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Critical analysis of the topology and rooting of the parabasalian 16S rRNA tree☆

Vladimír Hampl,* Ivan Cepicka, Jaroslav Flegr, Jan Tachezy, and Jaroslav Kulda

Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic

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Abstract

The morphological classification of the protozoan phylum Parabasala is not in absolute agreement with the 16S rRNA phylogeny. However, there are strong indications that tree-construction artifacts play a considerable role in the shaping of the 16S rRNA tree. We have performed rigorous analyses designed to minimize such artifacts using the slow–fast and taxa-exclusion methods. The analyses, which included new sequences from the genera *Monocercomonas* and *Hexamastix*, in most respects confirmed the previously suggested tree topology and polyphyly of Hypermastigida and Monocercomonadidae but detected one artificial cluster of long branches (Trichonymphidae, Pseudotrichonymphidae, *Hexamastix*, and *Tricercomitus*). They also indicated that the rooting of the phylum on the trichonymphid branch is probably wrong and that reliable rooting on the basis of current data is likely impossible. We discuss the tree topology in the view of anagenesis of cytoskeletal and motility organelles and suggest that a robust taxonomic revision requires extensive analysis of other gene sequences.

Keywords: Parabasala; Phylogeny; 16S rRNA; Long-branch attraction; Slow–fast method; Taxa-exclusion method; Classification; Anagenesis; Hypermastigida; Trichomonadida; Monocercomonadidae; *Monocercomonas*; *Hexamastix*

1. Introduction

The phylum Parabasala is comprised of anaerobic amitochondriate flagellates. Characteristic features of the phylum are: parabasal apparatus (Golgi complex associated with parabasal fibers), presence of a double membrane bounded organelle named the hydrogenosome, and cell division by semiopen pleuromitosis with extranuclear spindle. The vast majority of parabasalid species live endobiotically either as harmless intestinal commensals of various animal hosts, or as intestinal symbionts (mutualists) in termites and wood-eating cockroaches. The pathogenic parasites represent a tiny part of the parabasalian species diversity, however, some of them such as—*Trichomonas vaginalis*, *Tritrichomonas foetus*, and *Histomonas meleagridis*—are of considerable medical or veterinary importance. There are only four known free-living species in this phylum—*Pseudotrichomonas keilini*, *Ditrichomonas honigbergii*, *Monotrichomonas carabina*, and *Monotrichomonas* sp. These species live in anoxic habitats in salt or fresh water.

Traditionally, the phylum is divided into two orders, Trichomonadida and Hypermastigida (Corliss, 1994). The order Hypermastigida typically comprises large forms (hundreds of micrometers long) equipped with many flagella. Although this order covers a significant part of the morphological and species diversity of Parabasala, all its members live exclusively as intestinal symbionts of insects. The typical representatives of the second order Trichomonadida have smaller cells (not longer than 20 μm) with up to six flagella, excepting the polymonad family Calonymphidae. The order Trichomonadida encompasses the whole ecological diversity of Parabasala, including free-living, endosymbiotic, commensal, and pathogenic species.

Typical members of the order Trichomonadida are classified into the family Trichomonadidae. Their char-

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* Corresponding author.
E-mail address: vlada@natur.cuni.cz (V. Hampl).

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acteristic feature is the presence of an undulating membrane and costa. The undulating membrane, an important motility organelle of trichomonads, is formed by recurrent flagellum adhering to a fold of cytoplasmic membrane. The costa is a striated root fiber attached to the basal bodies complex that underlies the undulating membrane, presumably providing its mechanical support (Kulda et al., 1988). There are two types of costae (A, B) that differ in pattern of their striation, resulting from different organization of the fiber substructures. The A type banding pattern is also found in the ubiquitous parabasalian striated roots—parabasal fibers that maintain Golgi dictyosomes in perinuclear position (Brugerolle and Viscogliosi, 1994). Despite the differences between costa A and B, Viscogliosi and Brugerolle (1994) demonstrated that the main protein compounds of both types are related. Their results also suggest the presence of some common epitopes between the costae and parabasal fibers. In contrast, absence of immunological cross-reactivity and different molecular mass range indicates lack of relatedness between the proteins of costae and constituents of striated roots such as assemblin, centrin or the kinetodesmal protein, known to occur in other protists. Many species of the order Trichomonadida lack either the costa, or both costa and undulating membrane. These two structures are absent or modified in the families Devescovinidae and Calonymphidae which differ substantially from the basic morphological pattern of the Trichomonadidae. However, the costa is also absent and the undulating membrane reduced, or absent, in some species that otherwise conform to the basic Trichomonadidae morphology. The lack of costa and undulating membrane in these species was regarded to be a taxonomically important character substantiating their classification into the separate family Monocercomonadidae. The four-family classification of Trichomonadida (Trichomonadidae, Monocercomonadidae, Calonymphidae, and Devescovinidae) proposed by Honigberg (1963) with the addition of the fifth single-genus family Cochlosomatidae (Pecka et al., 1996) is still widely used.

A scheme of evolution in the phylum Parabasala based on light microscopic morphology was proposed by Honigberg (1963) and later amended by inclusion of ultrastructural data (see Brugerolle, 1976). In this proposal, the family Monocercomonadidae was regarded as ancestral to the whole phylum as it is morphologically simple. The type genus *Monocercomonas* was considered to represent the ancestral form, from which radiated other genera of Monocercomonadidae. The Trichomonadidae were also thought to have arisen from one such lineage by stepwise development of undulating membrane and costa. The current genera *Hypotrichomonas* and *Pseudotrichomonas*, possessing undulating membrane but lacking costa, were regarded as descendants of an intermediate form in this transformation. According to this scenario the Trichomonadidae lineage split to yield the trichomonad and tritrichomonad branches and from tritrichomonads arose the families Devescovinidae and Calonymphidae. These were regarded as the ancestors of morphologically very complex Hypermastigida.

The advent of molecular techniques brought new insight into the relationships between the lineages in the phylum Parabasala. Phylogenetic trees based on gene sequences of 16S rRNA and those based on morphology have contradicted one another in many respects (Dacks and Redfield, 1998; Delgado-Viscogliosi et al., 2000; Edgcomb et al., 1998; Gerbod et al., 2000, 2001; Keeling et al., 1998; Ohkuma et al., 1998, 2000; Viscogliosi et al., 1999). First, the expected origin of Hypermastigida within the Trichomonadida clade was challenged by a 16S rRNA phylogeny that suggested an opposing scenario, in which the Trichomonadida diverge after several Hypermastigida branches. Furthermore, members of the family Monocercomonadidae form neither monophyletic nor basal branches of the Trichomonadida subtree. This suggests that their simplicity is not a primitive state and that the costa and undulating membrane probably disappeared secondarily in these species. Moreover, this loss probably happened several times in unrelated branches. Molecular analyses also indicated the polyphyletic or paraphyletic nature of the families Calonymphidae and Devescovinidae (Gerbod et al., 2002) and order Hypermastigida (Brugerolle and Patterson, 2001; Delgado-Viscogliosi et al., 2000; Frohlich and Konig, 1999; Gerbod et al., 2001, 2002; Keeling et al., 1998; Ohkuma et al., 2000).

