J. Eukaryot. Microbiol., 51(5), 2004 pp. 575–581
© 2004 by the Society of Protozoologists

Molecular Phylogenetic Analysis Places *Percolomonas cosmopolitus* within Heterolobosea: Evolutionary Implications

SERGEY I. NIKOLAEV,[a] ALEXANDRE P. MYLNIKOV,[b] CEDRIC BERNEY,[c] JOSE FAHRNI,[c] JAN PAWLOWSKI,[c] VLADIMIR V. ALESHIN[a] and NIKOLAY B. PETROV[a,1]

[a]*Department of Evolutionary Biochemistry, A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia, and*
[b]*Institute for Biology of Inland Waters, RAS, Yaroslavskaya obl., Borok, Russia, and*
[c]*Department of Zoology and Animal Biology, University of Geneva, Geneva, Switzerland*

ABSTRACT. *Percolomonas cosmopolitus* is a common free-living flagellate of uncertain phylogenetic position that was placed within the Heterolobosea on the basis of ultrastructure studies. To test the relationship between *Percolomonas* and Heterolobosea, we analysed the primary structure of the actin and small-subunit ribosomal RNA (SSU rRNA) genes of *P. cosmopolitus* as well as the predicted secondary structure of the SSU rRNA. *Percolomonas* shares common secondary structure patterns of the SSU rRNA with heterolobosean taxa, which, together with the results of actin gene analysis, confirms that it is closely related to Heterolobosea. Phylogenetic reconstructions based on the sequences of the SSU rRNA gene suggest *Percolomonas* belongs to the family Vahlkampfiidae. The first Bayesian analysis of a large taxon sampling of heterolobosean SSU rRNA genes clarifies the phylogenetic relationships within this group.

Key Words. Helix 17, Heterolobosea, phylogeny, SSU rRNA, V3 region.

THE free-living flagellate *Percolomonas cosmopolitus* is common and widespread in surface coastal and oceanic waters. Initially, Ruinen (1938) described this organism as a representative of the heterolobosean genus *Tetramitus* on the basis of morphological and ecological characters, such as general body form, flagellation, dimensions, and trophic behaviour. More recently, on the basis of ultrastructural evidence, Fenchel and Patterson (1986) established for it a new genus *Percolomonas* of uncertain phylogenetic position within the class Heterolobosea. Ultrastructural features of *Percolomonas* were considered primitive for eukaryotes by Cavalier-Smith (1993), who proposed a new class Percolomonadea, constituting, together with the classes Heterolobosea and Lyromonadea, a new phylum Percolozoa. Later, the class Percolatea was erected to unite *Percolomonas* and *Stephanopogon* (Cavalier-Smith 2004). It has been suggested that *Percolomonas*, lyromonads and (other) heteroloboseids belong to the taxon Excavata (Cavalier-Smith 2002; Simpson and Patterson 1999), a group otherwise encompassing a diversity of aerobic and anaerobic flagellates.

The class Heterolobosea was established by Page and Blanton (1985) to unify Acrasida (former slime molds) and Schizopirenida (amoeboflagellates)—amoeboid organisms that share eruptive movement in the amoeboid stage, discoid mitochondrial cristae, and lack typical dictyosomes. Many heteroloboseans are capable of altering from amoeboid to flagellate stages; some are solely amoeboid. Now, it is clear that some organisms known only as flagellates also belong to this group (Fenchel and Patterson 1986). Based on the capacity to form so-called "fruiting bodies" or not, the class Heterolobosea was separated into two orders, Acrasida and Schizopyrenida, respectively. The order Schizopyrenida is traditionally divided into two families, Vahlkampfiidae and Gruberellidae, on the basis of the presence of a flagellate stage, the trophozoite morphology, and mode of mitosis. The family Vahlkampfiidae includes several genera, among which are *Vahlkampfia*, *Tetramitus*, *Naegleria*, and *Heteramoeba*. The genus *Vahlkampfia* was primarily established for vahlkampfiid amoebas without a flagellate stage in their life cycle (Page and Blanton 1985). Later, two species (i.e. *Paravahlkampfia ustiana* and *Neovahlkampfia damariscotta*) were excluded from the genus due to their high rates of morphological and genetic divergence (Brown and De Jonckheere 1999). Some authors included the anaerobic and amitochondriate *Psalteriomonas lanterna* in the class Heterolobosea, as a member of the order Schizopyrenida (Broers et al. 1990). Later, the class Lyromonadea was especially established to include *P. lanterna* (Cavalier-Smith 1993). Molecular phylogenetic studies revealed a high rate of divergence of the small-subunit ribosomal RNA (SSU rRNA) gene of *P. lanterna* compared to vahlkampfiids and have shown its basal position to the Vahlkampfiidae (Weekers, Kleyn, and Vogels 1997). Two anaerobic amoebae, *Monopylocystis visvesvarai* and *Sawyeria marylandensis*, were shown to be related to *P. lanterna* on the basis of SSU rRNA gene analyses (O'Kelly et al. 2003; Silberman et al. 2002).

Complementary to molecular evolution studies, there are works on the organization of the flagellar apparatus of heteroloboseans (Broers et al. 1990; Brugerolle and Simpson 2004; Fenchel and Patterson 1986; Simpson 2003). In some *Percolomonas* species, the striated ciliary roots, which for a long time were thought to be absent in this genus, were described. As striated ciliary roots are known for all other flagellated Heterolobosea, the authors disputed the validity of such groups as the Percolomonadea, Lyromonadea, and Percolozoa (Brugerolle and Simpson 2004).

