Department of Parasitology

Faculty of Science, Charles University in Prague

Ph.D. thesis:

Molecular Phylogeny of Selected Protists

Martin Kostka

Ph.D. supervisor: Prof. RNDr. Jaroslav Flegr, CSc.

Prague and České Budějovice, 2008

Za to, že tato práce mohla vůbec vzniknout a že ji mohu předložit, vděčím mnoha lidem. Mé díky patří především mému školiteli, Jardovi Flegrovi, a mým nejbližším spolupracovníkům, jmenovitě Ivanovi Čepičkovi, Vláďovi Hamplovi, Martinovi Kolískovi a Magdě Uzlíkové. Dále pak děkuji všem dalším členům a studentům Katedry parazitologie za skvělé prostředí. Děkuji také prof. Ivě Dykové a pracovníkům Biologického centra AV ČR i zemědělské fakulty JU v Českých Budějovicích, kde se začínají psát nové kapitoly mého profesního života. Davidovi Modrému děkuji za žáby. Za to, že jsem plně pocítil, že existují i významnější věci, než jsou např. tituly, děkuji s láskou svojí rodině.

Výsledky prezentované v dizertační práci Mgr. Martina Kostky jsou společným dílem pracovníků Laboratoře molekulární fylogenetiky na katedře Parazitologie UK a také pracovníků Oddělení eukaryotických mikroorganismů infikujících ryby Biologického centra AV ČR. Prohlašuji, že autor se na jejich získání zasloužil významným dílem.

Data presented in this thesis resulted from the team collaboration at the laboratory of Molecular phylogenetics of protozoa (Dept. of Parasitology, UK) and at the laboratory of Eukaryotes infecting fish (AS CR). I declare that author substantially contributed to the obtaining of these results.

Prof. RNDr. Jaroslav Flegr, CSc.

Contents

1. Review

1.1. Protists and phylogenetics

Modern phylogenetics, based on sofisticated phylogenetic analyses predominantly of molecular data, enabled us to understand relationships within and among groups with hitherto unknown phylogenies. Thank to these methods we are now certain of phylogenetic position of many traditionally problematic taxa (just a few examples: Myxozoa are cnidarians [1], Rafflesiaceae evolved from within Euphorbiaceae [2], etc.), many traditional groups were abandoned (e.g., Articulata, Anthophyta, …) and new higher taxa were defined (e.g., Afrotheria [3], Ecdysozoa [4], ...). Although we have witnessed a true revolution in this field, there are still numerous gaps in our understanding phylogeny of living creatures. In some areas, the gaps are more abundant and one of these areas is phylogeny of eukaryotes. 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 (there are much more zoologists and botanists than protozoologists). 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. Recently, the author collaborated on the reconstruction phylogeny of aquatic amoebae. His another contribution is programming of the program SlowFaster, a tool for slow-fast analysis of sequence datasets. More detailed introduction to all these themes follows.

1.2. Slopalinids and *Blastocystis*

Opalinids are protists known since the times of van Leeuwehoek. They are common in intestine of frogs and can be found in other amphibians and some fish. They are quite unusual, in several ways. They are large – up to millimeters, they are multiciliated and multinucleated. Five genera are recognized within the family: two are truly multinucleated with up to hundreds of nuclei (*Opalina* and *Cepedea*), the remaining three genera are binucleated (*Protozelleriella*, *Zelleriella* and *Protoopalina*) [e.g., 5]. All the mentioned exceptionalities of opalinids are also found in ciliates and this group was initially thought to be a sister group of opalinids. Opalinids were viewed as primitive relatives of ciliates without e.g. cytostome or nuclei differentiated in micro- and macronucleus. This putative position was further strengthened by the existence of astomatids – ciliates sharing several features with opalinids: astomatids lack cytostomes and are gut commensals (of molluscs and annelids). Later, this close relationship between astomatids and opalinids was doubted, most notably by Metcalf, who separated opalinids from all other ciliates including astomatids [6, according to 7]. Subsequently, the concept of monophyletic Opalinidae + Ciliata was abandoned and different authors classified opalinids as an isolated group within Zooglagellata [8], Sarcomastigophora [9] or as a separate phylum [10].