This molecular-based phylogeny not only contradicts the morphology-based concept of parabasalid evolution but it is also incongruent with the current classification of the phylum Parabasala, because it suggests the polyphyly of several taxa. Therefore, many authors have pointed out the need to revise the classification in order to make it consistent with molecular data (Delgado-Viscogliosi et al., 2000; Gerbod et al., 2001; Keeling, 2002; Keeling et al., 1998; Viscogliosi et al., 1999). Brugerolle and Patterson (2001) have made the first step towards revision in proposing the division of the phylum into three orders: Trichomonadida, Cristamonadida, and Trichonymphida instead of the current two Trichomonadida and Hypermastigida.

Molecular phylogenetic trees are prone to several types of artifacts that may result in a potentially misleading topology. The long-branch attraction (LBA) artifact results in the artificial clustering of taxa whose branch lengths noticeably exceed those of other organisms (Felsenstein, 1978). Such long-branch taxa may not be closely related and their increased length can be caused, for example, by higher mutational rates. The parabasalian 16S rRNA tree includes many such long branches indicating the possible influence of LBA on the tree topology.

In this paper, we present rigorous molecular phylogenetic analyses of the phylum Parabasala based on currently available 16S rRNA sequences and 10 new sequences from the genera *Monocercomonas* and *Hexamastix* (Hampl et al., Manuscript in preparation). The analysis is aimed at critical assessment of tree topologies produced from 16S rRNA alignments by investigating different subsets of characters and taxa sampling.

2. Materials and methods

The sequences of 16S rRNA were determined for the strain of *Monocercomonas* spp. (PYR-1-1, EUMM, HAD, VAR-1, R208, TSC, and R183 isolated from various reptile species), *Monocercomonas ruminantium* (strains HER5 and KOJ 14 isolated from cattle), and *Hexamastix* spp. (strains CYCL and T isolated from reptiles). Accession numbers of the sequences are AY319267–AY319280 and AY321149, the detailed information on the origin of the strains is given in Hampl et al. (Manuscript in preparation). The secondary structure-based alignment of 16S rRNA sequences of parabasalids and outgroups was downloaded from the ribosomal RNA database (http://rrna.uia.ac.be). The sequences of *Dientamoeba fragilis* and *H. meleagridis* and the new sequences were appended to this alignment and realigned using the function "realign selected sequences" in ClustalX 1.81 (Thompson et al., 1997). The alignment was then manually refined in the Bioedit sequence alignment editor (Hall, 1999). This full set of taxa was used only in the first complete analysis (Fig. 1). As isolates of *Monocercomonas* spp. PYR-1-1, EUMM, HAD, VAR-1, R208, TSC, and R183 were very similar to the database sequence of *Monocercomonas* sp. Ns-1PRR (ATCC 50210) and isolate KOJ14 identical to isolate HER5, only strains Ns-1PRR and HER5 were included to all further analyses to save computational effort.

Phylogenetic trees were constructed using the maximum likelihood (ML), maximum parsimony (MP), and Fitch–Margoliash with Logdet distance (LD) methods implemented in PAUP* 4.0 (Swofford, 1998) and by the Bayesian method implemented in the program MrBayes (Huelsenbeck and Ronquist, 2001).

Maximum likelihood trees in PAUP* were constructed using the best substitution models as determined by hierarchical nested likelihood ratio test implemented in Modeltest version 3.06 (Posada and Crandall, 1998). Except for some slow–fast analyses the trees were searched using heuristic method with 10 replicates each. The starting tree was constructed by random taxa addition. To save computer time, in some slow–fast analyses only one replicate of heuristic searches was used with "as-is" addition of sequences. For the maximum parsimony tree construction, the heuristic searches with 10 replicates and random taxa addition were used. One replicate of heuristic searches was used for the LD method and the starting tree was constructed by neighbor-joining. In order to lower the violation of the rate homogenity across sites assumption, constant positions were excluded from the alignment before performing the LD analyses (Waddell and Steel, 1997).

In MrBayes, base frequencies, rates for six different types of substitutions, number of invariant sites, and shape parameter of the gamma correction for rate heterogeneity with four discreet categories were allowed to vary. Usually 200,000 generations of the Markov Chain Monte Carlo were run by using the default setting (four simultaneous chains, heating temperature 0.2). The first 250–1000 trees were discarded as the "burnin."

The χ^2 test of deviation from the expected nucleotide composition for outgroup sequences was performed using Treepuzzle (Strimmer and vonHaeseler, 1996). Testing of phylogenetic hypotheses was performed by using program Consel v0.1f (Shimodaira and Hasegawa, 2001) that includes, among others, KH and SH tests, weighted KH and SH tests, and approximately unbiased test (Shimodaira, 2002).

Rooting was performed in PAUP* 4.0 (Swofford, 1998) by the outgroup and midpoint methods, and by using maximum likelihood with an enforced molecular clock.

The slow–fast (S–F) method (Brinkmann and Philippe, 1999) was used to estimate, and lower the effect of long-branch attraction (LBA) artifact by sequential removal of positions with higher mutational rate that are supposedly responsible for stochastic information noise. In the S–F method the positions in the alignment were divided into 11 (0–10) classes according to their increasing mutational rate. The mutational rate was estimated in the following way: the sum of the number of changes for each position within the well-supported, distinct clades comprising higher number of OTUs (in our case clades 1, 2, 7, and 12 in Fig. 1) was calculated by using maximum parsimony in PAUP*. The new alignments (s0, s1, s2, …, s9) were created from the complete alignment, in which only positions with 0, up to 1, up to 2, …, up to 9 changes, respectively, were included. All gaps were removed from the alignments and new phylogenetic trees were constructed from these alignments.

The exclusion of the positions with high mutational rate lowers LBA artifact, but at the same time increases the influence of stochastic effects at the tree topology as a result of the decreasing amount of data. At a certain point the stochastic effects overweight the information contained in the sequences and the topology collapses. To be able to consider the influence of both conflicting effects, the tree robustness and number of included positions for each level of S–F was graphically visualized