There is a relatively large SSU rRNA database for representatives of the Heterolobosea (Brown and De Jonckheere 1999; De Jonckheere and Brown 1999; Sogin et al. 1996; Weekers, Kleyn, and Vogels 1997), while the Discicristates (i.e. Euglenozoa and Heterolobosea) are poorly represented in the actin gene database. In the current study, in order to ascertain the taxonomic position of *Percolomonas*, we obtained the actin and SSU rRNA gene sequences of *P. cosmopolitus* and performed phylogenetic analyses including all heterolobosean sequences available to date. To overcome the problems caused by the high level of substitution rate heterogeneity across taxa in the SSU rRNA (Petrov and Aleshin 2002; Philippe and Germot 2000; Rokas and Holland 2000), conventional methods of phylogenetic inference were coupled with a comparative analysis of molecular synapomorphies at the level of the SSU rRNA secondary structure.

MATERIALS AND METHODS

Cell cultures and DNA extraction, amplification, cloning, and sequencing. The cells of *Percolomonas cosmopolitus* (Ruinen 1938) were obtained from White Sea coastal waters. They have been cultivated on the mineral Schmaltz-Pratt medium (artificial sea water) at 20 °C, 20‰ salinity, with the bacterium *Aerobacter aerogenes* as a food source. DNA was isolated by phenol extraction and precipitated with ethanol. A partial fragment of the actin gene was amplified using the forward primers Act-F1 (5′-CNG ARG CDC CAT TRA AYC-3′) and

[1] Corresponding Author: N. PETROV—Telephone number: 7-095-9391440; FAX number 7-095-9393181; E-mail: Petr@belozersky.msu.ru

Act-N2 (5′-AAC TGG GA(CT) GA(CT) ATG GA-3′) and the reverse primer Act-1354r (5′-GGA CCA GAT TCA TCA TAY TC-3′). The entire SSU rRNA gene was amplified using universal eukaryotic primers for nuclear SSU rRNA coding regions (Medlin et al. 1988). PCR products were purified by agarose gel electrophoresis, cloned in the pGEM-T Vector System (Promega), and manually sequenced on both strands using the *fmol* DNA sequencing system (Promega). The length of the amplified sequences of actin and SSU rRNA genes of *P. cosmopolitus* were 784 and 1,810 nucleotides, respectively. The sequences have been submitted to the GenBank database under accession numbers AY283751 for the actin gene and AF519443 for the SSU rRNA gene.

Phylogenetic and secondary structure analyses. The partial actin sequence of *P. cosmopolitus* was manually fitted to an alignment of 15 eukaryotic actin sequences. In the analyses, 254 amino acid positions were included. A Bayesian analysis (BA) of the data was conducted using MrBayes, version 2.01 (Huelsenbeck and Ronquist 2001) with four simultaneous runs of the Markov chain Monte Carlo algorithm. The chains were run for 600,000 generations with sampling every 10 generations. The states of the chains before they reached stationarity were discarded as the burn-in (burn-in = 20,000). The JTT model of amino acid substitutions (Jones, Taylor, and Thornton 1992) was used, with a proportion of invariable sites and a gamma-shaped distribution of the rates of substitution across sites, with 8 rate categories.

The complete SSU rRNA gene sequence from *P. cosmopolitus* was manually fitted to an alignment of a set of eukaryotic SSU rRNA gene sequences based on a universal model of eukaryotic SSU rRNA secondary structure (Van de Peer et al. 2000). Preliminary analyses revealed the approximate phylogenetic position of *P. cosmopolitus* within Discicristata, near the Heterolobosea (data not shown). An alignment of 44 sequences was constructed, including the sequence of *P. cosmopolitus*, 24 sequences from the Heterolobosea, and 19 sequences from other eukaryotes. In the phylogenetic analyses, 1301 unambiguously aligned positions were used, 278 of which were constant, and 939 of which were phylogenetically informative. Phylogenetic trees were inferred by the maximum likelihood (ML) method (Felsenstein 1981) and neighbor-joining (NJ) method (Saitou and Nei 1987) using PAUP* (Swofford 1998) and the maximum parsimony (MP) method using DNApars (Felsenstein 1993). The reliability of internal branches was assessed using the bootstrap method (Felsenstein 1985) with 1,000 replicates for NJ analyses, 100 replicates for ML analyses, and 500 replicates for MP analyses. All parameters were estimated from the dataset using Modeltest (Posada and Crandall 1998). NJ analyses with ML distances and ML analyses were performed using the GTR model of evolution (Lanave et al. 1984, Rodriguez et al. 1990), taking into account the proportion of invariables sites, and a gamma distribution of the rates of substitution for the variable positions, with 4 rate categories (GTR+G+I).

The most parsimonious trees for each MP bootstrap replicate were determined using a heuristic search procedure with 10 random-addition-sequence replicates and global rearrangements of branches. All characters were equally weighted. BA of the data was conducted as described above. The likelihood parameters for BA corresponded to the GTR model of DNA substitution (Lanave et al. 1984; Rodriguez et al. 1990), with a proportion of invariable sites and a gamma-shaped distribution of the rates of substitution, with 8 rate categories. Different topologies were tested with Kishino–Hasegawa (K-H) (Kishino and Hasegava 1989), Shimodaira–Hasegawa (S-H) (Shimodara and Hasegava 1999) tests using PAUP* (Swofford 1998) and approximately unbiased (AU) test (Shimodaira 2002) using CONSEL (Shimodaira and Hasegawa 2001). Elements of the SSU rRNA molecule secondary structure were modeled with *mfold* (Zuker, Mathews, and Turner 1999) and visualized with RnaViz (De Rijk and De Wachter 1997). Actin and SSU rRNA alignments are available upon request from the authors. Species names and corresponding GenBank accession numbers of all taxa included in our analyses are given in Fig. 1 and 2.

