootstrap support of 99% or higher and their posterior probability was 1.00. All branches are drawn to scale, only the length of *Centropyxis laevigata* branch was halved.

Figures:

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5
6
500 nm
7
200 nm
2 µm
8
9
10
2 µm

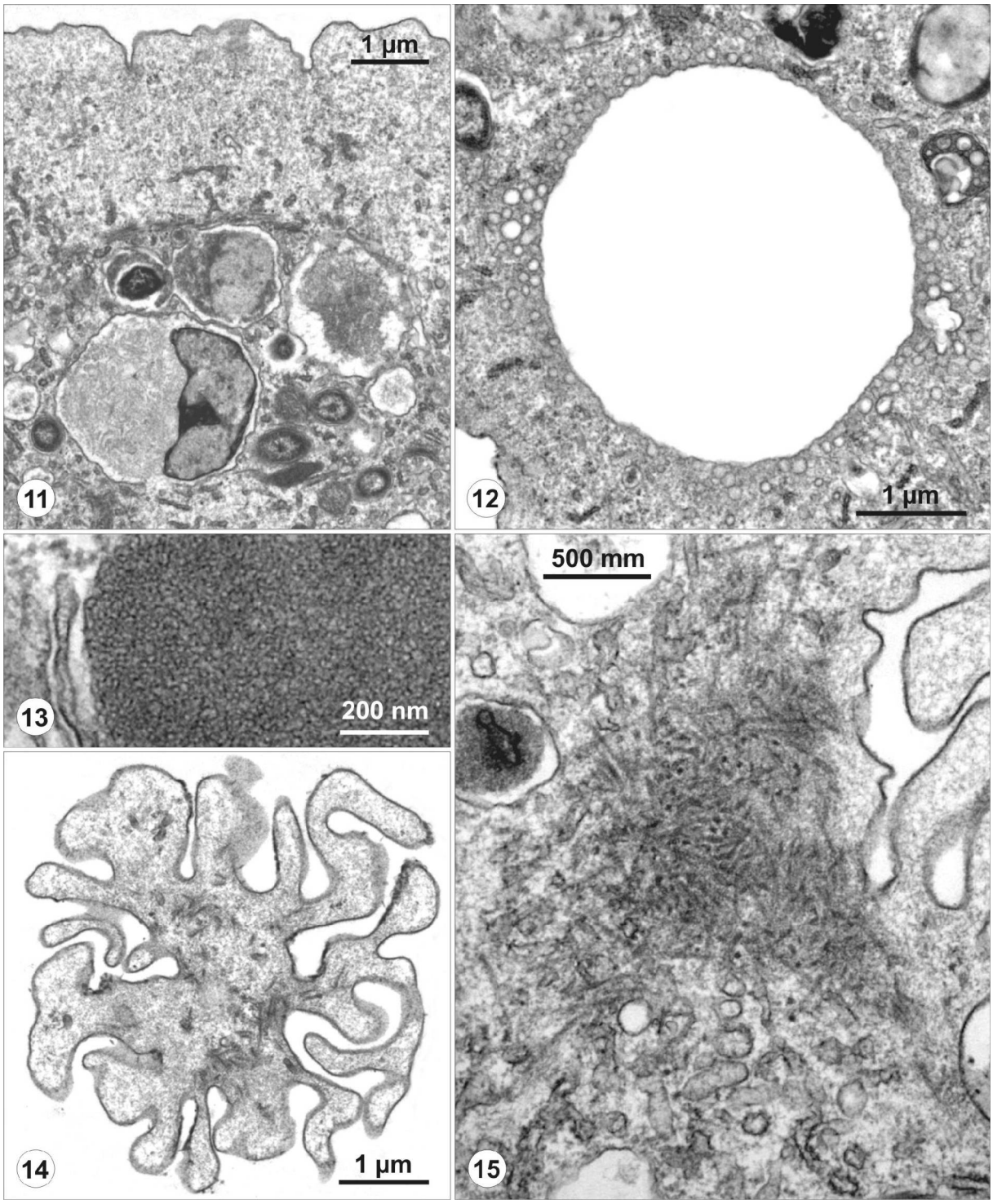
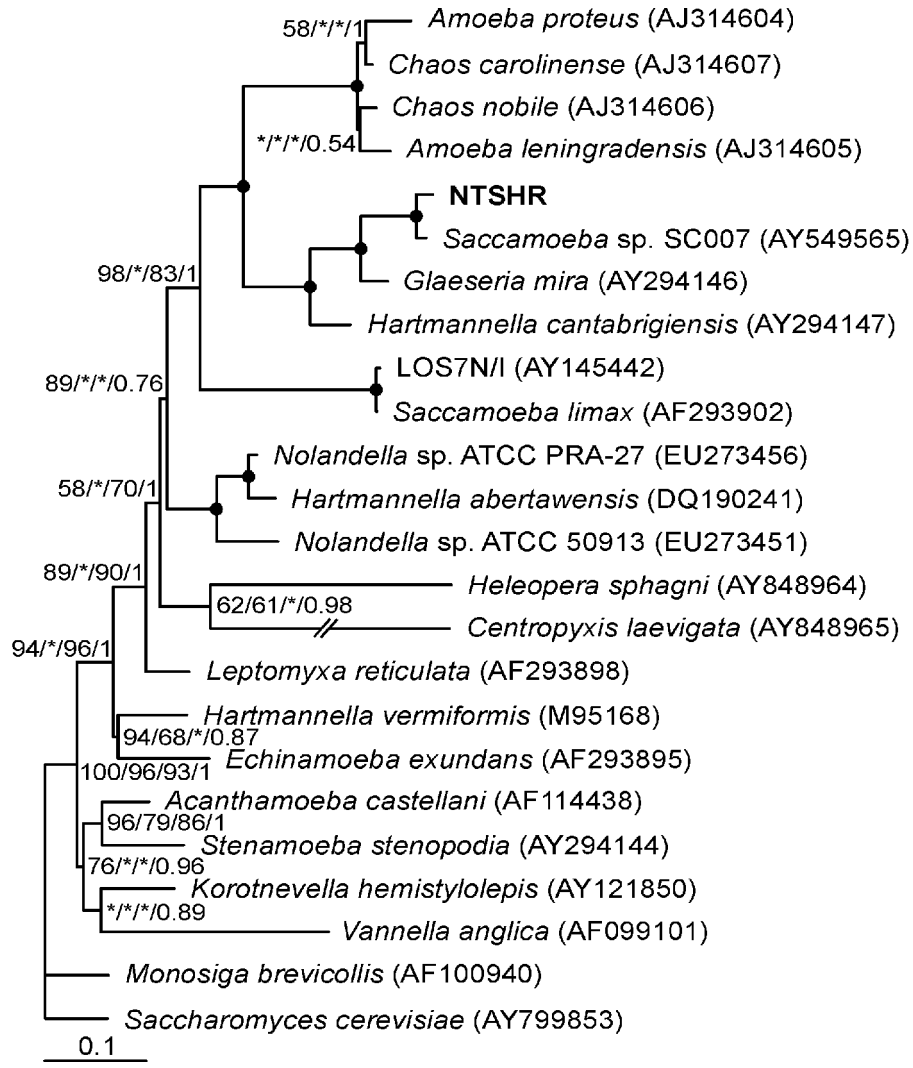