No hypothesis on closer relationships of these interesting organisms had been postulated until 1985, when Patterson [11] noticed several ultrastructural features that opalinids and proteromonadids had in common. Proteromonadidae is a small family of flagellates comprising two genera – *Proteromonas* and *Karotomorpha*. Both are commensal to vertebrates: frogs (*Karotomorpha*) or caudate amphibians, reptiles and mammals (*Proteromonas*). *Proteromonas* possesses two flagella, whereas *Karotomorpha* four. Their ultrastructure was published earlier [12]. The details of their basal bodies

morphology, e.g., the structure called double transitional helix, closely resemble those of opalinids. Another remarkable common feature of *Karotomopha* and opalinids is their heavily folded surface. In both taxa, the folds are supported by ribbons of microtubules. It is worth mentioning that light interference on these folds causes opalescence of opalinids, a phenomenon that gave them their name. The cell surface of *Proteromonas* is also folded, but the folds are not so prominent and are supported by single microtubules only. These, and same other, more general similarities, led Patterson to unite the two families in one monophyletic group – Slopalinida. He also stated that *Karotomorpha* is closer to opalinids than to *Proteromonas*, the family Proteromonadidae would then be paraphyletic. Further, he suggested that slopalinids could be relatives of heterokont algae: they also possess transitional helix in their basal bodies (although it is single-stranded in their case) and fine hair on their flagellum, mastigonemes, resemble somatonemes covering posterior part of *Proteromonas* cell. Although some authors sought other affinities of slopalinids (see, e.g., the Cavalier-Smith's phylum Opalozoa [13]), it was later shown by phylogenetic study [14] containing SSU rDNA sequence of *Proteromonas*, that Patterson was right and that *Proteromonas*, at least, belongs among heterokonts, or, more specifically, stramenopiles (meaning heterokonts without haptophytes and cryptophytes). The diverse group of stramenopiles comprise autotrophs (e.g., brown algae, diatoms, chrysophytes), fungus-like organisms (Oomycetes, Hyphochytriomycetes, Labyrinthulomycetes), heterotrophic flagellates (bicosoecids, Placididea) and other organisms, e.g., the actinophryid "heliozoans".

The aforementioned study [14] also revealed that another enigmatic organism is sister group of slopalinids: the genus *Blastocystis.* Protists of this genus are non-flagellated gut commensals/parasites of a wide spectrum of hosts, including man. Their cells are spherical and contain several nuclei. Their phylogenetic position was uncertain until their

molecular data were analysed. Affinity of *Blastocystis* with parabasalids, fungi and later with amoebae or Apicomplexa had been proposed, but nobody guessed these strange creatures might have evolved from within stramenopiles.

Despite some attempts (see sequences AF141969, -70 and -74, or AF147882 deposited in GenBank), no molecular data were available for opalinids themselves, or for *Karotomorpha*. That disqualified phylogenetic studies from answering several interesting questions, most importantly whether Patterson's group Slopalinida, based on morphological data only, was truly monophyletic. Another issue was the proposed paraphyly of Proteromonadidae. These two questions were central to two papers of Kostka and his coworkers (see appendix 1 and 2). In them, the authors showed that SSU rDNA phylogeny clearly supports both Patterson's hypotheses.

Another recent attempt to establish phylogenetic position of opalinids, the work of Nishi et al. [15], resulted in somewhat controversial conclusions: SSU rDNA-based phylogeny yielded outcomes similar to those of our team, i.e., reconstructed opalinids as stramenopiles, but α- and β-tubulin sequences suggested different phylogenetic affinities. In tubulin phylogenies, opalines belonged to another group, although related to stramenopiles, to alveolates. Alveolata comprise most notably ciliates, Apicomplexa and dinoflagellates. It seems that the phylogenetic position of opalinids is once again uncertain. However, when results of Nishi et al. are inspected in detail, one can notice a relatively low bootstrap support in their tubulin phylogenies. The highest bootstrap value contradicting opalinid affinity to stramenopiles is 55 % and 70 % in maximum likelihood α-tubulin and β-tubulin analysis, respectively. Moreover, the latter value goes for ((*Colpoda* + opalinids) + Apicomplexa) with *Tetrahymena* and a group of other ciliates as successive sister groups. That would suggest that apicomplexans were derived ciliates, which is very improbable. Nishi et al. also notice that AU tests based on tubulin data reject

the results of SSU rDNA phylogeny, and vice versa.