RESULTS

Morphology. The cultured strain of *P. cosmopolitus* used in this study fully corresponds morphologically to the one examined in a previous ultrastructural work (Fenchel and Patterson 1986), which provided the detailed description of the species.

Phylogenetic analyses. Our BA of actin genes (Fig. 1) reveals a close relationship between *P. cosmopolitus* and two species of the vahlkampfiid, heterolobosean genus *Naegleria*, with a posterior probability (PP) of 0.94. Moreover, the *Percolomonas* + *Naegleria* clade forms a sister-group to the Euglenozoa (PP of 1.00), supporting the monophyly of discicristates. However, because no other actin genes of Heterolobosea are available to date, this analysis does not allow us to ascertain the position of *P. cosmopolitus* more precisely.

Phylogenetic analyses of SSU rRNA sequences confirm the close relationship between *P. cosmopolitus* and heteroloboseans (Fig. 2). In the SSU rRNA gene tree, the monophyly of Heterolobosea including *Percolomonas* is supported by a PP of 1.00 in BA and by 99%, 98%, and 99% bootstrap values (BV) in ML, NJ, and MP analyses, respectively. Again, Euglenozoa form the sister-group to Heterolobosea, with a PP of 0.99 but with lower BV (67% for ML analyses, 86% for NJ analyses, 53% for MP analyses). The discicristates branch with the excavate *Jakoba incarcerata*, but this relationship is supported only in BA (PP of 1.00). Evidence for relationship with all jakobids is still weaker.

Among the Heterlobosea, *Macropharyngomonas halophila* forms the first diverging taxon (PP of 1.00), while all other heterolobosean species are divided into two main, but weakly supported clades. The first of them includes representatives of the lyromonads (i.e. *Psalteriomonas*, *Sawyeria*, *Monopylocystis*, and *Pseudomastigamoeba*) and Gruberellidae (i.e. *Stachyamoeba*) with two basal lineages consisting of *Paravahlkampfia ustiana* + the environmental clone RT5in38, and *Heteramoeba clara* + *Plaesiobystra hypersalinica*. The second clade includes *Neovahlkampfia damariscottae*, *Acrasis rosea*, and two clusters of vahlkampfiid amoeboflagellates with *P. cosmopolitus* branching between them. The first of these clusters, called here the *Naegleria* clade, includes the genera *Naegleria* and *Willaertia*, and the second one, called here the *Vahlkampfia* clade, includes the genera *Vahlkampfia*, *Tetramitus*, *Didascalus*, *Paratetramitus*, *Singhamoeba*, and *Learamoeba*. *Percolomonas* occupies a sister position to the *Vahlkampfia* clade. Although the SSU rRNA gene sequence of *P. cosmopolitus* obtained in this study is quite different from a sequence of the same species available in GenBank under Accession number AF011464, they comprise a highly reliable cluster independently of the method of analysis. Due perhaps, to the high SSU rRNA gene divergence of these two *P. cosmopolitus* sequences, MP analyses place them at the base of Heterolobosea, as the second branching taxon after *M. halophila*. To test the reliability of the position of *Percolomonas* within the vahlkampfiid clade we performed 5 BA using different starting topologies, where *Percolomonas* was placed in different nodes within the Heterolobosea. The position of *Percolomonas* appeared stable whatever starting tree was used. Then, we tested further the position of *Percolomonas* by performing K-H, S-H, and S-H AU tests on