1 µm
11
12
1 µm
13
200 nm
500 mm
14
1 µm
15

Fig. 8:


58/*/*/1
Amoeba proteus (AJ314604)
Chaos carolinense (AJ314607)
Chaos nobile (AJ314606)
//*/0.54
Amoeba leningradensis (AJ314605)
NTSHR
Saccamoeba sp. SC007 (AY549565)
98/*/83/1
Glaeseria mira (AY294146)
Hartmannella cantabrigiensis (AY294147)
LOS7N/I (AY145442)
89/*/*/0.76
Saccamoeba limax (AF293902)
Nolandella sp. ATCC PRA-27 (EU273456)
58/*/70/1
Hartmannella abertawensis (DQ190241)
Nolandella sp. ATCC 50913 (EU273451)
89/*/90/1
Heleopera sphagni (AY848964)
62/61/*/0.98
Centropyxis laevigata (AY848965)
94/*/96/1
Leptomyxa reticulata (AF293898)
Hartmannella vermiformis (M95168)
94/68/*/0.87
Echinamoeba exundans (AF293895)
100/96/93/1
Acanthamoeba castellani (AF114438)
96/79/86/1
Stenamoeba stenopodia (AY294144)
76/*/*/0.96
Korotnevella hemistylolepis (AY121850)
//*/0.89
Vannella anglica (AF099101)
Monosiga brevicollis (AF100940)
Saccharomyces cerevisiae (AY799853)
0.1

SlowFaster, a user-friendly program for slow-fast analysis and its application on phylogeny of *Blastocystis*

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Abstract

Background

Slow-fast analysis is a simple and effective method to reduce the influence of substitution saturation, one of the causes of phylogenetic noise and long branch attraction (LBA) artifacts. In several steps of increasing stringency, the slow-fast analysis omits the fastest substituting alignment positions from the analysed dataset and thus increases its signal/noise ratio.

Results

Our program SlowFaster automates the process of assessing the substitution rate of the alignment positions and the process of producing new alignments by deleting the saturated positions. Its use is very simple. It goes through the whole process in several steps: data input – necessary choices – production of new alignments.

Conclusions

SlowFaster is a user-friendly tool providing new alignments prepared with slow-fast analysis. These data can be used for further phylogenetic analyses with lower risk of long branch attraction artifacts.

Background

The long branch attraction (LBA) artifact [1] still remains one of important causes of biases and mistakes in phylogenetic analyses of sequence data [2]. LBA causes taxa with long branches to be artifactually grouped with or attracted to other long branched taxa (i.e., fast evolving taxa or taxa evolving for a long time separate from other groups, e.g. outgroups). An important source of LBA is substitution saturation of positions in alignment (the term “mutational saturation” is also used, although it is not correct in this context). It would be ideal to have positions that underwent a single or a few changes during evolution, but many positions in real alignments are subject to

multiple substitutions. This subset of rapidly evolving positions is the source of stochastic noise rather than useful signal. However, these saturated positions are responsible for a major part of information used in phylogenetic analyses [3], which could confuse most of the tree-reconstructing methods. Because there are only four possible states for nucleic acid data (20 for amino acids), it is probable that a part of saturated positions will evolve randomly – convergently into the same state. It could then be erroneously judged as a synapomorphy. LBA can thus be a major problem especially in maximum parsimony, but occurs also in other analyses [4]. Maximum likelihood can, under an appropriate model of evolution, deal better with saturated positions, but datasets containing sites with different rates of substitution across the tree (covarion-like) may still be problematic [5]. Besides LBA, a high level of saturation in the dataset may cause signal simply to be overwhelmed by noise at least at some points of the tree topology. Such nodes could be resolved incorrectly or (at least) with a low statistical support.