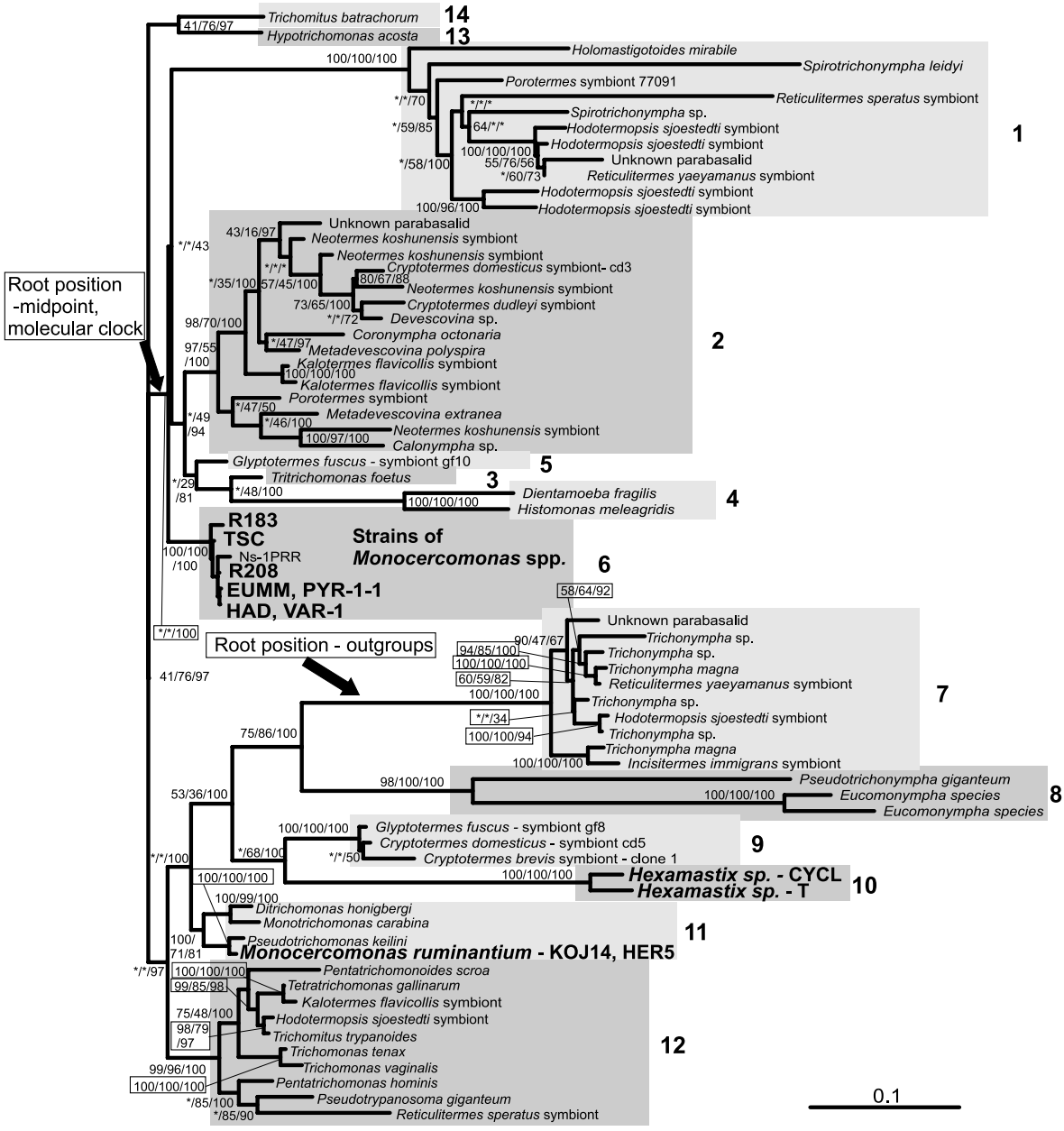

Fig. 1. Phylogenetic tree of Parabasala based on 16S rRNA gene sequences, the tree was constructed by the ML method using TrN + I + G model of substitution. The values at the nodes indicate statistical support for node estimated by three methods (LD bootstrap/MP bootstrap/MrBayes posterior probability). Bar indicates the branch length corresponding to 10% of sites that underwent substitution event. The shaded boxes and numbers indicate the well-supported clades: (1) Spirotrichonymphidae, Holomastigotoididae, and termite symbionts; (2) Calonymphidae and Devescovinidae and termite symbionts; (3) *Tritrichomonas foetus*; (4) *Dientamoeba fragilis* and *Histomonas meleagridis*; (5) Gf10 termite symbiont; (6) eight reptile isolates of *Monocercomonas* sp.—Ns-1PRR (ATCC 50210), PYR-1-1, EUMM, HAD, VAR-1, R208, TSC, and R183; (7) Trichonymphidae and termite symbionts; (8) Eucomonymphidae and termite symbionts; (9) termite symbionts (Gf8, Cd5, and Cb symbiont 1) indirectly assigned to genus *Tricercomitus* or *Hexamastix* (Keeling et al., 1998; Ohkuma et al., 2000); (10) isolates of *Hexamastix* sp.—T and CYCL; (11) free-living trichomonads (*Ditrichomonas*, *Monotrichomonas*, and *Pseudotrichomonas*) and isolates of *M. ruminantium*—HER5, KOJ14; (12) Trichomonadina, Trichomitopsiinae, Pentatrichomonoidinae, and termite symbionts; (13) *Hypotrichomonas acosta*; and (14) *Trichomitus batrachorum*. New sequences are printed in bold. The arrows indicate the position of the root as inferred by the outgroup method, midpoint method, and maximum likelihood with molecular clock.

(supplementary material Fig. s1). The tree robustness was expressed as the average of the posterior probabilities calculated in MrBayes (APP) or average of bootstrap values (ABV). The APP was calculated as the average of posterior probability values in the tree. To lower the effect of random fluctuation, the Markov Chain Monte Carlo analysis was run three times for each alignment and for each run the APP was calculated. Because different runs in some cases produced slightly different topologies, all three replicates were

plotted in the graph. ABV was calculated as the average of MP and LD bootstrap values in the tree (300 bootstrap replicates). Because the S–F method is based on the gradual exclusion of exactly those positions that carry information on the internal topology of clades 1, 2, 7, and 12, and hence the bootstraps of these nodes decrease and reach zero at the s0 level, these nodes were not included in calculating ABV and APP.

3. Results and their interpretation

3.1. Phylogenetic relationships among parabasalids based on 16S rRNA gene sequences

A phylogenetic tree of Parabasala based on 16S rRNA gene sequences was constructed by using the maximum likelihood method (ML) with the TrN + I + G model of nucleotide change. The Parabasala formed 14 distinct clades (Fig. 1). These 14 clades were revealed in all analyses despite using different tree-construction methods. The new sequences, from *Monocercomonas* and *Hexamastix*, are printed in bold.

The relationship between the 14 parabasalid clades varied with the tree-construction method used. The tree constructed from the same data set by Bayesian method had essentially the same topology as that shown in Fig. 1. In the phylogenetic tree constructed by maximum parsimony (MP) the clades 7, 8, 9, and 10 (trichonymphids, eucomonymphids, *Hexamastix*, and putative *Tricercomitus*) clustered with the clade 1 (spirotrichonymphids) and formed a sister branch to *Trichomitus batrachorum* and *Hypotrichomonas acosta*. In Fitch–Margoliash Logdet distance tree (LD) the clades 7, 8, 9, and 10 clustered with clades 1 and 4 (spirotrichonymphids and dienthamoebids) and formed the sister branch to clades 2, 3, 5, and 6 (devescovinids, *Tritrichomonas*, reptile monocercomonads, and termite symbiont Gf10).

Because the clades 1, 4, 7, 8, 9, and 10 represented the longest branches in the tree by far, their clustering may be a result of the long-branch attraction (LBA) artifact (Felsenstein, 1978). To investigate the influence of LBA on the position of these branches and on the topology of parabasalian tree in general, we used the S–F method (Brinkmann and Philippe, 1999) and the taxa-exclusion method, an approach, in which we performed separate analyses for each single long branch.