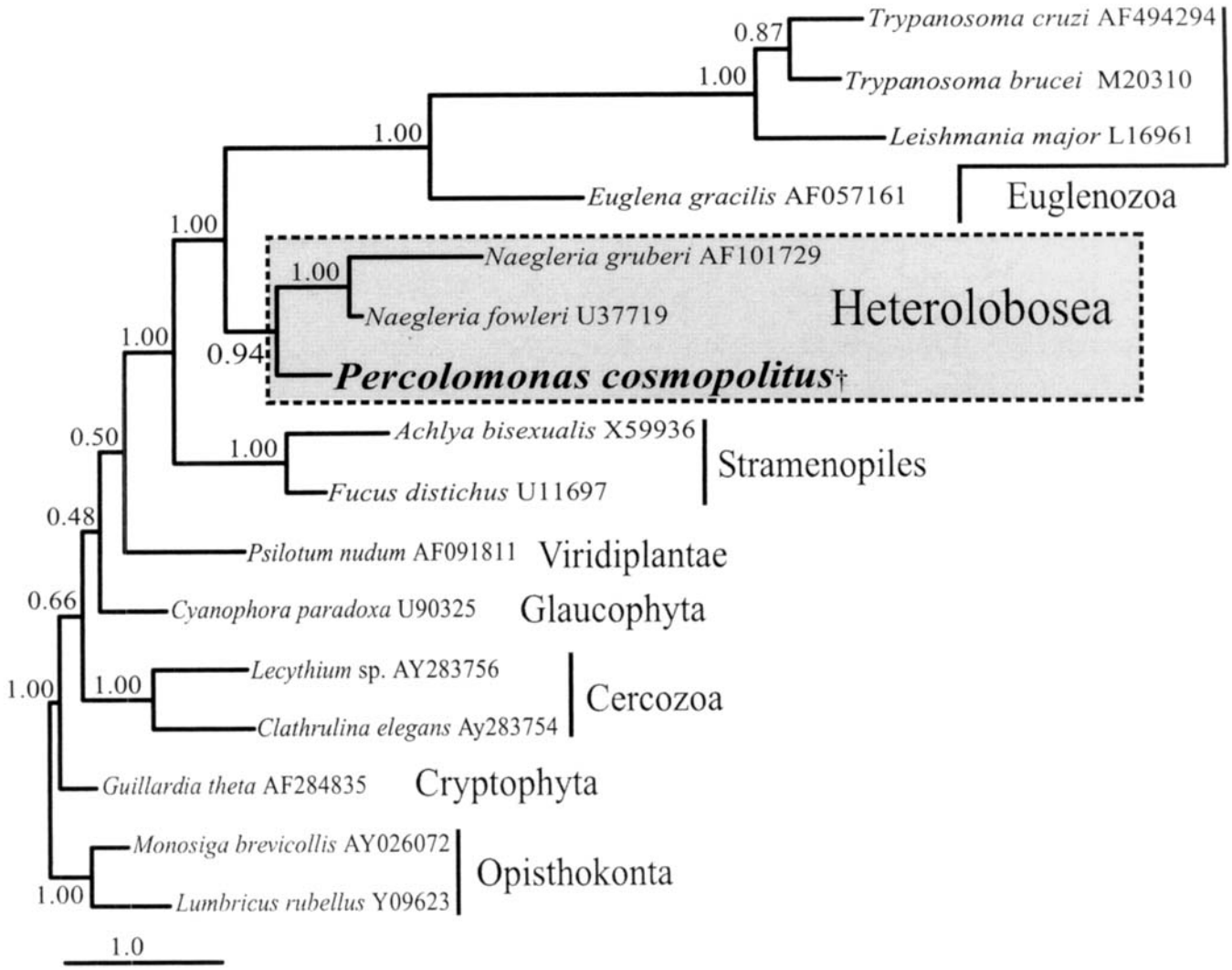

Fig. 1. Phylogenetic position of *Percolomonas cosmopolitus* based on actin gene sequences. The tree was constructed by Bayesian analysis (BA) using the JTT + G + I model with 4 categories of substitution rates (alpha = 0.80; proportion of invariable sites = 0.34). Posterior probabilities for BA are indicated at the appropriate nodes. The tree was rooted on opisthokonts according to the hypothesis of Stechmann and Cavalier-Smith (2002).

these five trees and the non-forced topology shown in Fig. 2. All tests revealed that the tree shown in Fig. 2 has a significantly better likelihood than a tree constraining the basal position of *Percolomonas* as a sister-group to Heterolobosea, although a position of *Percolomonas* as a sister-group to the Vahlkampfiidae or to the Vahlkampfiidae + Acrasida could not be rejected.

Secondary structure analyses. Comparative analysis of putative molecular synapomorphies uncovered an insertion in the V3 region of the *P. cosmopolitus* SSU rRNA molecule (Fig. 3). Recently, this insertion was shown to be a unique feature of Heterolobosea (Wuyts, Van de Peer, and De Wachter 2001). Modeling the secondary structure of the corresponding part of the SSU rRNA molecule revealed that this insertion corresponds to an additional helix situated between helices 17 and 18 of the predicted model, which are regions of unambiguous positional homology among all taxa included in the dataset. The position of helix 17_1 corresponds to the unpaired purine base found in most other eukaryotic taxa available to date. Interestingly, this insertion and additional helix 17_1 is absent in *M. halophila*, the first diverging member of Heterolobosea in the SSU rRNA phylogeny (Fig. 2).

DISCUSSION

For a long time, the position of *Percolomonas* was a key question in the phylogeny of Discicristates. *Percolomonas* was initially described as a member of the family Vahlkampfiidae, then considered as incertae sedis within the class Heterolobosea. On the basis of ultrastructural characters, Cavalier-Smith (1995) proposed a separate class Percolomonadea for the only genus *Percolomonas*, which, together with the classes Heterolobosea and Lyromonadea, would form the phylum Percolozoa. Our analyses clearly show that *P. cosmopolitus* belongs to the Heterolobosea, as *P. cosmopolitus* and heterolobosean sequences form a clade confirmed by high PP and/or BV in both actin and SSU rRNA gene trees (Fig. 1, 2). The actin phylogeny (Fig. 1) shows a close relationship between *P. cosmopolitus* and *Naegleria*. The poor sampling of actin sequences in heterolobosean impedes more precise conclusions, but our results suggest that this gene marker might be informative for the phylogeny of

