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Short Communication

Non-monophyly of Retortamonadida and high genetic diversity of the genus *Chilomastix* suggested by analysis of SSU rDNA

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1. Introduction

Retortamonads (Retortamonadida) are a small group of protists comprising flagellates living mostly as intestinal commensals of both vertebrates and invertebrates (Kulda and Nohýnková, 1978), although free-living representatives have been also found (Bernard et al., 1997). Potential pathogenicity has been reported for some species from vertebrates. Although medically unimportant, retortamonads have attracted attention because of their evolutionary history. Their cells lack some typically eukaryotic organelles, the mitochondrion in particular, and retortamonads were once considered to be one of a series of eukaryotic lineages – 'Archezoa' – that had diverged before the acquisition of the mitochondrial organelle (Cavalier-Smith, 1983, 1987). However, this hypothesis has fallen into disfavor, as relict mitochondria have been found in most of the putative archezoan groups (see Simpson and Roger, 2004). Although retortamonads are one of the last eukaryotic groups for which no sign of a mitochondrial past has yet been found, it has been shown that they are closely related to diplomonads (Silberman et al., 2002; Hampl et al., 2008; Kolisko et al., in press), whose cells do possess a mitochondrial remnant, the 'mitosome' (Tovar et al., 2003). It is, therefore, generally assumed that retortamonads are also secondarily amitochondriate.

Although numerous species of retortamonads have been described they are assigned to just two genera, the biflagellated *Retortamonas* and the quadriflagellated *Chilomastix* (Kulda and Nohýnková, 1978). The characteristic features of retortamonads include four basal bodies arranged in two pairs, two or four flagella, one of them being directed posteriorly and associated with well-developed cytostome, which continues as a curving cytopharynx. There is also a microtubular corset underlying the cell surface (Brugerolle, 1973, 1977, 1991; Kulda and Nohýnková, 1978; Bernard et al., 1997). The morphological synapomorphies of Retortamonadida were defined by Simpson and Patterson (1999). Retortamonad cells also possess all features typical for "true excavates" (Simpson and Patterson, 1999; Simpson, 2003) and are currently classified into the eukaryotic supergroup Excavata (Cavalier-Smith, 2002; Simpson, 2003; Adl et al., 2005).

Retortamonadida was proposed as a holophyletic lineage. Until the present study, molecular data of a single retortamonadid genus, *Retortamonas*, have been available (Silberman et al., 2002; Hampl et al., 2008; Kolisko et al., in press) and *Chilomastix* was always assumed to be sister to *Retortamonas*. Molecular phylogenetic studies have shown clearly that retortamonads are closely related to diplomonads (Diplomonadida), *Carpediemonas* and *Dysnectes* (Silberman et al., 2002; Simpson et al., 2002; Kolisko et al., 2005; Yubuki et al., 2007), together forming the monophyletic group Fornicata (Simpson, 2003). Fornicate morphological synapomorphies have been defined recently (Adl et al., 2005; Yubuki et al., 2007).

Retortamonads have often been regarded to be closely related to diplomonads on the basis of the ultrastructure of the flagellar apparatus and the presence of cytostomes and cytopharynges (Brugerolle, 1977, 1991; Cavalier-Smith, 1993). Together, they were named Eopharyngia (Cavalier-Smith, 1993) though eopharyngian morphological synapomorphies have not been defined so far (see Simpson, 2003). The close relationship between the two groups has been further supported by molecular phylogenetics (Silberman et al., 2002; Hampl et al., 2008; Kolisko et al., in press). Diplomonads, the largest fornicate group, comprise both parasitic and free-living flagellates. The most remarkable difference

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between retortamonads and diplomonads is that while retortamonads are unizoic (their cells contain a single set of organelles), diplomonads are mostly diplozoic (their cells contain two axially symmetrical sets of organelles including doubled nucleus and cytoskeleton). On the basis of the presence or absence of cytostomes (Kulda and Nohýnková, 1978), use of canonical vs. non-canonical genetic code (Keeling and Doolittle, 1997) and molecular phylogenetic studies (Kolisko et al., 2005; Keeling and Brugerolle, 2006; Jørgensen and Sterud, 2007), diplomonads have been divided into two monophyletic groups, Hexamitinae and Giardiinae. Interestingly, unizoic enteromonads, which had been hypothesized as ancestors of diplozoic diplomonads (Siddall et al., 1992), branch within the Hexamitinae indicating that either diplomonads arose several times independently from unizoic cells or that unizoic enteromonads arose from diplozoic diplomonads (Kolisko et al., 2005, in press). It has been recently established that the symmetry of diplomonad cells is, at least in case of *Giardia intestinalis*, only superficial and that there is considerable asymmetry in the karyotypes and behavior of the two nuclei during the cell cycle (Tůmová et al., 2007). Moreover, mastigonts of a single *Giardia* cell exchange a flagellum during each cell cycle (Nohýnková et al., 2006).