Reconstructing the phylogeny of *Blastocystis* was part of our work which was the basis for our another paper (see appendix 6). Nowadays, there are many SSU rDNA sequences of various *Blastocystis* isolates from different hosts. Analyses of these data show that the genus *Blastocystis* is very diverse and contain several well supported lineages. Interrelationships among them are, however, not resolved very well. Although the host specificity of at least some lineages is seemingly quite low, some of the lineages contain mainly or exclusively strains isolated from reptiles and amphibians, or birds. If we understood the phylogeny of *Blastocystis* better, we could, study host switching / coevolution of the parasite with its host, etc. We tried to increase the resolution of some nodes of the phylogeny with the use of slow-fast method [16] (see chapter V. Data filtering). Our results support a basal clade of amphibian / reptile isolates and a crown-group of strains inhabiting mainly endothermic vertebrates.

1.3. Basal eopharyngians

The taxon Eopharyngia comprises several traditional groups of mainly commensal/parasitic secondarily amitochondriate flagellates: retortamonads (*Retortamonas*, *Chilomastix*), diplomonads (e.g., *Hexamita*, *Spironucleus*, *Giardia*, *Octomitus*) and enteromonads (e.g., *Enteromonas*, *Trimitus*). Diplomonads are probably their best known representatives. They are interesting in having double karyomastigont in their cells – two nuclei with 2 × four basal bodies and their roots. In Hexamitiinae, posterior pair of flagella is associated with cytopharynges, in Giardiinae no cytostomes and cytopharynges are present and axonemae of the posterior flagellar pair are located in cytoplasm. Morphology of enteromonads resemble that of diplomonads but they possess

one karyomastigont only. Enteromonads were often interpreted as more primitive ancestors of diplomonads, which arose from them via heterochrony (karyokinesis that was not followed with cytokinesis) [17]. It have been shown, however, that this view of evolution is too simple. Molecular studies suggest that enteromonads are an inner group of Hexamitiinae and are probably even polyphyletic within diplomonads [18]. That would mean that either cells of enteromonads are secondarily simplified or diplomonads arose several times from enteromonad-like ancestors.

Retortamonads were usually considered relatives of diplomonads (including 'enteromonads') and they together form the taxon Eopharyngia. The two groups share some morphological similarities: retortamonads also have karyomastigonts with four basal bodies (but only two flagella in *Retortamonas*), the most posterior flagellum is associated with a cytostome. All eopharyngians lack some typical eukaryote organelles, namely typical mitochondria, Golgi complex and peroxysomes. However, mitochondrial remnants, mitosomes, were found in *Giardia* [19]. Molecular data also corroborated relationship between *Retortamonas* and diplomonads [20]. Two free-living flagellates, *Carpediemonas* and *Dysnectes,* were recently identified as close relatives of eopharyngians, forming the group of Fornicata with them [21 and 22, respectively].

However, there were no molecular data available for *Chilomastix*, the second retortamonad genus. See appendix 3 for our paper on the first phylogenetic analysis of molecular data for two *Chilomastix* isolates. We show that *Chilomastix* does not form a monophylum with *Retortamonas* in SSU rDNA trees, which was quite surprising. Rather, *Retortamonas* and *Chilomastix* are successive outgroups to diplomonad clade, Retortamonadidae is thus a paraphyletic group. If this topology was correct, it would have some implications for the view of eopharyngian evolution (to put it simply, the ancestor of diplomonads would be retortamonadid-like). The two examined *Chilomastix* isolates

display remarkable differences in SSU rRNA gene length and composition, although they are robustly clustered with each other. Nonetheless, this implies that there might be hidden more independent lineages within genera *Retortamonas* and *Chilomastix*, as there are many unexamined species described inhabiting various hosts.

1.4. Amoebozoa

Amoebozoa is one of several eukaryotic supergroups [23], which can be recognized in the phylogenetic tree of eukaryotes. It comprises mainly various amoeboid organisms, such as true amoebae (e.g., *Amoeba*), testate lobose amoebae (*Arcella*), pelobionts (*Entamoeba*), slime molds (*Dictyostelium*) and others. Our knowledge of phylogeny and even recognition of the group itself depend mainly on molecular data – morphological characters that could be used to define synapomorphies of higher amoebozoan groups are rather scarce. Amoebozoan phylogeny, which is based predominantly on SSU rDNA data, is still very unclear, although some groupings are quite well supported (e.g., Tubulinea, Flabellinea, pelobionts) [e.g., 24].