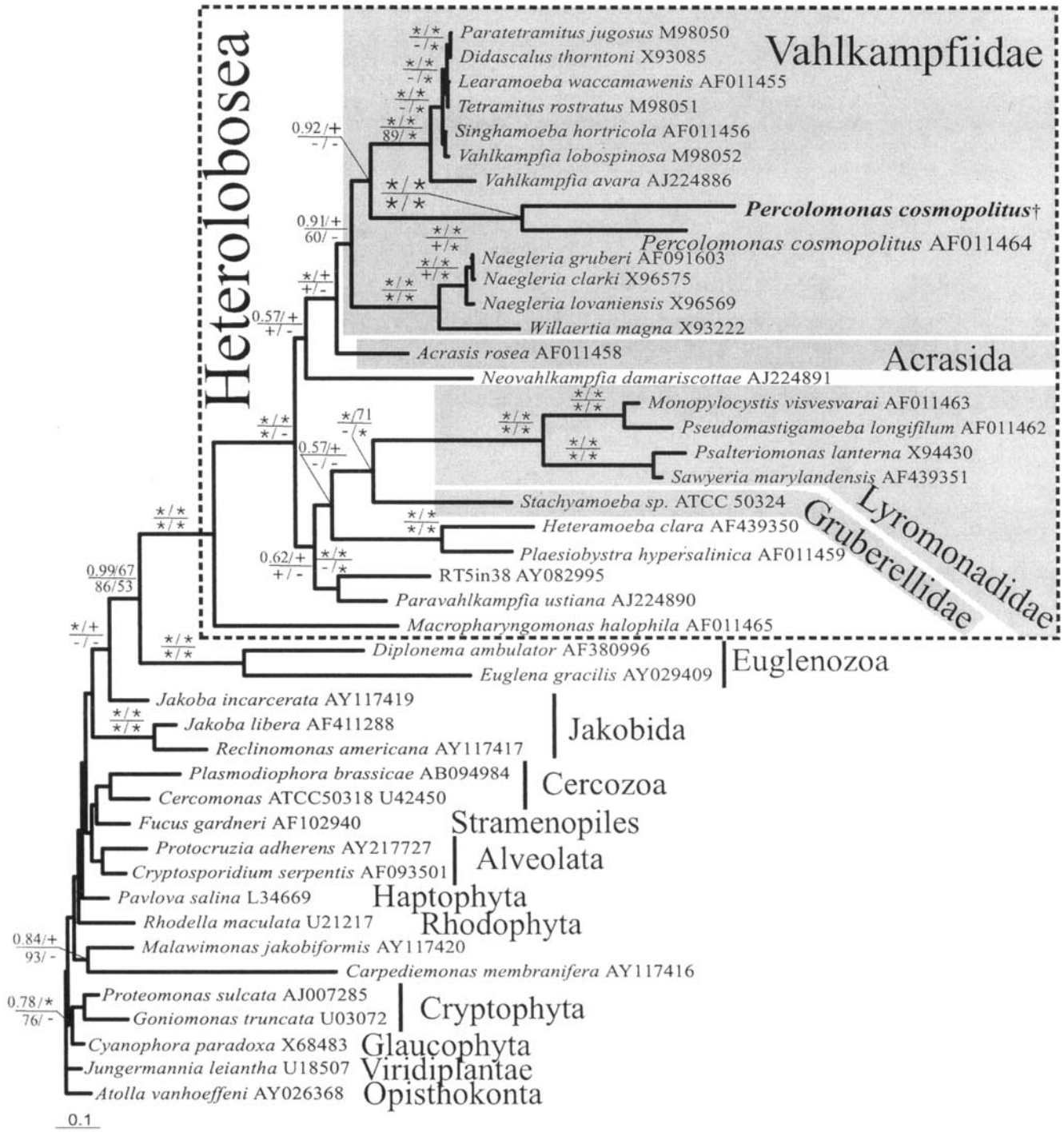

Fig. 2. Phylogenetic position of *Percolomonas cosmopolitus* based on small subunit rRNA gene sequences. The tree was constructed by Bayesian analysis (BA) using the GTR + G + I model with 8 categories of substitution rates (alpha = 0.34; proportion of invariable sites = 0.21). The numbers at nodes indicate posterior probabilities (PP) for BA (up, left) and bootstrap values (BV) for ML (up, right), NJ (down, left), and MP (down, right) analyses. Stars represent PP of 0.95–1.00 or BV of 95–100%. Pluses indicate BV < 50%; dashes indicate the absence of the node in NJ or MP analyses. Number of bootstrap replicates, evolutionary models employed, and BA parameters are described in the text. Branches are drawn to scale. The tree was rooted as in Fig. 1.

Heterolobosea because of the short branches leading to its representatives. However, the congruence of SSU rRNA and actin genes data increases the reliability of the position of *Percolomonas*. In BA of SSU rRNA gene sequences, *Percolomonas* is placed in the crown of the Heterolobosea clade, within the family Valkampfiidae. Thus, molecular data presented in this study do not support such a high degree of taxonomic distinction between *Percolomonas* and other heteroloboseans. A striated flagellar root that is homologous to the heterolobosеid rhizoplast was recently described for *Percolomonas* (Brugerolle and