3.2. Slow–fast analysis

The most remarkable topological change induced by gradual removal of fast evolving sites was the splitting of the long-branch cluster 7–8–9–10 in Bayesian and ML trees, and similarly, cluster 1–7–8–9–10 in MP trees. The topology of the Bayesian trees down to s6 level did not differ in important features from the topology of the tree constructed from the total data. However, the number of excluded positions down to s6 was rather low. At the s5–s2 levels, the number of excluded positions considerably increased and the support for the clustering of clades 7, 8, 9, and 10 decreased concomitantly. The position of clades 9 and 10 remained the same, while clades 7 and 8 moved to the position of sister group to *T. batrachorum* and *H. acosta*. Corresponding to the topological changes, the average tree robustness decreased at s5 level, but then began to rise and at s3 level reached a value equal to or even a little higher than the value with all sites included. Prominent clustering of almost all long branches was observed in the s0 and s1 tree, but at these levels the tree robustness considerably decreased—probably because of the low amount of data—and the tree topologies are therefore unreliable. Trees inferred by maximum likelihood were similar to the Bayesian topologies. However, the 7–8–9–10 cluster was split only in the s3 tree. The splitting of the 1–7–8–9–10 cluster in maximum parsimony trees followed, almost exactly, the pattern observed in the Bayesian trees. The topology at the s3 level was virtually identical to the Bayesian tree. In the LD trees, the cluster of long branches 1–4–7–8–9–10 was not split at any level. The tree topologies and robustness for all levels using the S–F method are summarized in supplementary material (Figs. s1 and s2).

The Bayesian, ML, and MP methods converged to the same topology at the s3 level after removal of fast evolving sites (Fig. 2). Because it was inferred from slowly evolving sites, that tree may more correctly reflect true phylogenetic relationships among parabasalian clades than the tree inferred by using the complete alignment.

3.3. Taxa-exclusion analysis

The second approach that was used to reduce the effect of the LBA artifact, was to first exclude all unstable long-branch-forming clades (1, 4, 7, 8, 9, and 10) from the data set and then perform a series of analyses that re-included only one of these clades at a time. This taxa-exclusion method allowed us to infer the relationship of the long branch to other OTUs without any artificial attraction to other long branches. In each case we also performed S–F analyses from s6 to s1 level.

The position of the *D. fragilis–H. meleagridis* clade 4 and putative *Tricercomitus* clade 9 in the reduced trees was the same as that in Fig. 1. The statistical support for dientamoebids as a sister branch to *T. foetus* obtained in trees using all nucleotide positions was 70, 65, and 100 in MP (bootstrap), LD (bootstrap), and MrBayes (posterior probability) analyses, respectively. Similarly, the statistical support for *Tricercomitus* as a sister branch to the free-living trichomonad branch was 81, 71, and 100, respectively. These results strongly support the position of these clades as shown in Fig. 1.

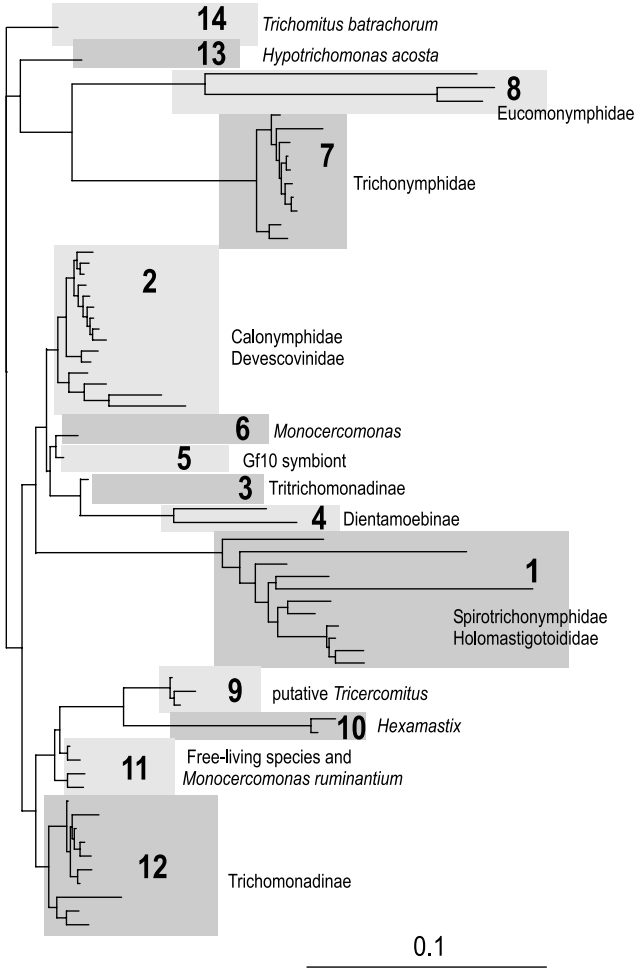

Fig. 2. Result of slow–fast analyses. The tree based on the most consistent s3 level of slow–fast was constructed by MrBayes. Identical topology was revealed also by MP and ML methods. The numbering of clades corresponds to Fig. 1.

The situation for the other four long-branching clades was more complicated. Clade 1 (Spirotrichonymphidae and Holomastigotoididae) appeared, except for in the s2 tree for MP, within or at the base of the *Tritrichomonas–Monocercomonas*–Calonymphidae–Devescovinidae branch. The statistical support for this position in the tree (using all nucleotide positions) was 80, 62, and 99 in MP (bootstrap), LD (bootstrap), and MrBayes (posterior probability) analyses, respectively. These results indicated that spirotrichonymphids and holomastigotoidids belong to the *Tritrichomonas–Monocercomonas*–Calonymphidae–Devescovinidae subtree as revealed in Fig. 1. However, they failed to determine the exact branching point of this clade.

Clades 7 (Trichonymphidae) and 8 (Eucomonymphidae) did not branch as a sister group to the free-living trichomonads and *M. ruminantium*, i.e., at position congruent to the ML tree in Fig. 1. In most analyses, they appeared as a branch related to *T. batrachorum* and *H. acosta*. The statistical support of the *T. batrachorum–H. acosta*–Trichonymphidae cluster in the tree obtained by using all nucleotide positions was 80, 52, and 96 in MP (bootstrap), LD (bootstrap), and MrBayes (posterior probability) analyses, respectively. For the *T. batrachorum–H. acosta*–Eucomonymphidae cluster the statistical support was 59, less than 50 and 99, respectively. These results contradicted the tree in Fig. 1 and suggest that the position of these clades in Fig. 1 may be the result of LBA. Because both clades were placed in a similar position in the reduced trees, they may form a monophyletic group.

The position of clade 10 (*Hexamastix*) was rather unstable, but its placement next to the free-living trichomonad branch as in the complete ML tree (Fig. 1) was favored by most analyses, especially those with more strict S–F levels (including the most consistent s3 level). These observations suggest to us that the sister position of *Hexamastix* to free-living trichomonads revealed in the tree in Fig. 1 may be correct. Because the placement of clade 10 corresponds to the placement of putative *Tricercomitus* clade 9, the two clades are probably related as shown in Fig. 1. The results of taxa-exclusion analyses of clades 1, 7, 8, and 10 are illustrated in detail in the supplementary material (Fig. s3).