Insufficient resolution of SSU rDNA- or other genes-based phylogenies is not the only reason why the reliability of amoebozoan phylogeny is low. There is another general problem accentuated in Amoebozoa. The mentioned lack of readily accessible morphological data and considerable shape plasticity of many amoebae make troublesome both finding synapomorphies of higher taxa and identification of species or genera of amoebae. However, precise determination of organisms is crucial for further work, especially in phylogenetic studies. Some sequences of amoebae that are used in phylogenetic studies originate from insufficiently described or even misidentified organisms [25, 26]. It would be very useful to document all newly published sequences

with detailed morphological study so that the true identity of the source organism could be verified.

Two papers (see appendices 4 and 5) on which the author participated dealt with this problem in two amoebozoan genera. In both cases, the amoebae were characterized molecularly by sequencing their SSU rRNA gene, but great effort was dedicated to maintain their stable cultures, provide their detailed description based on both light and electron microscopy and to determine them most accurately.

In the former of these papers, authors characterized and phylogeneticaly analysed a strain of *Mayorella gemmifera*. *Mayorella* is an example of amoeba with uncertain phylogenetic position within Amoebozoa. The only other representative of this genus in phylogenetic analyses , *Mayorella* sp. JJP-2003, was never thoroughly characterized morphologically. Although the two *Mayorella* strains formed a monophyletic clade (of unclear affinities to other amoebozoans), they also exhibited a great difference in SSU rRNA gene sequence length, mainly due to a long insertion in V2 region of *M. gemmifera* SSU rRNA.

The latter paper deals with a new isolate of *Saccamoeba limax.* We show that sequence No. AF293902 deposited in GenBank, ascribed to *Saccomoeba limax* and frequently used in phylogenetic analyses, probably does not represent this species or even genus. Unfortunately, there are no morphological data of this organism available, so our assumption can hardly be confirmed or refuted on the basis of other then sequence data. Otherwise, our analysis further corroborates that some other genera (*Hartmannella*, *Nolandella*, *Amoeba* and *Chaos*) and higher taxa of amoebae are probably paraphyletic or even polyphyletic, or that some published sequences originate from another misidentified organisms. In any case, careful revision of published data related to amoebae and careful examination of data yet to be published is of essence.

1.5. Data filtering

The paper that can be found in appendix 6, is different from other papers introduced in this work. Its main issue is not phylogeny of a protist taxon, although *Blastocystis* alignment is used as an exemplary dataset there, but rather a software carrying out so called slow-fast analysis. Slow-fast analysis [16] is purposed to reduce the number of too variable positions in alignments. It is well documented, that high proportion of substitutionally saturated positions may cause artifacts in reconstructed tree topology – long branch attraction (LBA) is an important example of these [27]. One way to suppress these negative effects is to analyse those alignment positions only, that substitute more rarely. In saturated positions, the phylogenetic signal is overwhelmed by noise, so the deletion of such positions improves the signal/noise ratio of a dataset.

Slow-fast analysis requires several subbranches of well known topology to be *a priori* chosen, then uses parsimony to count changes of each position within these branches. The numbers of changes are estimates of saturation rates of alignment positions. Positions with highest rates are then successively deleted from original alignment and the new datasets with smaller and smaller numbers of variable positions can be used to reconstruct interrelationships among the input branches. The risk of LBA should be lower.

Manual deletion of variable positions indicated by slow-fast analysis can be very time consuming, especially in larger datasets. On the other hand, it can be relatively easily automatized. Nevertheless, except for rather complex package MUST [28], which is not easily operated under MS Windows, no program for this type of analysis was available. This was the reason why we decided to create such a software on our own: our user-friendly program SlowFaster is an easily-operated, step-by-step tool to conduct slow-fast analysis. It also offers several unique additional features. Substitution rates of alignment

positions may differ in different tree branches. That is one of the reasons why several branches are chosen as input taxa in which number of changes is determined. However, in real alignments, some branches are more taxon-rich than others – and their influence on computation of change numbers is thus greater. SlowFaster allows user to weight the importance of input branches for these counts – the more taxa in a branch, the lower is the weight. Another useful function of SlowFaster relates to the fact, that as new datasets with less and less positions are examined, we will probably reach the step where the advantage from lowering noise level in dataset will be outweighed by loss of information. The resolution of topology nodes (estimated by, e.g., bootstrap values) then gets worse. SlowFaster offers the possibility to produce new alignments of the same length as the alignments without the most variable positions, but positions to delete from these are chosen randomly. One can then compare e.g. mean bootstrap value in trees constructed on the basis of slow-fast datasets and randomly-shortened datasets. When support for the former tree topology is higher, it can be interpreted as benefit from lowering noise level, whereas an opposite case would mean that we are already loosing more useful information than noise.