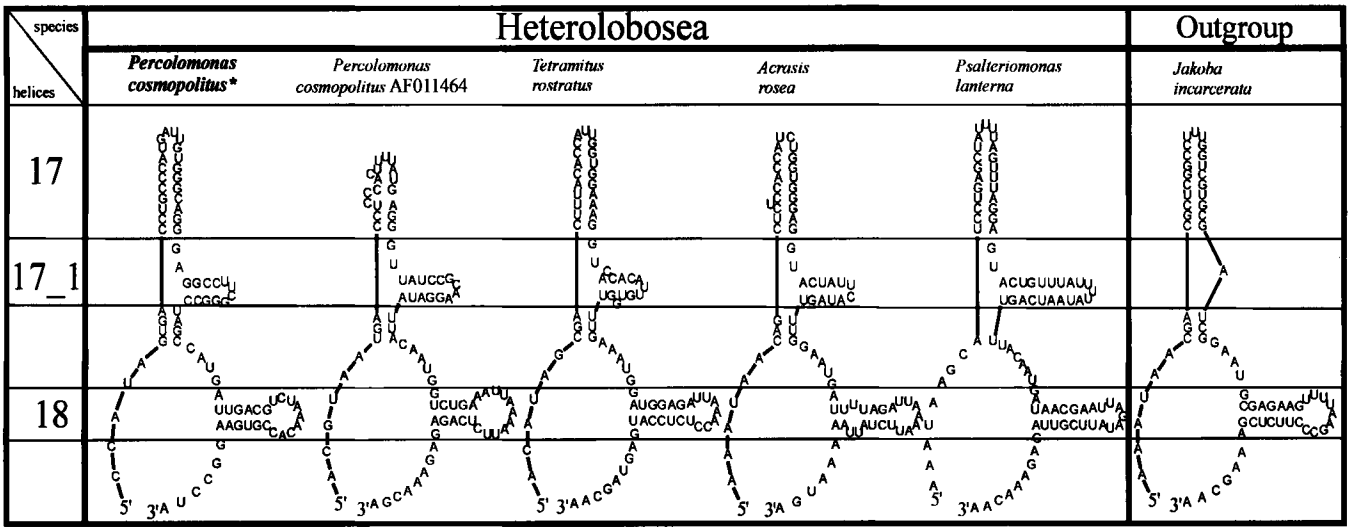

Fig. 3. Secondary structure of helices 17, 17_1, and 18 of small subunit (SSU) rRNA given in comparative representation for *Percolomonas cosmopolitus* and various heterolobosean taxa. *Jakoba incarcerata* represents an outgroup condition. Horizontal lines serve as boundaries between homologous regions of the SSU rRNA secondary structure.

Simpson 2004), and this is consistent with the phylogenetic position determined in this study. Our SSU rRNA gene data are congruent with the views of Fenchel and Patterson (1986), who proposed a close relationship of *Percolomonas* and *Tetramitus*. Thus, the term Percolozoa should probably become the junior synonym of the term Heterolobosea, although for the moment the exact position of *Stephanopogon*, the other enigmatic discicristate protist is not established.

In our SSU rRNA phylogenetic analyses (Fig. 2), the sequence of *P. cosmopolitus* obtained in our study groups with high support with a SSU rRNA gene sequence of the same species (Sogin et al. 1996) available in GenBank. However, its SSU rRNA gene sequence differs sharply from our isolate, indicating that they were possibly obtained from different *Percolomonas* strains. Furthermore, both sequences are highly divergent relative to other heterolobosean sequences, and in the tree they have extremely long branches. NJ and MP analyses placed *Percolomonas* in a more basal position within the Heterolobosea (see also Sogin et al. 1996), but as the two SSU rRNA gene sequences of *P. cosmopolitus* are clearly fast evolving, we suspect this basal position is artifactual, and the topology obtained with BA and ML more likely reflects the true relationships among heterolobosean. Furthermore, the K-H, S-H, and S-H AU tests strongly contradict the basal position of *Percolomonas* among Heterolobosea.

The additional helix 17_1 detected in *P. cosmpolitus* and all other available representatives of Heterolobosea, except the basal *Macropharyngomonas halophila*, strongly supports the monophyly of this subclade (Fig. 3). We consider this short expansion segment as a derived, rather than ancestral character state, because all species outside Heterolobosea, including prokaryotes do not possess this character. The absence of this unique heterolobosean additional helix 17_1 in *M. halophila* also confirms the basal position of this recently described heterolobosean on the SSU rRNA gene tree (Fig. 2) and suggests that helix 17_1 appeared after the divergence of *M. halophila* from all other heterolobosean.

Our BA of a large taxon sampling of heterolobosean SSU rRNA gene sequences shows clear divergence of *Neovahlkampfia*, *Paravahlkampfia*, and the anaerobic lyromonads (*Psalteriomonas*, *Sawyeria*, *Monopylocystis*, and *Pseudomastigamoeba*) from the rest of the family Vahlkampfiidae. Thus, the family Vahlkampfiidae appears to be paraphyletic. The lyromonads form a very distinct clade in the SSU rRNA phylogeny, and should probably be considered as a separate family. The SSU rRNA gene sequence of *Pseudomastigamoeba longifilum* (Accession number AF011462) was removed from GenBank at the submitter's request, because the source organism could not be confirmed, so this sequence should be labelled as 'undetermined lyromonad'. The gruberellid *Stachyamoeba* sp. seems to be closely related to the lyromonads, and the order Schizopyrenida does not constitute a natural assemblage, since members of its two families (i.e. Vahlkampfiidae and Gruberellidae) branch separately on the SSU rRNA gene tree. However, this tree topology is congruent with such features as the disintegration of the nucleolus during mitosis: promitosis is known for Acrasidae and Vahlkampfiidae, while in Gruberellidae the nucleolus disintegrates during mitosis (Page 1987). Finally, the unpublished SSU rRNA gene sequence of the slime mold *A. rosea* belonging to the order Acrasida reveals its relationship to the vahlkampfiids. This probably reflects a true branching order, because morphological data indicate that trophozoites of Acrasida are monopodial amoebas with eruptive pseudopodia, and are indistinguishable from those of Vahlkampfiidae (Page and Blanton 1985).