Fig. 3 shows a scheme of relationships among groups of Parabasala that summarizes the results of taxa-exclusion analyses. The scheme is congruent with tree from the s3 level of S–F analysis (Fig. 2), and differs from the ML tree in Fig. 1 in the position of the hypermastigid clades 7 (Trichonymphidae) and 8 (Eucomonymphidae). The color of each clade in the figure reflects their family or order classification.

3.4. Rooting of the parabasalian tree

To investigate the position of the root of the parabasalian tree, we chose 12 sequences from various eukaryotic groups, that did not significantly differ in their base composition from the parabasalian sequences and used them as outgroups—*Cryptomonas* sp., *Fucus gardneri*, *Chlorarachnion reptans*, *Oryza sativa*, *Phreatamoeba balamuthi*, *Naegleria gruberi*, *Ceramium rubrum*, *Prymnesium parvum*, *Saccharomyces cerevisiae*, *Euglena gracilis*, *Hexamita inflata*, and *Eimeria necatrix*. The relationship among parabasalid groups in the rooted tree constructed by maximum likelihood method (TrN + I + G model of nucleotide change) was generally in agreement with the unrooted tree in Fig. 1. The root was situated on the Trichonymphidae branch inside the cluster 7–8–9–10 (Fig. 1, arrow). A similar topology was also recovered by maximum parsimony. In the tree inferred by the LD method, clade 1 (Spirotrichonymphidae and Holomastigotoididae) joined the 7–8–9–10 cluster at the root.

The affinity of particular long branches to the outgroups could be caused by LBA artifact. To investigate

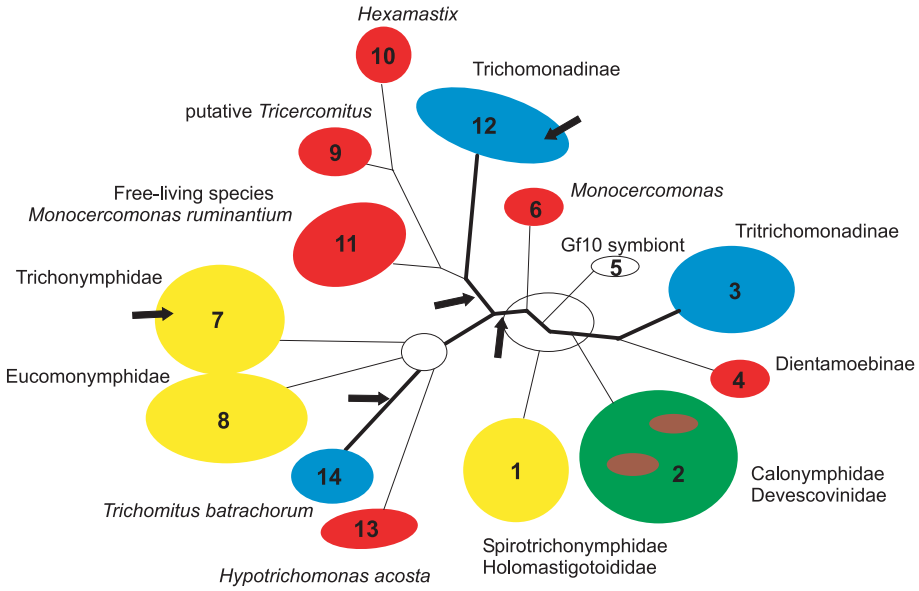

Fig. 3. Result of taxa-exclusion analyses. The tree is a composition based on results of taxa-exclusion analyses. The colors of clades indicate their taxonomic classification: red, family Monocercomonadidae; blue, family Trichomonadidae; green, family Devescovinidae; brown, family Calonymphidae; and yellow, order Hypermastigida. The thick lines indicate the branches connecting the Trichomonadidae. The forms at these branches probably possessed costa (see Section 4). The arrows indicate the root positions that were tested (see Section 3).

this, we used four methods: the S–F method, the exclusion of long parabasalian branches, the use of single outgroups, and testing of constrained trees.

The removal of fast evolving sites during the S–F analysis had no significant influence on the root position. The root was situated either inside the 7–8–9–10 cluster, at its base or, in case of the s4 analysis, on the Eucomonymphidae branch (clade 8) separate from the 7–9–10 cluster.

As the next approach, we excluded all the long-branch taxa (clades 1, 4, 7, 8, 9, and 10) from the tree so they could not attract outgroups by LBA. If the root was truly located at Trichonymphidae or another excluded clade, it should appear in the position, where this clade emerged in the complete tree (i.e., in the *T. batrachorum–H. acosta* clade in case of Trichonymphidae). We used four tree-construction methods (ML, Bayesian method, MP, and LD) and performed S–F analyses for each of them. Results are summarized in Fig. 4. The position of the root varied with the tree-construction method and in most analyses was inconsistent with the position in the complete tree.

To investigate the influence of each outgroup sequence on the position of the root, we performed separate analyses with each single outgroup. The results are summarized in Fig. 5. The position of the root varied considerably with the different outgroups and with tree-construction method used.

The topology of the ML tree obtained by using the complete set of taxa (Fig. 1) is probably wrong, and this can affect the rooting. Thus, in the next analysis, we used the complete set of taxa but constrained the topology to the topology of Parabasala as it is predicted in Fig. 3. Then we tested likelihood differences between the five most probable positions of the root (Fig. 3, arrows). We performed this test on the complete alignment, as well as with the s3 alignment. We used either the set of 12 outgroups, or *Hexamita* as a single outgroup. Neither an approximately unbiased test, or other tests implemented in Consel showed statistically significant differences between likelihoods of the positions tested.

We also used rooting methods that do not require outgroups—the midpoint method, and ML with an enforced molecular clock. Using these methods we estimated the root position in the complete tree and the tree with long branches excluded. The root inferred by these methods appeared, in both cases, between *Monocercomonas* sp. and the *H. acosta–T. batrachorum* branch (Fig. 1 arrow and Fig. 4 shade box).

Although some analyses placed the root at the base of the Trichonymphidae or at a position congruent with such rooting, most of them favored other positions. Our analyses, thus, challenged the Trichonymphidae rooting but did not suggest any other robust position for the root.

3.5. Testing of polyphyly of the order Hypermastigida and family Monocercomonadidae

The representatives of the orders Hypermastigida and Trichomonadida, and families Monocercomonadidae and Trichomonadidae appeared to be polyphyletic in the trees. However, the evidence on the polyphyly of these groups was not strong.