Although phylogenetic analyses based on the SSU rDNA gene sequences strongly support monophyly of Eopharyngia, the exact relationship between retortamonads and diplomonads remains unclear. Phylogenetic studies analysing the SSU rDNA gene are undecided as to whether retortamonads are sister to the Giardiinae lineage, making diplomonads paraphyletic, or whether diplomonads are monophyletic and retortamonads form their sister branch. (Silberman et al., 2002; Simpson et al., 2002; Kolisko et al., 2005; Keeling and Brugerolle, 2006; Yubuki et al., 2007). On the other hand, analyses of HSP90 gene sequences support monophyly of diplomonads to the exclusion of retortamonads (Kolisko et al., in press) which corresponds with analyses based on ultrastructural data (Siddall et al., 1992; Simpson, 2003). Apart from Eopharyngia, two free-living excavate flagellate genera, *Carpediemonas* and *Dysnectes*, belong to Fornicata. However, both ultrastructural and molecular-phylogenetic approaches have not fully resolved phylogenetic relationships between the three fornicate lineages (Yubuki et al., 2007).

So far, hypotheses on retortamonad evolutionary history have been based solely on sequence data from the genus *Retortamonas*. Although *Chilomastix*, the second of the two retortamonad genera, has not been forgotten by protozoologists, the unavailability of isolates made most research impossible. We have cultured two different *Chilomastix* species, *C. mesnili* and *C. wenrichi*, have sequenced their SSU rDNA and performed phylogenetic analyses. The present paper represents the first phylogenetic study that includes sequences from the genus *Chilomastix*. Our data strongly suggest that Retortamonadida are not monophyletic, but that they are paraphyletic and that diplomonads branch inside them. We, therefore, propose a new scenario of evolution of Eopharyngia. Our study also reveals considerable genetic diversity within *Chilomastix*.

2. Materials and methods

2.1. Organisms

Chilomastix wenrichi isolate CAVIA2 was obtained from the large intestine of a Guinea pig (*Cavia porcellus*). *Chilomastix mesnili* isolate FAB was obtained from feces of a human patient suffering from diarrhea who had recently returned to the Czech Republic from South America. The isolates were xenically cultured with bacteria (FAB) or with bacteria and *Blastocystis* sp. (CAVIA2) in Dobell and Leidlaw's biphasic medium (Dobell and Leidlaw, 1926) at 37 °C and were maintained by serial transfer every 2–4 days. The isolates are deposited in the culture collection of the Department of Parasitology of Charles University in Prague, Czech Republic. To confirm species identity of the *Chilomastix* isolates, their morphology was examined on protargol-stained preparations. Moist films spread on coverslips were prepared from pelleted cultures obtained by centrifugation at 500 g for 8 min. The films were fixed in Bouin-Hollande's fluid for 15 h, were washed with 70% ethanol, and were stained with 1% protargol (Bayer, I. G. Farbenindustrie AG, Germany) following the Nie's (1950) protocol.

2.2. DNA isolation, amplification, cloning and sequencing

Genomic DNA was isolated using the High pure PCR template preparation kit (Roche Applied Science). Eukaryote-specific primers MedlinA (CGTGTTGATCCTGCCAG) and MedlinB (TGATCCTTC TGCAGGTTCACCTAC) (Medlin et al., 1988) were used to amplify SSU rDNA with an annealing temperature of 45 °C. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and were cloned into the pGEM®-T EASY vector using the pGEM®-T EASY VECTOR SYSTEM I (Promega). Eleven clones from three independent PCRs of the isolate CAVIA2 were partially sequenced using the primer MedlinA. Two of the obtained sequences were SSU rDNA of *Blastocystis* sp. The other nine sequences, which were almost identical, were ascribed to *Chilomastix wenrichi* and were further sequenced. Four clones of the *C. mesnili* isolate FAB originating from two independent PCRs were sequenced. All clones were sequenced bidirectionally by primer walking. Sequence data reported in this paper are available in GenBank under accession numbers EF450168 and EU009463–EU009466.