Data on the phylogeny of the Heterolobosea warrant re-evaluation of the taxonomical value of many morphological features characterizing the group. The peculiarities of the life cycle in the Heterolobosea seem to have low taxonomic value. Our data suggest that a complex life cycle that includes amoeboid and flagellate stages might be ancestral for the Vahlkampfiidae. Subsequent reduction of the flagella as in *Vahlkampfia* spp. or the reduction of the amoeboid stage in *Percolomonas* and some *Tetramitus* species might account for the chaotic distribution of these features among vahlkampfiids.

Our analyses show that the anaerobic Lyromonadidae, possessing hydrogenosomes instead of mitochondria, are close to the mitochondria-bearing Gruberellidae, *Heteramoeba*, and *Paravahlkampfia*. This is consistent with numerous losses of mitochondria documented already in anaerobic fungi, archamoebae, and other protists (Arisue et al. 2002; Edgcomb et al. 2002; Seravin 1992; Voncken et al. 2002).

ACKNOWLEDGMENTS

We acknowledge L. Yu. Roussine, E. M. Merzliak, and E. E. Kulikov for their support throughout this work. This research was supported by RFBR grant 02-04-48958, RFBR grant 02-04-48265, and grant SSc 1712.2003.4 of the State Committee of Education of Russia, as well as the Swiss National Science Foundation grants 3100-064073.00 and 7SUPJ062343.

LITERATURE CITED

Arisue, N., Hashimot, T., Lee, J. A., Moore, D. V., Gordon, P., Sensen, C. W., Gaasterland, T., Hasegawa, M. & Müller, M. 2002. The phylogenetic position of the pelobiont *Mastigamoeba balamuthi* based on sequences of rDNA and translation elongation factors EF-1alpha and EF-2. *J. Eukaryot. Microbiol.*, **49**:1–10.

Broers, C. A. M., Stumm, C. K., Vogels, G. D. & Brugerolle, G. 1990. *Psalteriomonas lanterna* gen. nov., spec. nov., a free-living amoeboflagellate isolated from freshwater anaerobic sediments. *Eur. J. Protozool.*, **25**:369–380.

Brown, S. & De Jonckheere, J. F. 1999. A reevaluation of the amoeba genus *Vahlkampfia* based on SSU rDNA sequences. *Eur. J. Protistol.*, **35**:49–54.

Brugerolle, G. & Simpson, A. G. B. 2004. The flagellar apparatus of Heterolobosea. *J. Eukaryot. Microbiol.*, **51**:96–107.

Cavalier-Smith, T. 1993. Percolozoa and the symbiotic origin of the metakaryote cell. *In:* Ishikawa, H., Ishida, M. & Sato, S. (ed.), Endocytobiology. Tübingen University Press, Tübingen. **5**:399–406.

Cavalier-Smith, T. 1995. Zooflagellate phylogeny and classification. *Tsitologyia*, **37**:1010–1029.

Cavalier-Smith, T. 2002. The phagotrophic origin of eukaryotes and phylogenetic classification of Protozoa. *Int. J. Syst. Evol. Microbiol.*, **52**:297–354.

Cavalier-Smith, T. 2004. The excavate protozoan phyla Metamonada Grassé emend. (Anaeromonadea, Parabasalia, *Carpediemonas*, Eopharyngia) and Loukozoa emend. (Jakobea, *Malawimonas*): their evolutionary affinities and new higher taxa. *Int. J. Syst. Evol. Microbiol.*, **53**:1741–1758.

De Jonckheere, J. F. & Brown, S. 1999. SSU rDNA analysis reveals the existence of a second *Naegleria* sp. with dividing flagellates: *N. robinsoni* sp. nov. *Eur. J. Protistol.*, **35**:264–266.

De Rijk, P. & De Wachter, R. 1997. RnaViz, a program for the visualisation of RNA secondary structure. *Nucleic Acids Res.*, **25**:4679–4684.

Edgcomb, V. P., Simpson, A. G. B., Amaral Zettler, L., Nerad, T. A., Patterson, D. J., Holder, M. E. & Sogin, M. L. 2002. Pelobionts are degenerate protists: insights from molecules and morphology. *Mol. Biol. Evol.*, **19**:978–982.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *J. Mol. Evol.*, **17**:368–376.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**:783–791.

Felsenstein, J. 1993. PHYLIP: phylogeny inference package, version 3.5. University of Washington, Seattle.

Fenchel, T. & Patterson, D. J. 1986. *Percolomonas cosmopolitus* (Ruinen) n. gen., a new type of filter-feeding flagellate from marine plankton. *J. Mar. Biol. Ass. U.K.*, **66**:465–482.

Jones, D. T., Taylor, W. R. & Thornton, J. M. 1992. The rapid generation of mutation data matrices from protein sequences. *Comput. Appl. Biosci.*, **8**:275–282.

Huelsenbeck, J. P. & Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, **17**:754–755.

Kishino, H. & Hasegawa, M. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order of the Hominoidea. *J. Mol. Evol.*, **29**:170–179.

Lanave, C., Preparata, G., Saccone, C. & Serio, G. 1984. A new method for calculating evolutionary substitution rates. *J. Mol. Evol.*, **20**: 86–93.

Medlin, L., Elwood, H. J., Stickel, S. & Sogin, M. L. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene*, **71**:491–499.