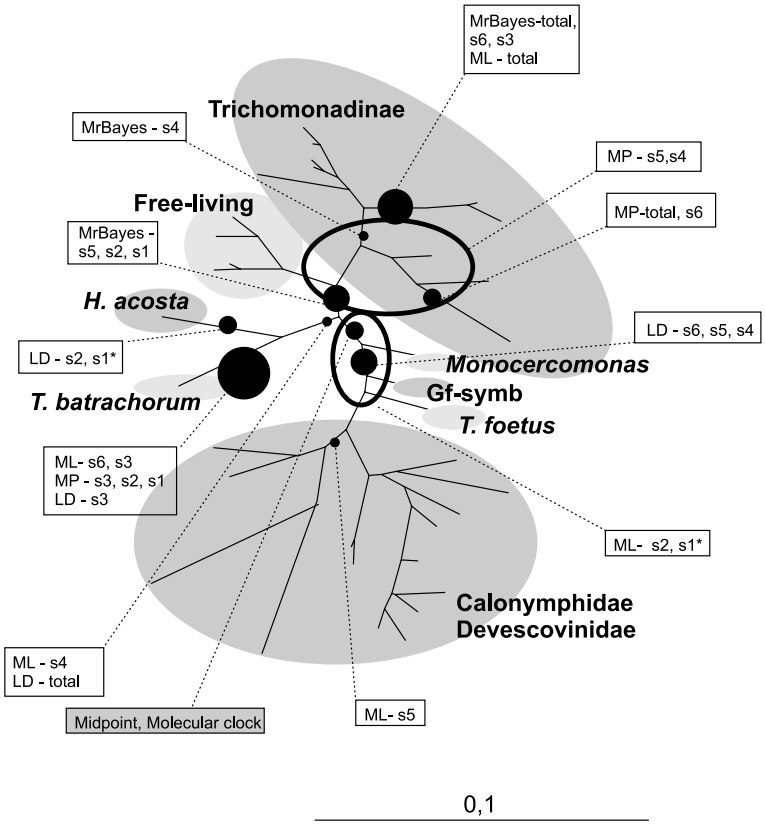

Fig. 4. Rooting of the tree of Parabasala after exclusion of long branches. The tree of Parabasala without long branches was rooted using 12 eukaryotic outgroups. The position of the root for various methods and S–F analyses is indicated. The methods and levels of S–F are listed in boxes. "Total" designates the analysis based on all nucleotide positions (not S–F). The diameter of the dot corresponds to the number of methods that placed the root in the particular position. If the topology of the reduced tree slightly changed, and it was not possible to place the branch in the figure exactly, the region where the branch should belong was marked by an ellipse.

Because all taxa in the tree are members of either Hypermastigida or Trichomonadida, the problem of polyphyly of Hypermastigida and Trichomonadida is interconnected; either Hypermastigida or Trichomonadida (or both) are polyphyletic. To test the significance of polyphyletic nature of Hypermastigida/Trichomonadida we constructed a phylogenetic tree based on 16S rRNA gene sequences in which monophyly of Hypermastigida/Trichomonadida was constrained, and then used tests implemented in the program Consel v0.1f to test the significance of the difference between the likelihood value of the constrained tree and that of the best tree. All tests showed that this difference is not significant at the 5% level (approximately unbiased test $p = 0.122$, $SE = 0.006$).

Analogously, we tested the polyphyly of the family Monocercomonadidae. Because the classification of *H. acosta* to the family Monocercomonadidae can be wrong (Kulda, 1965), we performed two separate tests. In one, we regarded *H. acosta* as a member of Monocercomonadidae and in the other we tested the monophyly of Monocercomonadidae without this species. In both cases all the tests implemented in Consel v0.1f showed that the overall best tree is significantly better than the tree with monophyletic family Monocercomonadidae at the 0.01 level (approximately unbiased test $p = 0.01$, $SE \leqslant 0.002$ for both constrained topologies).

We tested the polyphyly of the family Trichomonadidae in the same way. Again we performed two separate tests with *H. acosta* either included or excluded from the family Trichomonadidae. In both cases all the tests showed that the overall best tree is significantly better than the tree with monophyletic family Trichomonadidae at 0.02 level (approximately unbiased test, *Hypotrichomonas* included: $p = 0.003$, $SE = 0.001$; *Hypotrichomonas* excluded: $p = 0.005$, $SE = 0.001$).

Because the alignment at the s3 level of S–F probably contained a less biased phylogenetic signal, we performed all these tests again using this alignment. The results were identical and p values were similar to those based on the non-reduced alignment.

4. General discussion

4.1. Phylogenetic relationships in the phylum Parabasala

Results of our analyses concerning the relationship among parabasalid taxa are generally consistent with

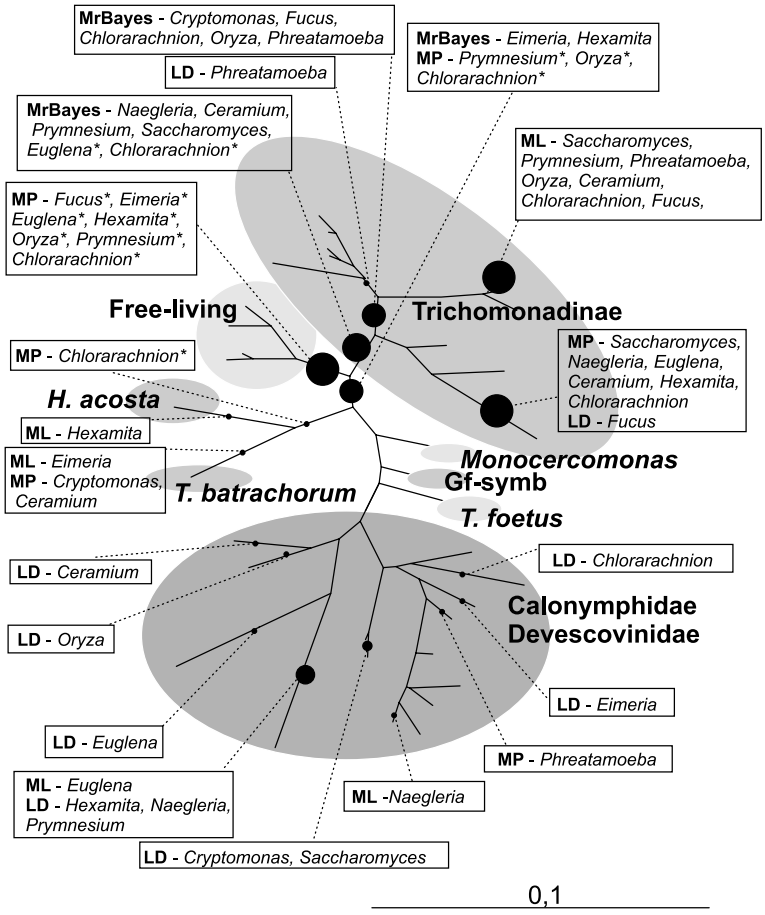

Fig. 5. Rooting of the tree of Parabasala after exclusion of long branches with each single outgroup independently. The diameter of the dot corresponds to the number of outgroups that placed the root in the particular position. *indicate the cases, in which the topology of the reduced tree was changed but it was still possible to place the root in the figure.

the previous results (Delgado-Viscogliosi et al., 2000; Edgcomb et al., 1998; Gerbod et al., 2000, 2001, 2004; Keeling et al., 1998; Ohkuma et al., 2000; Viscogliosi et al., 1999). However, the positions of certain clades in the comprehensive analysis (Fig. 1) were unstable and method-dependent. All clades in question formed long branches resulting from a high divergence of their 16S rRNA gene sequences. The position of these branches could, therefore, be influenced by stochastic effects and artifacts of the tree-construction methods.

We reanalyzed the position of these branches using the S–F (Brinkmann and Philippe, 1999) and taxa-exclusion methods, both designed to minimize the attraction between long branches in the tree. As expected, results from both methods led to similar conclusions (Figs. 2 and 3).