We hope that publication of our software will enable other teams to better exploit their datasets and help them in revealing new phylogenetic hypotheses.

1.6. Literature cited

[1] Jiménez-Guri E, Philippe H,Okamura B, Holland PWH (2007): Buddenbrockia Is a Cnidarian Worm. *Science* 317: 116-118.

[2] Davis ChC, Latvis M, Nickrent DL, Wurdack KJ, Baum DA (2007): Floral Gigantism

in Rafflesiaceae. *Science* 315: 1815.

[3] Stanhope MJ, Waddell WG, Madsen O, de Jong W, Hedges SB, Cleven GC, Kao D, Springer MS (1998): Molecular evidence for multiple origins of Insectivora and for a new order of endemic African insectivore mammals. *Proc Natl Acad Sci U S A* 95: 9967–9972.

[4] Aguinaldo AMA, Turbeville JM, Linford LS, Rivera MC, Garey JR, Raff RA, Lake JA (1997): Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature* 387: 489-493.

[5] Delvinquier BLJ, Patterson DJ (1993): The Opalines. In *Parasitic Protozoa* (Kreier JP, Baker JR, eds.), Volume 3, pp. 247-325. Academic Press, San Diego.

[6] Metcalf MM (1909): Opalinata. Its anatomy and reproduction, with a description of infection experiments and a chronologicalreview of the literature. *Arch Protistenkd* 13: 195-375.

[7] Corliss JO (1955): The Opalinid Infusorians: Flagellates or Ciliates? *J Protozool* 21: 107-114.

[8] Grassé PP (1952): Traité de zoologie, Vol. 1. Masson, Paris.

[9] Corliss JO, Balamuth W (1963): Consideration of the Opalinids as a New Superclass in the Subphyllum Sarcomastigophora. *J Protozool* 10 (Suppl.): 26.

[10] Wessenberg H (1978): Opalinata. In *Parasitic Protozoa*, *Intestinal Flagellates*, *Histomonads*, *Trichomonads*, *Amoeba*, *Opalinids*, *and Ciliates*, (Kreier, J. P., ed.), Vol. 2, pp. 551-581. Academic Press, New York-London.

[11] Patterson DJ (1985): The Fine Structure of Opalina ranarum (family Opalinidae): Opalinid Phylogeny and Classification. *Protistologica* 4: 413-428.

[12] Brugerolle G, Joyon L (1975): Étude cytologique ultrastructurale des genres Proteromonas et Karotomorpha (Zoomoastigophorea Proteromonadida Grassé 1952). *Protistologica* 11: 531-546.

[13] Cavalier-Smith T (1993): The Protozoan Phylum Opalozoa. *J Euk Microbiol* 40: 609-615.

[14] Silberman JD, Sogin ML, Leipe DD, Clark CG (1996): Human Parasite Finds Taxonomic Home. *Nature* 380: 398.

[15] Nishi A, Ishida K, Endoh H (2005): Reevaluation of the Evolutionary Position of Opalinids Based on 18S rDNA, and α- and β-Tubulin gene Phylogenies. *J Mol Evol* 60: 695-705.

[16] Brinkmann H, Philippe H: Archaea Sister Group of Bacteria? Indications from Tree Reconstruction Artifacts in Ancient Phylogenies. *Mol Biol Evol* 1999, 16: 817-825.

[17] Siddall ME, Hong H, Desser SS (1992): Phylogenetic Analysis of the Diplomonadida

(Wenyon, 1926) Brugerolle, 1975: Evidence for Heterochrony in Protozoa and Against Giardia lamblia as a "Missing Link". *J Euk Microbiol* 39: 361-367.

[18] Kolisko M, Cepicka I, Hampl V, Leigh J, Roger AJ, Kulda J, Simpson AGB Flegr J (2008): Molecular phylogeny of diplomonads and enteromonads based on SSU rRNA, α-tubulin and HSP90 genes: implications for the evolutionary history of the double karyomastigont of diplomonads. *BMC Evolutionary Biology* (accepted).