It has been shown that in real alignments, LBA can be a major problem [2]. An effective way to estimate and reduce the effect of substitution saturation and LBA is removal of fast-evolving data. One such method is slow-fast analysis of the dataset [6]. The positions of the alignment are divided into several classes according to their substitution rate (estimated within *a priori* defined monophyletic groups). Several new alignments are then created, which contain only positions with a substitution rate lower than several thresholds, ranging from maximum to minimum rate. Thus the signal/noise ratio of the alignments successively increases, however, on the expense of amount of positions included in the alignment. Technically, the Slow-Fast method needs some input tree topology to work with. The topology must be provided by primary phylogenetic analysis of the dataset or by another independent method. This

topology is needed for recognition of some monophyletic groups (whose relative positions on the tree is not necessary to know before slow-fast analysis). Maximum parsimony is then used to determine the number of changes for each position within the monophyletic subgroups. Substitution rates assigned to positions are thus independent from interrelationships among the monophyletic groups, and therefore, these interrelationships may in turn be investigated without the fear of circularity. When each position is assigned its number of changes, those with the highest substitution rate are gradually omitted from new alignments. The following phylogenetic analyses of these new datasets (starting from the dataset containing the positions with the highest substitution rate) then provide results based on decreasing number of sequence data, however, with decreasing risk of artefactual groupings of long branches. There are several good examples of successful use of slow-fast analysis, see e.g. [6-11].

Although the slow-fast analysis is relatively powerful and very simple in principle, it is quite demanding when one wants to determine the number of changes for individual alignment positions (e.g., with the help of PAUP [12], using the "describetree" command) and the manual procedure of deleting of positions by editing the dataset is especially very time consuming. We believe that this is one of the most important reasons why this method is used relatively scarcely. Clearly, a computer program that provides this evaluation of positions and which produces new alignments would be handy. To our knowledge, the only software providing slow-fast analysis have been MUST [13]. MUST is a complex package, yet it still does not provide a quick and easily operated tool for this type of analysis. This is what our program SlowFaster does. It is a user-friendly tool to conduct slow-fast analysis and produce a set of new alignments without fast evolving positions. It have several

additional functions. Note that another program for slow-fast analysis was presented recently [11].

Implementation

SlowFaster was programmed in Borland Delphi and runs under MS Windows. Both the executable file [see Additional file 1] and the source code [see Additional file 2] are available as supplements. The program leads the user in several steps through the process of generating new datasets. Original alignment is loaded in FASTA, Phylip or NEXUS format. The program works with both nucleic acid and amino acid alignments and supports usual ambiguity coding. The topology needed for the recognition of monophyla is loaded as a tree in the Newick ("bracketed") format (PAUP users can use "savetree format=phylip" command to obtain tree in Newick format). After choosing the monophyletic groups by simply clicking on the branches of the depicted tree, parsimony is used to count the number of changes of every alignment position within the selected groups. Finally, new alignments are produced (in FASTA, Phylip or NEXUS format). Each of the new datasets has a number which is a threshold: positions with greater number of changes were omitted from this dataset. As the threshold gets lower and lower, the datasets contain fewer and fewer data because the more saturated positions were deleted from them. These datasets can be then further analysed to obtain phylogenies with a lower risk of LBA. During the whole process, there are hints shown in a window, telling the user what to do in the given step.

The software was tested thoroughly on several model datasets [see Additional file 3] and also on dataset of Hampl *et al*. [10]. In this latter case, we obtained the same new datasets with our program (Hampl *et al*. obtained them with the help of PAUP and through careful manual deletion of positions).

An interesting alternative to slow-fast method is using substitution rates estimated with maximum likelihood (ML). Although ML estimates are not implemented in SlowFaster, this program enables production of alignments without positions with high rates through the "Load changes" button. The rates can be counted in another software. E.g. Tree-Puzzle [14], if rate heterogeneity is selected, gives information on the rate category of each position in its outfile under "Combination of categories that contributes the most to the likelihood". These data can be simply copied in a file which is then loaded in SlowFaster. New alignments are then produced directly from these data. More generally, any sequence of any (even real) numbers can be loaded and the software will divide positions in rate categories (their number is specified by the user) based on these values.

The program also creates a log file which contains useful information, most notably groups used for changes counting, list of positions with certain number of changes and number of changes for all positions from the first one to the last.

Results and discussion

Sample Data

As an example, we analysed an alignment of 34 SSU rDNA sequences of 31 isolates of *Blastocystis* + 3 outgroups. *Blastocystis* is an unusual protist, a sister group of slopalinids (used as the outgroup) within the group of stramenopiles. See e.g. [15 - 16] for a review of *Blastocystis*. Although these nonflagellated, multinucleated gut commensals comprise a single genus, their SSU rDNA phylogeny shows clearly that they are rather long branched taxa in comparison to other stramenopiles. Their branches are even longer than, for example, branches separating classes of autotrophic stramenopiles. This group is therefore suspected of a high level of substitution saturation. We sequenced SSU rRNA genes of five *Blastocystis* isolated from

tortoises to improve taxon sampling by increasing the number of non-mammalian and non-bird isolates in the analysis (the vast majority of *Blastocystis* sequences available in GenBank are from bird or mammalian isolates). The accession numbers of the five new sequences (GERA3b, GERA3a, GECA2, KINIX2 and GEPA2) are [GenBank:EF209016], [GenBank:EF209017], [GenBank:EF209018], [GenBank:EF209019] and [GenBank:EF209020], respectively.