Both analyses cast doubt on the phylogenetic relationship of Trichonymphidae and Eucomonymphidae (clades 7 and 8) to the 9–10–11 cluster. The affinity of these clades to 9–10–11 cluster in Fig. 1 probably results from LBA between clades 7, 8 and 9, 10. Because only few published analyses have included the *Tricercomitus* clade 9 (Delgado-Viscogliosi et al., 2000; Keeling, 2002; Keeling et al., 1998), the affinity of spirotrichonymphids and eucomonymphids to *Tricercomitus* has not previously attracted much attention. Moreover, two of these analyses (Delgado-Viscogliosi et al., 2000; Keeling et al., 1998) used distance or quartet puzzling methods that in our opinion may have introduced even more serious biases that obscured the relationships of parabasalian clades (e.g., clustering of *Dientamoeba* with hypermastigids), so this artifact remained hidden. But the recent ML tree of Keeling (2002) is virtually identical with our ML tree in Fig. 1 and also includes this artificial cluster.

4.2. The root of Parabasala

Previous molecular analyses that attempted to determine the root of Parabasala (Delgado-Viscogliosi et al., 2000; Keeling et al., 1998; Ohkuma et al., 2000) used the outgroup method with several eukaryotic taxa. In detailed analyses focused on the rooting of Parabasala (Keeling et al., 1998; Ohkuma et al., 2000) the authors used the KH test for testing of the various hypotheses of the root position. Most of the analyses favored rooting at the Trichonymphidae branch.

Our results strongly challenged this hypothesis but did not suggest any robust alternative position of the root. A major problem of rooting for the Parabasala is probably the lack of any close outgroup species. The phylum stays as a separate branch with no clear and close relationship to any other eukaryote group in virtually all analyses concerning eukaryotic phylogeny (e.g., Edgcomb et al., 2001; Sogin and Silberman, 1998). Some evidence suggests (Baldauf et al., 2000; Henze et al., 2001) that diplomonads may represent the nearest, but still very distant, sister taxon to Parabasala. The large genetic distances between ingroup and outgroup complicate the identification of the root position (Huelsenbeck et al., 2002). It has been shown that as the length of the branch leading to outgroup sequence increases, the ability of the outgroup method to determine the root decreases. As the length approaches infinity, the position of the root becomes essentially random (Huelsenbeck et al., 2002). Additionally, several groups of Parabasala have highly divergent 16S rRNA sequences and form long branches in the parabasalian tree. The presence of long branches probably enhances the influence of artifacts of the tree-construction methods such as LBA. Long ingroup branches can be strongly attracted to the long branch of outgroup in the absence of phylogenetic signal, as probably happened in case of the Trichonymphidae. LBA also affects statistical tests based on comparing of likelihoods.

The basal position of multiflagellate parabasalids like the Trichonymphidae is difficult to explain from a morphological point of view. Polymastigont organization is not very common in other taxa of flagellates and all potential relatives of Parabasala typically possess four-kinetosome mastigont (doubled in Diplomonadida). The four-kinetosome mastigont is also regarded as plesiomorphic for Trichomonadida. The basic set of four privileged kinetosomes with characteristic cytoskeletal appendages can be identified even in genera with complex mastigonts supplemented by numerous additional kinetosomes (Brugerolle, 1991). The most parsimonious scenario would therefore predict that the Parabasala evolved from four-kinetosome flagellates rather than trichonymphid-like ones.

4.3. Polyphyly of the orders Hypermastigida and Trichomonadida, families Monocercomonadidae and Trichomonadidae, and anagenesis of cytoskeletal and motility organelles

Family Trichomonadidae was split into three distinct clades in our analyses (trichomonads, tritrichomonads, and *Trichomitus*) and its polyphyly was statistically significant ($p < 0.02$). This fact could, however, reflect the polyphyly of Monocercomonadidae or Hypermastigida or both (see below). In other words, the common ancestor of all parabasalids could theoretically be a Trichomonadidae-like protozoan. In this case, Trichomonadidae would be paraphyletic rather than polyphyletic. To distinguish between para- and polyphyly it would be necessary to know the morphotype of the last common ancestor.

Morphologically the Trichomonadidae family is characterized by the presence of undulating membrane and costa. The independent origin of the undulating membrane in three separate Trichomonadidae branches is relatively plausible, because the membranes of each group differ in their ultrastructure (Brugerolle, 1976). Moreover, analogous undulating membranes are also present in unrelated protozoa (e.g., trypanosomes). In contrast, the triple independent origin of the costa appears to be less probable. The costa, a dominant striated root fiber of the Trichomonadidae, might have been evolved from parabasal fibers that are present in virtually all representatives of Parabasala. Indeed, there is a close similarity in ultrastructure between the parabasal fiber and the costa of *Trichomitus* and tritrichomonads, both possessing the A type pattern of banding. The B type costa of Trichomonadinae and *Trichomitopsis* shares, with the A type, a 42 nm periodicity of the major repetitive bands, but contains additional longitudinal filaments providing its characteristic lattice appearance in longitudinal sections. There are also minor differences in the topology of attachment to kinetosomes. Despite these differences, comparative morphological studies on trichomonad mastigonts (Brugerolle, 1976) revealed a common basic pattern of organization of kinetosome associated fibers in Trichomonadidae, and substantiated homology of the main components of the mastigont, including the costa. Moreover, the available immunocytochemical and protein analyses (Viscogliosi and Brugerolle, 1994) suggest that the major proteins of both types of costa belong to a common protein family. These results favor the hypothesis that costa originated only once in the common ancestor of Trichomonadidae, thus implying that the family is paraphyletic rather than polyphyletic.

The polyphyletic nature of family Monocercomonadidae has been proposed in several studies. In our analyses (Fig. 3), the representatives of this family formed four groups. Although the bootstrap support for nodes separating Monocercomonadidae is very low, all tests support the hypothesis of Monocercomonadidae polyphyly.

The polyphyly of Monocercomonadidae implies multiple origin of forms without costa and with a reduced, or absent, undulating membrane. This scenario seems plausible, because multiple losses, or reductions, of functional structures can be easily explained, for example, by the loss of selectable advantages that they bring to the organisms experiencing new ecological conditions.

The order Hypermastigida appeared in our analyses as diphyletic. Families Trichonymphidae + Eucomonym-

phidae represented the first, though weakly supported, clade and Spirotrichonymphidae + Holomastigotoididae the second well-supported clade, as previously suggested by Gerbod et al. (2001). Although the hypothesis of diphyletic Hypermastigida was not statistically significant and was not supported by high bootstraps, it was the most probable hypothesis. It is important to mention that sequences for *Koruga bonita* and *Joenina* sp. are missing in our analyses. We did not include them, because the 16S rRNA gene sequence of *K. bonita* is only partial and the other sequence was only putatively ascribed to the genus *Joenina*. However, it has been shown by other authors that both sequences branch inside the Calonymphidae and Devescovinidae clade with high bootstrap support (Frohlich and Konig, 1999; Gerbod et al., 2002), which is in accordance with morphological observations (Brugerolle and Patterson, 2001). These results clearly show the polyphyly of the order.

