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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.

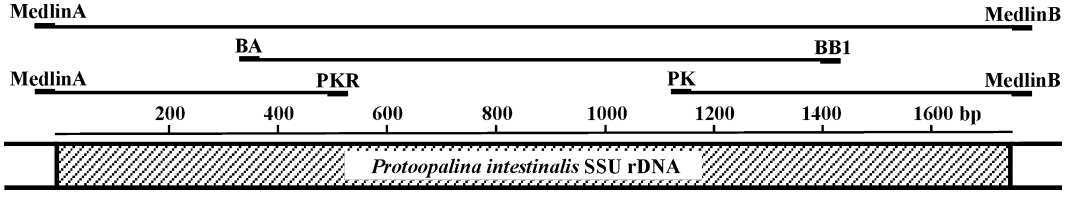

Fig. 1. The graphic representation of the SSU rDNA gene of *Protoopalina intestinalis*. Locations of the used primers and the lengths of the corresponding PCR products including their overlapping parts are indicated.

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

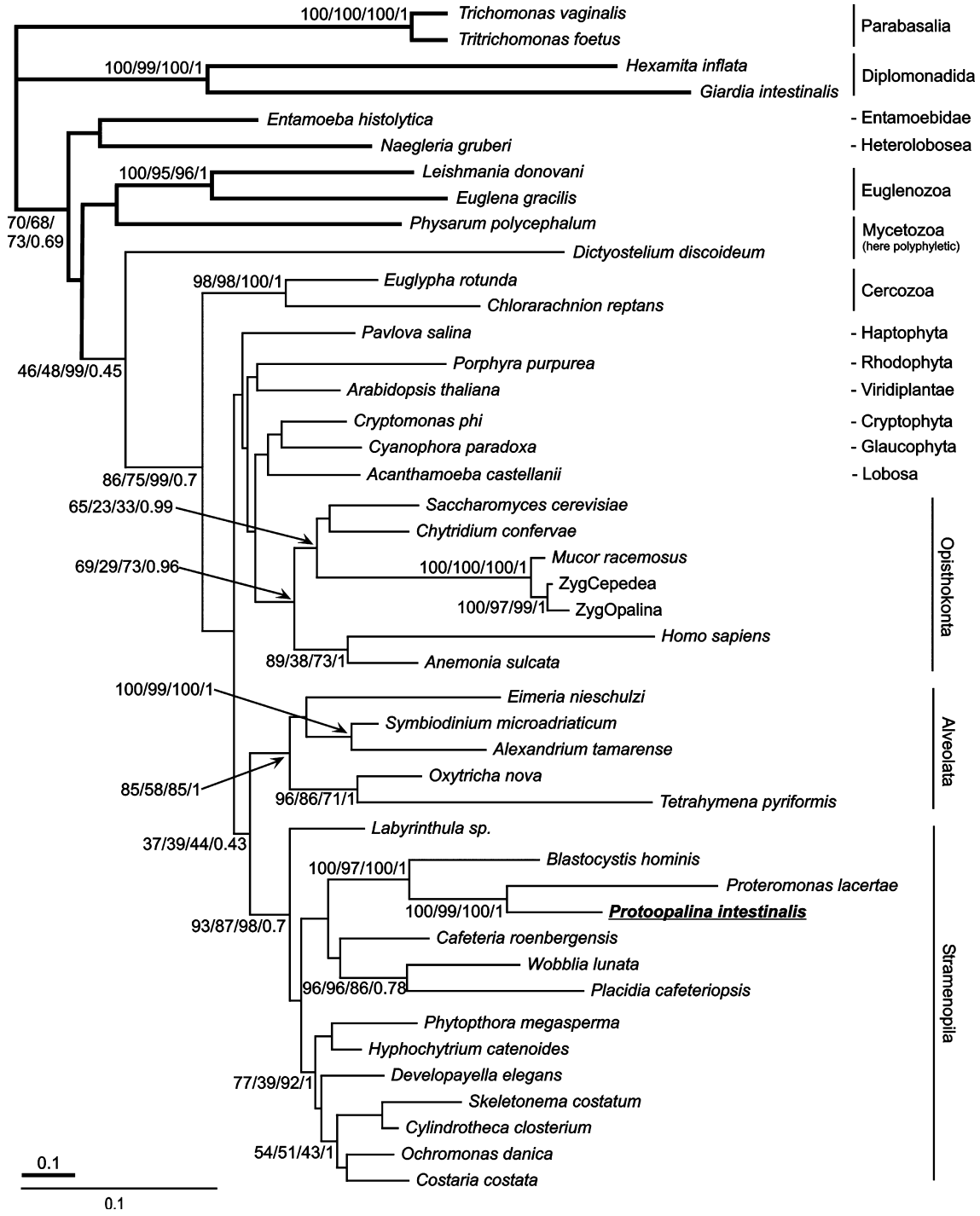

Fig. 2. Maximum likelihood tree showing relationships among 44 eukaryotic taxa. Bootstrap values from maximum likelihood (100 replicates), maximum parsimony (1000 replicates), LogDet (1000 replicates), and bayesian posterior probabilities, respectively, are shown at the nodes. Bootstrap values are not 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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