The alignment was prepared with ClustalX [17] and ambiguous parts with many indels were deleted from the alignment in the program BioEdit [18]. The resulting alignment contained 1471 positions. PAUP 4.0β10 [12] was used to analyse the dataset employing maximum likelihood (ML), maximum parsimony (MP), the Fitch-Margoliash method with LogDet distances (LD) and maximum likelihood distances (MD). Appropriate models for maximum likelihood were chosen with the help of Modeltest [19]. The robustness of each obtained topology was tested by bootstrapping (1000 replicates for all methods except for ML, for which 100 replicates were used). Phylogenetic analyses resulted in the tree shown in fig. 1. Two deep nodes of the phylogeny were resolved with low bootstrap support and/or resolved differently by different methods and were therefore depicted and treated as unresolved trichotomies.

Use of SlowFaster

At this time, our SlowFaster program was employed to perform the slow-fast analysis. First, the alignment used in our analyses was loaded via the “Load alignment” button. Then the tree topology shown in fig. 1 was loaded via the “Load tree” button. In typical slow-fast analyses, several monophyletic subgroups are chosen in this step. We decided to select the single subtree of all *Blastocystis* isolates. This arrangement was enabled by the fact that we were mostly interested in resolving the two nodes represented in the input tree by trichotomies. Assigning substitution rates to alignment

positions was thus independent from the true topology of these nodes. When the *Blastocystis*-containing subgroup was chosen in the tree window of SlowFaster program, new datasets in NEXUS format were created by clicking the "New alignments" button. Also, alignments of the same length as these new datasets, but shortened by random deletion of positions, were prepared by checking the "jackknives" checkbox on the program screen. These were used to test whether the loss of informative positions influences decrease of bootstrap support of the resulting tree topology more than shortening the datasets itself. We did not use the "Weights" feature of the program. When this checkbox is checked, the algorithm will assign different weights to changes within different chosen monophyletic groups. Changes within smaller groups would have assigned greater weight (if group A is twice as taxon-rich as group B, changes within it will have half the weight of the weight of changes in group B). The impact of large monophyla is then not dominant just because they contain more taxa.

The maximum number of observed changes in a position of our alignment was 9. Thus, nine new alignments were created. They were labeled BlastoS8 down to BlastoS0, where the number is the threshold. BlastoS0 alignment was of course of no use in this particular case (the analysis with just one monophyletic group) as it contained only those positions that did not change during the evolution of *Blastocystis*. All other alignments were analysed phylogenetically by all four methods (ML, MP, LD, MD) and topologies of the 32 resulting trees were bootstrapped.

It is highly probable that in some point of the slow-fast analysis, the profit from diminishing noise is lower than the loss from diminishing information. To roughly estimate the effect of the lack of information, we used average values of bootstraps as a measure of reliability of the alignments [10]. We found that this average value drops

suddenly for the alignment BlastoS1 which is therefore likely to suffer from lack of information and the resulting trees obtained from this dataset were not taken into account. To further prove this decision, "jackknifed" datasets of the same length but shortened by random deletion of position were also analysed. For each of eight datasets (Blasto_S1 to S8), ten of these randomly shortened datasets were analysed (80 alignments on the whole: Blasto_J1_1 to J1_10, J2_1 to J2_10, ... J8_1 to Blasto_J8_10). Within each dataset, the average value of bootstraps was determined and average of these averages for ten dataset of the same length were compared to average bootstrap value of the respective dataset resulting from slow-fast analysis. This comparison showed that the bootstrap values does not change much when analysing J8_x down-to J1_x datasets (e.g. all these average values ranged from 84.76 to 86.36 in ML analyses or from 90.15 to 91.5 in LD). On the contrary, the downfall of bootstraps was much more prominent in Blasto_S1 dataset when compared to Blasto_S2 – BlastoS8 datasets (e.g. 87.19 for original dataset, 86.90 for Blasto_S2, but 81.13 for Blasto_S1 in ML analyses, or 91.29 and 88.03 vs. 79.13, respectively, for LD).

Results concerning the two unresolved trichotomies are shown in Table 1. The isolate GERA3b grouped either with the basal branch of three reptile/amphibian isolates (1a, in fig. 1) or with the rest of *Blastocystis* (1b). In the original alignment, the former topology was very well supported by MP and LD, the latter was weakly supported by ML and MD. As the most saturated positions were deleted from alignment, the bootstrap support for topology "1a" decreased slightly in MP, but increased strikingly in MD and slightly in ML analysis (BlastoS1 not taken into account). The slow-fast analysis thus supports the "1a" topology. The second unresolved node concerned a branch of four reptile/amphibian isolates. Either it was

basal to two major branches of mostly mammal/bird isolates (2b; weakly supported by ML and MP in the original alignment), or it grouped with one of them (2a; weakly supported by LD and MD). After the slow-fast method was applied, both LD and MD favored the first possibility with reasonable bootstrap support for S3 and S2 datasets. However, MP and ML were unable to decide on the two possibilities. We conclude that the "2b" topology is probably correct, although the certainty is not high. For other nodes, decrease/increase of their bootstrap support from datasets S3 and S2 is marked in fig. 1.

Conclusions

Overall, the slow-fast analysis, provided by the program SlowFaster, proved to be a useful tool to solve uncertain phylogenies by increasing the signal/noise ratio. In the *Blastocystis* SSU rDNA tree it was able to make a choice among competing hypotheses and add more confidence in some other cases. Our software automates quite time-consuming slow-fast analysis.

Availability and requirements

Project name: SlowFaster

Project home page: http://natur.cuni.cz/flegr/programs/slowfaster.htm

Operating system: MS Windows

Programming language: Borland Delphi

Any restrictions to use by non-academics: none

The software can be accessed through the project home page and its current version is included with the manuscript as an additional file.