The polyphyly of Hypermastigida implies multiple origin of a complex Hypermastigida morphology. Other than the large cell size and higher cell complexity in general, the hypermastigid clades differ in the organization of the mastigont and cytoskeleton (Brugerolle and Lee, 2000). Moreover, the trend in Trichomonadida to multiply flagella or mastigonts in certain cases is well known, so it is not difficult to imagine multiple origins of polymastigont or multiflagellated forms in the evolution of this order.

The second order, Trichomonadida, is also polyphyletic in our analyses (Fig. 3). Because all taxa in the tree belong either to order Trichomonadida or Hypermastigida, the polyphyly of both orders has the same statistical significance and has probably the same cause. In our opinion, Trichomonadida are paraphyletic rather than polyphyletic. As we concluded in the previous section, their common ancestor probably morphologically resembled the family Trichomonadidae. Therefore, we favor the hypothesis that the polyphyletic distribution of Hypermastigida/Trichomonadida is due to multiple origins of the hypermastigote morphology, which is easier to explain.

The previous discussion is summarized in Fig. 3, in which a thick line between Trichomonadidae branches indicates the presence of a costa and (supposedly) undulating membrane. The monocercomonad- and hypermastigid-like morphology originated several times from these costa bearing parabasalids. Because we do not know the position of the root, we can only speculate whether the Trichomonadidae morphology is plesiomorphic for the whole order, or whether it originated from simpler (Monocercomonadidae) or more complex (Hypermastigida, Calonymphidae, and Devescovinidae) morphotypes.

Unlike the costa and undulating membrane characters, the morphology of the pelta–axostylar complex is reflected in some respects by the topology of the tree in Fig. 3. Species in the right half of the tree (*Tritrichomonas*, *Monocercomonas* spp., Devescovinidae, Calonymphidae, and Joeniidae) possess a stout hyaline axostyle. The trunk of their axostyle has more or less uniform diameter along its whole length and tapers abruptly at the posterior end. The microtubular sheet forming this axostyle is rolled up in a tube-like fashion, not cone-like as it is in most of other Trichomonadida. In Calonymphidae, Devescovinidae, and Joeniidae (Brugerolle and Patterson, 2001) the microtubular sheet is more spiralized and fills the internal space of the axostylar trunk. In Calonymphidae, the multiple axostyles either form one central bundle (*Calonympha*, *Snyderella*), or stretch separately in the cytoplasm (*Coronympha*, *Metacoronympha*). These two types of organization correspond to the existence of two unrelated clades of Calonymphidae in the tree (Gerbod et al., 2002). Exceptions to the rule in this part of the tree are axostyles of dientamoebids. *Dientamoeba* has lost the pelta and axostyle completely and the axostyle in *Histomonas* is reduced.

In the left part of the tree, the branch of Trichomonadidae, *Hexamastix*, free-living trichomonads, and *M. ruminantium* shares, with few exceptions, a relatively slender type of axostyle. The axostyles of *H. acosta* and *T. batrachorum* are stouter but, similar to the previous group, they are formed by cone-like rather than tube-like coiling of the microtubular sheet.

An exception to the scheme is the presence of multiple axostyles formed by microtubular bands in two unrelated groups—Trichonymphidae/Eucomonymphidae and Spirotrichonymphidae/Holomastigotoididae.

Molecular phylogeny sheds new light on the polymorphism of the shape of the axostyle in the genera *Monocercomonas* and *Hexamastix*. Honigberg (1963) suggested that the wide polymorphism of these usually conservative structures is the result of a primitive evolutionary status of this genus. However, the results of molecular analyses indicate that it is an artifact of the polyphyly of the genus. For example, in the set of currently available taxa, the representatives of the genus *Monocercomonas* split into two unrelated groups in agreement with the morphology of their axostyles: *Monocercomonas* spp. from reptilian hosts on one side and *M. ruminantium* on the other. A similar situation is, or could be, possible for the genus *Hexamastix*.

4.4. Taxonomy of the phylum Parabasala

Several authors have pointed out the need to revise the classification of the phylum Parabasala on the basis of phylogenetic analyses of molecular data (Delgado-Viscogliosi et al., 2000; Gerbod et al., 2001; Keeling et al., 1998; Ohkuma et al., 2000; Viscogliosi et al., 1999). To reconcile the classification to the current knowledge of parabasalian phylogeny, Brugerolle and Patterson (2001)

proposed a new classification of Parabasala at the ordinal level. They divided the phylum into three orders (Trichonymphida, Cristamonadida, and Trichomonadida) instead of the current two (Hypermastigida and Trichomonadida). The newly created order Cristamonadida comprises the families Devescovinidae and Calonymphidae—currently classified to the order Trichomonadida—and the families Joenidae, Lophomonadidae, Deltotrichonymphidae, Rhizonymphidae, and Kofoididae—currently classified under the order Hypermastigida. For the remaining hypermastigid families they created the order Trichonymphida, that they regarded as basal within the phylum Parabasala. This modification reflected the growing molecular and ultrastructural evidence that some representatives of Hypermastigida are related to Calonymphidae and Devescovinidae (Frohlich and Konig, 1999; Gerbod et al., 2002).

However, this proposed classification is still incongruent with molecular and morphological data in several respects. First, the monophyletic nature of the order Trichonymphida is doubtful. Our analyses suggest that the families Spirotrichonymphidae and Holomastigotoididae do not form a clade with the families Eucomonymphidae and Trichonymphidae. Although we cannot exclude the possibility that the proposed order Trichonymphida is monophyletic, to establish this taxon at the current stage of knowledge is, in our opinion, premature. Second, the designation of Cristamonadida and Trichomonadida as sister orders does not correspond with current views, or with the results of our study. Although current data fully support the monophyly of the order Cristamonadida, creation of this order causes the paraphyly or polyphyly of the order Trichomonadida. Both molecular and morphological data indicate that the clade Cristamonadida arose from one lineage of the order Trichomonadida. The closest relatives to Cristamonadida are probably the subfamily Tritrichomonadinae and genera *Dientamoeba*, *Histomonas*, and *Monocercomonas*. To accommodate the classification to the phylogeny either the aforementioned taxa must be included within the order Cristamonadida, or the group Cristamonadida must be reclassified as a member (perhaps family or suborder) of the order Trichomonadida with its current species composition.

The classification of an organismal group should reflect the phylogenetic relationships among the species. Although the available data are clearly in conflict with the current classification, they are still not sufficient to understand the phylogenetic relationships among the species. Based on analyses of 16S rRNA gene sequences, we were able to identify 14 robust clades and to reconstruct the possible relationship among them (Fig. 3). However, this tree is based on the single well-sampled gene, has low support for deep nodes, and some key information, for example, the root position, cannot be inferred reliably. In our opinion, any taxonomic revision may be premature and risky at this stage. We suggest that future work in this field should be focused on verification of the relationships among the robust clades as deduced from the 16S rRNA by gathering and analyzing sequences of another independent gene. The first serious move in this direction was made by Gerbod et al. (2004). Until a robust parabasalian phylogeny is recovered we suggest the retention of the current classification system for the orders Hypermastigida (revised by Hollande and Carruette-Valentin, 1971), and Trichomonadida (revised by Honigberg, 1963, and modified by Brugerolle, 1976, 1980; Camp et al., 1974; Honigberg and Kuldová, 1969; Pecka et al., 1996).

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