O'Kelly, C. J., Silberman, J. D., Amaral Zettler, L. A., Nerad, T. A., & Sogin, M. L. 2003. *Monopylocystis visvesvarai* n. gen., n. sp. and *Sawyeria marylandensis* n. gen., n. sp.: two new amitochondrial heterolobosean amoebae from anoxic environments. *Protist*, **154**:281–290.

Page, C. 1987. Transfer of *Stachyamoeba lipophora* to the class Heterolobosea. *Arch. Protistenkd.*, **133**:191–197.

Page, F. C. & Blanton, R. L. 1985. The Heterolobosea (Sarcodina: Rhizopoda), a new class uniting the Schizopyrenida and the Acrasidae (Acrasida). *Protistologica*, **21**:121–132.

Petrov, N. B. & Aleshin, V. V. 2002. Conditionally neutral phylogenetic markers of major taxa: a new aspect of the evolution of macromolecules. *Russ. J. Genet.*, **38**:877–894.

Philippe, H. & Germot, A. 2000. Phylogeny of eukaryotes based on ribosomal RNA: long-branch attraction and models of sequence evolution. *Mol. Biol. Evol.*, **17**:830–834.

Posada, D. & Crandall, K. A. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**:817–818.

Rodriguez, F., Oliver, J. L., Marin, A. & Medina, J. R. 1990. The general stochastic model of nucleotide substitution. *J. Theor. Biol.*, **142**:485–501.

Rokas, A. & Holland, P. W. 2000. Rare genomic changes as a tool for phylogenetics *Trends Ecol. Evol.*, **15**:454–459.

Ruinen, J. 1938. Notizen uber Salzflagellaten. II. Uber die Verbreitung der Salzflagellaten. *Arch. Protistenkd.*, **90**:210–258.

Saitou, N. & Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**:406–425.

Seravin, L. N. 1992. Eukaryotes devoid of the most important cellular organelles (flagella, Golgi apparatus, mitochondria) and the main task of organellology. *Tsitologyia*, **34**:3–33.

Shimodaira, H. 2002. An approximately unbiased test of phylogenetic tree selection. *Syst. Biol.*, **51**:492–508.

Shimodaira, H. & Hasegawa, M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol. Biol. Evol.*, **16**:1114–116.

Shimodaira, H. & Hasegawa, M. 2001. CONSEL: for assessing the confidence of phylogenetic tree selection. *Bioinformatics*, **17**: 1246–47.

Silberman, J. D., Simpson, A. G. B., Kulda, J., Cepicka, I., Hampl, V., Johnson, P. J. & Roger, A. J. 2002. Retortamonad flagellates are closely related to diplomonads—implications for the history of mitochondrial function in eukaryote evolution. *Mol. Biol. Evol.*, **19**: 777–86.

Simpson, A. G. B. 1997. The identity and composition of the Euglenozoa. *Arch. Protistenkd.*, **148**:318–328.

Simpson, A. G. B. 2003. Cytoskeletal organization, phylogenetic affinities and systematics in the contentious taxon Excavata (Eukaryota). *Int. J. Syst. Evol. Microbiol.*, **53**:1759–1777.

Simpson, A. G. B. & Patterson, D. J. 1999. The ultrastructure of *Carpediemonas membranifera* (Eukaryota) with reference to the excavate hypothesis. *Europ. J. Protistol.*, **35**:357–370.

Sogin, M. L., Silberman, J. D., Hinkle, G. & Morrison, H. G. 1996. Problems with molecular diversity in the Eukarya. *In:* Roberts, D. M., Sharp, P., Alderson, G. & Collins, M. A. (ed.), Society of General Microbiology Symposium: Evolution of Microbial Life. Cambridge University Press, Cambridge. p. 167–184.

Stechmann, A. & Cavalier-Smith, T. 2002. Rooting the eukaryote tree by using a derived gene fusion. *Science*, **297**:89–91.

Swofford, D. L. 1998. PAUP*: phylogenetic analysis using parsimony (* and other methods). Sinauer Associates, Sunderland, Massachusetts.

Van de Peer, Y., De Rijk, P., Wuyts, J., Winkelmans, T. & De Wachter, R. 2000. The European small subunit ribosomal RNA database. *Nucleic Acids Res.*, **28**:175–176.

Voncken, F., Boxma, B., Tjaden, J., Akhmanova, A., Huynen, M., Verbeek, F., Tielens, A. G. M., Haferkamp, I., Neuhaus, H. E., Vogels, G., Veenhuis, M. & Hackstein, J. H. P. 2002. Multiple origins of hydrogenosomes: functional and phylogenetic evidence from the ADP/ATP carrier of the anaerobic chytrid *Neocallimastix* sp. *Mol. Microbiol.*, **44**:1441–1454.

Weekers, P. H. H., Kleyn, J. & Vogels, G. D. 1997. Phylogenetic position of *Psalteriomonas lanterna* deduced from the SSU rDNA sequence. *J. Eukaryot. Microbiol.*, **44**:467–470.

Wuyts, J., Van de Peer, Y. & De Wachter, R. 2001. Distribution of substitution rates and location of insertion sites in the tertiary structure of ribosomal RNA. *Nucleic Acids Res.*, **29**:5017–5028.

Zuker, M., Mathews, D. H. & Turner, D. H. 1999. Algorithms and thermodynamics for RNA secondary structure prediction: A practical guide. *In:* Barciszewski, J. & Clark, B. F. C. (ed.), RNA Biochemistry and Biotechnology. NATO ASI Series, Kluwer Academic Publishers. p. 11–43.

Received 3/31/04; accepted 7/16/04