Authors' contributions

MK and JF designed the program and contributed bug fixes. MK developed the source code. IC and MU collected the data used as example and analysed them

together with MK. These three authors contributed to writing the manuscript. All authors read and approved the final manuscript.

Acknowledgements

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Figures

Figure 1 - MP tree of 31 *Blastocystis* isolates and 3 outgroups based on SSU rDNA sequences

Figure1: MP tree of 31 *Blastocystis* isolates (host in brackets) and 3 outgroups, based on SSU rDNA sequences. Bootstrap support values for four tree-reconstructing methods – ML, MP, LD and MD, respectively – are shown at the nodes. The symbol "+" is used for bootstrap support 99 and higher (in case only one "+" symbol is present, all methods scored such a high support). The effect of slow-fast analysis on nodes is represented by arrow symbols in the figure. Increase of an average bootstrap

support by more than 10% of one and more than one tree-reconstructing method in two datasets (BlastS3 and S2) is marked with "↑" and "↑↑", respectively. Similarly, the decrease of bootstrap support is marked with "↓" and "↓↓" at the particular nodes. Bootstraps of other nodes did not change dramatically. Except for our five new isolates (GERA3A, GERA3B, GEPA2, GECA2, KINIX2), *Blastocystis* isolates are labeled with accession numbers of their SSU rRNA gene sequences.

Tables

Table 1: Overview of results from slow-fast analysis of *Blastocystis* alignment.

Dataset	Posit	Length	1a				1b				2a				2b			
			ML	MP	LD	MD	ML	MP	LD	MD	ML	MP	LD	MD	ML	MP	LD	MD
Untr.	1471	1289		92	99		54			58	50	33					46	35
S8	1467	1250		93	99		51			54	45			30		34	43	
S7	1460	1187	57	96	99	54					42	34		36			37	
S6	1452	1121	62	96	99	48					48	35		38			38	
S5	1438	1026	61	91	97	55					54	42		34			35	
S4	1407	844	63	87	99	73					57			36		45	36	
S3	1371	674	59	82	97	85					–	38	67	73	–			
S2	1330	522	68	75	98	97					–	30	64	64	–			
S1	1258	343		49	92	90	71				–	–	90	76	–	–		
S0	1097	124																

For each dataset (the first column) ranging from untreated initial alignment (Untr.) to alignment BlastoS0, the number of alignment positions (Posit.) and the length of the most parsimonious tree (Length) are noted in the second and third columns,

respectively. In the remaining columns is given the bootstrap support from the four tree reconstructing methods for four topologies of interest. In some cases (marked with a dash) the method was unable to decide between the given node and its alternative.

Additional files

Additional file 1 – SlowFaster
This is the executable file of the application.

Additional file 2 – Source code
Zip archive containing Delphi source code of the program and additional Delphi files.

Additional file 3 – Sample data
Zip archive containing sample data – alignments in Phylip, FASTA and NEXUS format and tree files in Newick format.

