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Short communication

Phylogenetic position of *Karotomorpha* and paraphyly of Proteromonadidae

Martin Kostka [a,*], Ivan Cepicka [b], Vladimir Hampl [a], Jaroslav Flegr [a]

[a] *Department of Parasitology, Faculty of Science, Charles University, Vinicna 7, 128 44 Prague, Czech Republic*
[b] *Department of Zoology, Faculty of Science, Charles University, Vinicna 7, 128 44 Prague, Czech Republic*

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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

* Corresponding author. Fax: +420 224919704.
E-mail address: mkostka@centrum.cz (M. Kostka).

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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,

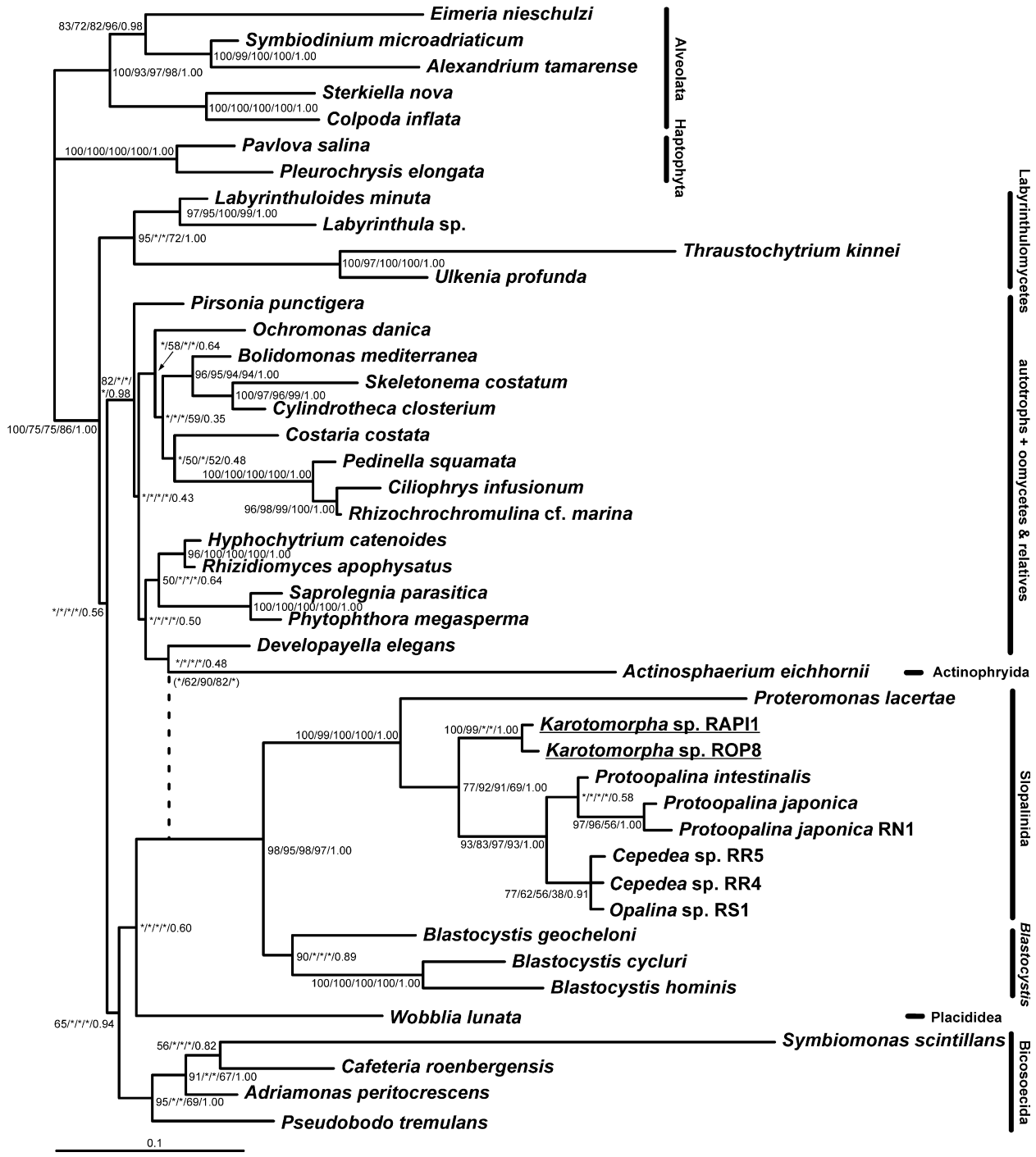

Fig. 1. Tree resulting from Bayesian analysis of SSU rDNA showing relationships among 36 stramenopile taxa + seven alveolate and haptophyte outgroups. Bootstrap values from maximum likelihood (100 replicates), maximum parsimony (1000 replicates), Fitch-Margoliash method with Log Det distances (1000 replicates), 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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