[19] Tovar J, Leon-Avila G, Sánchez LB, Šuťák R, Tachezy J, van der Giezen M, Hernandez M, Müller M and Lucocq JM: Mitochondrial remnant organelles of Giardia function in iron-sulfur protein maturation. *Nature* 2003, 426: 172-176.

[20] Silberman JD, Simpson AGB, Kulda J, Cepicka I, Hampl V, Johnson PJ, Roger AJ (2002): Retortamonad Flagellates are Closely Related to Diplomonads—Implications for the History of Mitochondrial Function in Eukaryote Evolution. *Mol Biol Evol* 19: 777-786.

[21] Simpson AGB, Roger AJ, Silberman JD, Leipe DD, Edgcomb VP, Jermiin LS, Patterson DJ, Sogin ML (2002): Evolutionary history of "early-diverging" eukaryotes: the excavate taxon Carpediemonas is a close relative of Giardia. *Mol Biol Evol* 19: 1782-1791.

[22] Yubuki N, Inagaki Y, Nakayama T, Inouye I (2007): Ultrastructure and ribosomal RNA phylogeny of the free-living heterotrophic flagellate Dysnectes brevis n. gen., n. sp., a new member of the Fornicata. *J Euk Microbiol* 54: 191-200.

[23] Cavalier-Smith T (1998). A revised system of life. *Biol Rev* 73: 203-266.

[24] Tekle YI, Grant J, Anderson R, Nerad TA, Cole JC, Patterson DJ, Katz LA (2008): Phylogenetic placement of diverse amoebae inferred from multigene analyses and assessment of clade stability within ‘Amoebozoa’ upon removal of varying rate classes of SSU-rDNA. *Mol Phyl Evol* 47: 339–352.

[25] Smirnov AV, Nassonova ES, Chao E, Cavalier-Smith T (2007): Phylogeny, Evolution, and Taxonomy of Vannellid Amoebae. *Protist* 158: 295-324.

[26] Smirnov AV, Nassonova ES, Cavalier-Smith T (2008): Correct identification of species makes the amoebozoan rRNA tree congruent with morphology for the order Leptomyxida Page 1987; with description of Acramoeba dendroida n. g., n. sp., originally misidentified as 'Gephyramoeba sp.'. *Eur J Protistol* 44: 35-44.

[27] Felsenstein J (1978): Cases in which parsimony or compatibility methods will be positively misleading. *Syst Zool* 27: 401-410.

[28] Philippe H (1993): MUST, a computer package of management utilities for sequences and trees. *Nucleic Acids Res* 21: 5264-5272.

2. Publications

This thesis is based on the following papers. Three of them are already published in the jornal Molecular Phylogenetics and Evolution. One is accepted by BMC Bioinformatics. Note that the program SlowFaster presented in the paper can be downloaded from http://www.natur.cuni.cz/flegr/programs/slowfaster.htm. Remaining two papers were sent to Acta Protozoologica.

Appendix 1:

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.

Appendix 2:

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

Appendix 3:

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.

Appendix 4:

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

Appendix 5:

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

Appendix 6:

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).

3. Conlusions

- Our team was the first to successfully sequence and phylogeneticaly analyse SSU rDNA of an opalinid (*Protoopalina*), *Karotomorpha* and *Chilomastix*, and to publish the results.
- We have shown that SSU rDNA phylogeny supports the hypotheses that
  - (1) opalinids, *Proteromonas* and *Karotomorpha* form a monophyletic group.
  - (2) slopalinids belong among stramenopiles and that *Blastocystis* is their sister group.
  - (3) Proteromonadidae is a paraphyletic group – *Karotomorpha* is more closely related to opalinids than to *Proteromonas*.
- We have shown that retortamonadids are also paraphyletic, *Chilomastix* is a sister group of *Retortamonas* + diplomonads (including enteromonads).
- We have obtained and analysed SSU rDNA sequence of two morphologicaly well-characterized amoebae.
- We have shown that ATCC 30942 strain denominated *Saccamoeba limax*, SSU rDNA of which is used in some analyses, is probably misidentified.
- We have programmed a user-friendly software for slow-fast analyses of molecular datasets.