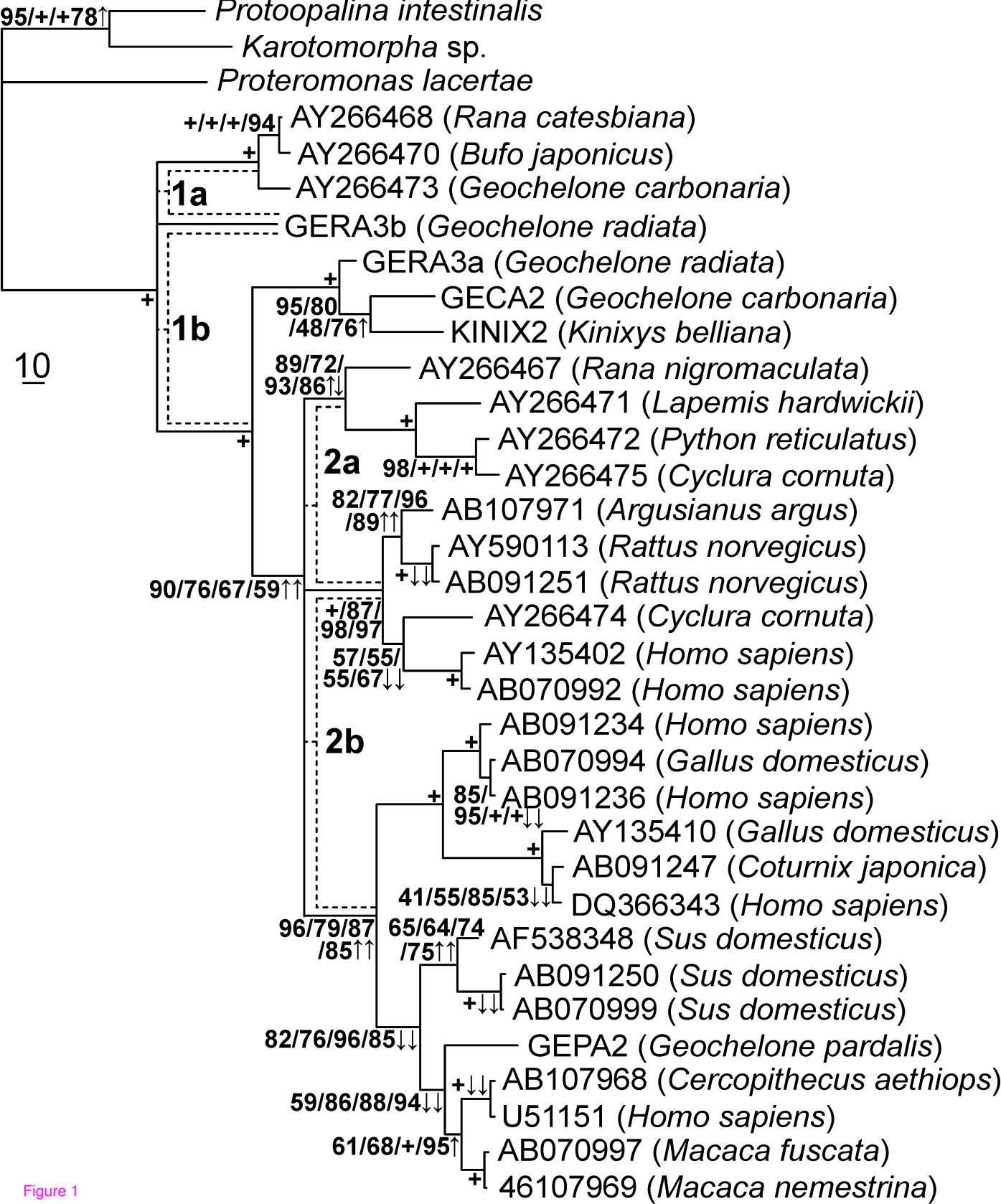
95/+/+78↑
Protoopalina intestinalis
Karotomorpha sp.
Proteromonas lacertae
+/+/+/94
AY266468 (Rana catesbiana)
+
AY266470 (Bufo japonicus)
AY266473 (Geochelone carbonaria)
1a
GERA3b (Geochelone radiata)
GERA3a (Geochelone radiata)
+
+
95/80
/48/76↑
GECA2 (Geochelone carbonaria)
KINIX2 (Kinixys belliana)
1b
10
89/72/
93/86↑↓
AY266467 (Rana nigromaculata)
AY266471 (Lapemis hardwickii)
+
AY266472 (Python reticulatus)
+
2a
98/+/+/+
AY266475 (Cyclura cornuta)
82/77/96
/89↑↑
AB107971 (Argusianus argus)
AY590113 (Rattus norvegicus)
+↓↓
AB091251 (Rattus norvegicus)
90/76/67/59↑↑
+/87/
98/97
AY266474 (Cyclura cornuta)
57/55/
55/67↓↓
AY135402 (Homo sapiens)
+
AB070992 (Homo sapiens)
2b
AB091234 (Homo sapiens)
+
AB070994 (Gallus domesticus)
+
85/
95/+/+↓↓
AB091236 (Homo sapiens)
AY135410 (Gallus domesticus)
+
AB091247 (Coturnix japonica)
41/55/85/53↓↓
DQ366343 (Homo sapiens)
96/79/87
/85↑↑
65/64/74
/75↑↑
AF538348 (Sus domesticus)
AB091250 (Sus domesticus)
+↓↓
AB070999 (Sus domesticus)
82/76/96/85↓↓
GEPA2 (Geochelone pardalis)
+↓↓
AB107968 (Cercopithecus aethiops)
59/86/88/94↓↓
U51151 (Homo sapiens)
61/68/+/95↑
AB070997 (Macaca fuscata)
+
46107969 (Macaca nemestrina)

Figure 1

Additional files provided with this submission:

Additional file 1: sf3.exe, 403K
http://www.biomedcentral.com/imedia/1369945795206817/supp1.exe
Additional file 2: sourcecode.zip, 39K
http://www.biomedcentral.com/imedia/1479834574217259/supp2.zip
Additional file 3: sampledata.zip, 22K
http://www.biomedcentral.com/imedia/1716081958217259/supp3.zip

Charles University

Faculty of Science

Department of Parasitology

Martin Kostka

Ph.D. thesis

MOLECULAR PHYLOGENY OF SELECTED PROTISTS

(MOLEKULÁRNÍ FYLOGENEZE VYBRANÝCH PRVOKŮ)

Thesis supervisor: Doc. RNDr. Jaroslav Flegr, CSc.

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Introduction

Although modern phylogenetics enabled us to understand the evolution of many traditionaly problematic groups, there are still some gaps and uncertainties in our knowledge of the whole “tree of life” topology. One of the areas where the gaps are relatively abundant is the eukaryote branch. The term “eukaryotes” is used here as the equivalent of “numerous and diverse lineages of protists plus a few multicellular groups”. The fact that our knowledge of protist phylogeny is not as robust as the knowledge of phylogeny of animals or plants, is not very surprising. First, there is a relative lack of interest. Second, unicellular organisms are often not so easily obtained and manipulated, thus the quantity of samples is lower. Third, there are many isolated, very old lineages of protists and the phylogeny-reconstructing methods may have problems with their substitutionally saturated sequences.

The author wants to introduce his and his colleagues' attempts to solve phylogenetic position of several protist taxa in this work. These are mainly parasitic/commensal protists. Opalinids are long known frog commensals. They are large, multiciliated and multinucleated. Nonetheless, the phylogenetic position of these interesting creatures was long uncertain. In 1985, Patterson noticed similarities in the ultrastructure of opalinids and a group of intestinal flagellates, the proteromonadids. He postulated that the two families belong to a common group (Slopalinida) and also that the latter is paraphyletic (i.e., that opalinids evolved from within proteromonadids). There are

molecular data available of *Proteromonas* only. In phylogenetic analyses, its sister group was rather enigmatic protist, *Blastocystis*. It is non-flagellated spherical organism inhabiting intestine of a wide spectrum of hosts. Another interesting protist group with hitherto no molecular data available is *Chilomastix*, a flagellate belonging among Eopharyngia with *Retortamonas* and diplomonads (including enteromonads). *Chilomastix* was believed to be a sister group of *Retortamonas*, but our results are in disagreement with this assumption.

Recently, the author collaborated on the reconstruction phylogeny of aquatic amoebae. They are more tricky than most of other eukaryotes as their identification might be problematic. It is, however, crucial for phylogenetics. There are several examples known of misidentified amoebae a sequence of which was used in phylogenetic analyses. The authors of the two "amoebae papers", on which the author of this thesis participated, dedicated great effort to determine and morphologicaly characterise the studied amoebae species.

Another contribution of the author and his colleagues is programming of the program SlowFaster, a tool for slow-fast analysis of sequence datasets. Slow-fast analysis is a method that is able to suppress negative effects of substitution saturation of alignment positions. Although lowering the risk of some artifacts, such as long branch attraction artifact, is very useful, this method can be quite time-consuming when applied manually.

Aims of the thesis

1. Use the methods of molecular phylogenetics to verify Patterson's hypotheses based on morphology:
    - Are slopalinids (Opalinidae + Proteromonadidae) a monophyletic group?
    - Are Proteromonadidae paraphyletic?
    - Do Slopalinids belong among stramenopiles?
2. Elucidate the position of *Blastocystis* with regard to Slopalinida
3. Check the phylogenetic position of *Chilomastix* within Eopharyngia and Fornicata.
4. Obtain and analyse molecular data from morphologically carefuly characterised amoebae.
5. Develop a software that will automate the slow-fast analysis that will allow deeper analyses of phylogenetic position of long-branched protists or other organisms.

Results

1. We sequenced SSU rDNA of *Protoopalina intestinalis*, representative of opalinids, and *Karotomorpha*, a proteromonadid with hitherto no molecular data available. Analyses of these data confirmed close affinity of the two families and also the paraphyly of proteromonadids – the resulting topology was (Proteromonas + (Karotomopha + Protoopalina)). Slopalinids belonged within stramenopiles in our analyses.
2. In our analyses, the genus *Blastocystis*, genetically quite variable, formed a sister group of slopalinids within the group of Stramenopila.
3. We sequences SSU rDNA of two *Chilomastix* isolates. The sequences differed substantially in length and composition, but formed a monophylum in resulting phylogenetic trees. Surprisingly, *Retortamonas* was not reconstructed as a sister group of *Chilomastix*, but the two genera formed a paraphyletic group from which the diplomonads evolved (with *Retortamonas* closer to diplomonads). This result implies that the ancestor of diplomonads (including enteromonads) was retortamonadid-like.
4. We sequenced and analyses SSU rDNA of two morphologically well defined amoebae, *Mayorella gemmifera* and *Saccamoeba limax*. We have found that another sequence ascribed to *S. limax* and used in some analyses probably originates from a

misidentified organism.

5. We have programmed the program SlowFaster. It is a unique user-friendly tool leading a user step-by-step through the whole process of slow-fast analysis. We believe that this tool will allow other authors to better exploit their datasets.

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List of Publications

Papers:

Kostka M, Hampl V, Cepicka I, Flegr J (2004): Phylogenetic position of *Protoopalina intestinalis* based on SSU rRNA gene sequence. *Molecular Phylogenetics and Evolution* 33: 220-224.

Kostka M, Cepicka I, Hampl V, Flegr J (2007): Phylogenetic position of *Karotomorpha* and paraphyly of Proteromonadidae. *Molecular Phylogenetics and Evolution* 43: 1167-1170.

Cepicka I, **Kostka M**, Uzlíková M, Kulda J, Flegr J (2008): Non-monophyly of Retortamonadida and high genetic diversity of the genus *Chilomastix* suggested by analysis of SSU rDNA. *Molecular Phylogenetics and Evolution* 48: 770-775.

Dyková I, Pecková H, **Kostka M** (2008): Introduction of *Mayorella gemmifera* Schaeffer, 1926 into phylogenetic studies of Amoebozoa. (Sent to *Acta Protozoologica*).

Dyková I, **Kostka M**, Pecková H (2008): SSU rRNA-based phylogenetic position of the genus *Saccamoeba* Frenzel, 1892 (Amoebozoa). (Sent to *Acta Protozoologica*).

Kostka M, Uzlíková M, Čepička I, Flegr J (2008): SlowFaster, a user-friendly program for slow-fast analysis and its application on phylogeny of *Blastocystis*. *BMC Bioinformatics* (accepted).

Selected abstracts:

Kostka M, Hampl V, Čepička I, Flegr J (2005): Phylogenetic position of Protoopalina intestinalis based on SSU rDNA sequence. *International Congress of Protozoology* 2005. Guangzhou, China.

Kostka M, Čepička I, Hampl V, Flegr J (2006): Phylogeny of slopalinids. 16th ISEP Meeting, Wroclaw, Poland.

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Úvod

Ačkoli díky moderní fylogenetice rozumíme evoluci mnoha tradičně problematických skupin, v celkovém obrazu „stromu života“ stále zůstávají bílá místa. Jednou z takových oblastí, kde jsou mezery v našem poznání fylogeneze hojnější, je větvení v rámci eukaryot. Pojem „eukaryota“ je zde použit ve významu „početné a rozmanité linie prvoků plus pár mnohobuněčných skupin“. To, že fylogenezi pvoků nerozumíme tak dobře jako třeba fylogenezi rostlin nebo živočichů, není zcela překvapivé – chybí zde takový zájem o prvoky, navíc jednobuněčné organizmy se často hůře získávají a špatně se s nimi manipuluje, takže není k dispozici takový počet vzorků. V neposlední řadě jsou ale problémy způsobeny také tím, že v rámci prvoků najdeme prastaré, izolované linie, jejichž sekvence mohou být substitučně saturované a působit tak vznik artefaktů při užití molekulárně fylogenetických metod.

Cílem autora je představit v této práci příspěvek týmu (resp. dvou týmů), jehož (jichž) je členem, k vyřešení problematiky fylogenetického postavení několikataxonů prvoků. Jedná se především o parazitické či komenzální prvoky. Opalinky jsou dlouho známí komenzálové žab. Jsou velké, mnohobičíkaté a mnohojaderné. Přesto, že jsou zvláštní a zajímavé, jejich fylogenetická pozice byla dlouho nejasná. Až v r. 1985 si Patterson povšimnul podobností v ulatrastruktuře opalinek a jedné skupiny střevních bičíkovců, proteromonadidů. Zařadil obě čeledi do jedné skupiny, Slopalinida, a

dále vyslovil domněnku, že čeleď Proteromonadidae je parafyletická – že se z ní vyvinuly opalinky. Molekulární data však byla k dispozici pouze z prvoka rodu *Proteromonas.* Ve fylogenetických analýzách se jako její sesterská skupina objevil poněkud záhadný prvok *Blastocystis.* Jedná se o bezbičíkaté sférické organismy žijící ve střevech širokého spektra hostitelů. Jinou zajímavou skupinou prvoků pro něž dosud nebyla k dispozici žádná molekulární data, je *Chilomastix*, bičíkovec patřící mezi Eopharyngia spolu s rodem *Retortamonas* a diplomonádami (včetně enteromonád). Věřilo se, že *Chilomastix* a *Retortamonas* jsou si vzájemně sesterské, naše výsledky však tento předpoklad nepotvrdily.

V poslední době spolupracuje autor na problematice fylogeneze vodních améb. Ty jsou oproti jiným prvokům zrádnější I díky tomu, že jejich přesná determinace může být problematická. je známo několik případů, kdy byly ve fylogenetických analýzách použity sekvence z nesprávně určených améb. Autoři dvou článků o amébách, na nichž se podílel I autor této práce, věnovali značné úsilí právě determinaci a morfologické charakterizaci studovaných améb.

Dalším příspěvkem autora a jeho spolupracovníků je naprogramování programu SlowFaster, což je nástroj pro slow-fast analýzu datasetů. Slow-fast analýza je metoda potlačující negativní vliv substituční saturace pozic v alignmentech. Ačkoli snížení rizika vzniku artefaktu přitahování dlouhých větví je velmi užitečné, je tato dosti časově náročná, je-li aplikována manuálně.

Cíle práce

1. S využitím molekulárně fylogenetických metod ověřit platnost Pattersonových hypotéz založených na morfologických datech:
    - Jsou slopalinidi (Opalinidae + Proteromonadidae) monofyletickou skupinou?
    - Je čeleď Proteromonadidae parafyletická?
    - Patří Slopalinida mezi Stramenopila?
2. Vyjasnit pozici *Blastocystis* vzhledem k slopalinidům
3. Ověřit fylogenetickou pozici rodu *Chilomastix* v rámci skupin Eopharingia a Fornicata
4. Získat a analyzovat molekulární data morfologicky dobře charakterizovaných améb
5. Vyvinout program pro automatizaci slow-fast analýzy, jež umožní hlubší analýzu fylogenetické pozice prvoků a jiných organismů s dlouhými větvemi

Výsledky

1. Získali jsme sekvenci SSU rDNA z prvoka *Protoopalina intestinalis*, reprezentujícího opalinky, a *Karotomorpha*, což je proteromonadid z něhož zatím nebyla žádná molekulární data k dispozici. Analýzy těchto dat potvrdily blízkou příbuznost obou skupin I parafylii proteromonadidů – výsledná topologie byla (*Proteromonas* + (*Karotomorpha* + *Protoopalina*)). Slopalinidi se v našich analýzách zařadili do skupiny Stramenopila.
2. Blastocystis, geneticky poměrně variabilní rod, se v našich analýzách vyskytoval jako sesterská skupina k řádu Slopalinida v rámci stramenopil.
3. Získali jsme sekvenci SSU rDNA ze dvou izolátů rodu *Chilomastix.* Obě sekvence se od sebe vzájemně dosti lišily délkou I složením, ale přesto ve fylogenetických stromech tvořily dobře podpořenou monofyletickou skupinu. Překvapivě však Retortamonas nebyla sesterskou skupinou tohoto rodu, byla bližší diplomonádám – čeleď Retortamonadidae zahrnující oba tyto rody je tedy zřejmě parafyletická. Takový výsledek by znamenal, že prapředek diplomonád (včetně enteromonád) byl retortamonadidního typu.
4. Získali jsme sekvence SSU rDNA dvou morfologicky dobře charakterizovaných améb, *Mayorella gemmifera* a *Saccamoeba limax*. Ukázali jsme, že sekvence připisovaná *S. limax* a používaná v některých fylogenetických analýzách, patří zřejmě

špatně určenému organismu.

5. Vytvořili jsme program SlowFaster. Jde o unikátní jednoduše ovladatelný nástroj, který uživatele provede celým procesem slow-fast analýzy. Věříme, že pomůže dalším autorům lépe využít jejich data.

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Kostka M, Čepička I, Hampl V, Flegr J (2006): Phylogeny of slopalinids. 16th ISEP Meeting, Wroclaw, Poland.

Obhajoba

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Práce bude obhajována v pondělí 15. září 2008 ve 13:00 na katedře parazitologi PřF UK, Viničná 7, Praha 2, ČR.