2.3. Phylogenetic analyses

Four data sets containing sequences of SSU rDNA were created. The first data set contained 9 sequences of fornicates including two *Chilomastix* species and 33 sequences representing a broad range of other eukaryotic taxa. The second data set contained 16 sequences of fornicates and 6 sequences of other excavates. The third data set contained only sequences of fornicate taxa. The fourth data set contained the same sequences as the second data set, plus fragments of SSU rDNAs obtained from fin whale (*Balaenoptera physalus*) feces (GenBank accession numbers AY392799, AY392812, AY392815, and AY392816) by Jarman et al. (2004). Sequences from each data set were aligned using the T-Coffee method (Notredame et al., 2000) with the help of the T-Coffee@igs server http://www.igs.cnrs-mrs.fr/Tcoffee/ (Poirot et al., 2003). To fit the requirements of the server, sequences of *Retortamonas* spp., *Chilomastix mesnili* and *Euglena gracilis*, which were longer than 2000 nucleotides, were shortened by deleting the most divergent parts of their long insertions not aligned with any other taxon by ClustalX 1.81 (Thompson et al., 1997). The resulting alignments were manually edited using BioEdit 7.0.4.1 (Hall, 1999). The fourth data set was then trimmed: the sites for which sequences obtained from whale feces had only gaps were omitted. The resulting alignments contained 1044, 1067, 1094, and 177 characters, respectively. The alignments are available from the corresponding author upon request.

Phylogenetic analyses were conducted using the maximum parsimony (MP), Fitch-Margoliash with Logdet (LD) distances, Fitch-Margoliash with maximum likelihood distances (MLdist), and maximum likelihood (ML) methods implemented in PAUP* 4.0b10 (Swofford, 2002), and by the Bayesian method implemented in MrBayes 3.1.1 (Ronquist and Huelsenbeck, 2003). The models of nucleotide substitution for the ML and MLdist analyses were chosen by hierarchical nested likelihood ratio tests implemented in Modeltest 3.06 (Posada and Crandall, 1998). The models were selected as follows: TrNef + I + Γ for the first and second data sets, TrN + I + Γ for the third data set, and TrNef + Γ for the fourth

data set. The proportion of invariable sites for LD analysis was estimated from a neighbor-joining tree. MP, LD, MLdist, and ML trees were constructed by ten replicates of a heuristic search in which the starting tree was obtained by the stepwise addition procedure with a random order of taxa addition and swapped using the tree bisection and reconnection (TBR) algorithm. The trees were bootstrapped with 1000 (300 for ML in case of the first data set) replicates, each with ten replicates of random taxon addition with TBR branch swapping. For the Bayesian analyses, base frequencies, rates for the six different types of substitution, the proportion of invariable sites, and the shape parameter of the gamma correction for the rate heterogeneity (approximated by four discrete categories) were allowed to vary. A covarion model was used to allow rate heterogeneity along the tree. The number of generations of Markov chain Monte Carlo was 10^6 for the second and fourth data set, 2×10^6 for the third data set, and 3×10^6 for the first data set (until average standard deviation of split frequencies was lower than 0.01) and the trees were sampled every 100 generations. First 2500 (second and fourth data set), 5000 (third data set) or 7500 (first data set) trees were discarded as burn-in.

Alternative positions of the genus *Chilomastix* were tested using AU tests implemented in consel 0.1i (Shimodaira and Hasegawa, 2001). The trees of highest likelihood whose topologies corresponded to the tested hypotheses were constructed by ten replicates of a heuristic search with TBR branch swapping under constraints defined by particular hypotheses. The trees were tested against the 500 trees of highest likelihood found during the heuristic search for the best tree. Site likelihoods were calculated using PAUP*.

3. Results

The SSU rDNA sequences of the two *Chilomastix* species were rather different in length and base composition. The first BlastN hit for *C. wenrichi* was *Octomitus intestinalis* with an *E* value of 10^{-82}; the first BlastN hit for *C. mesnili* was an uncultured eukaryote (GenBank accession number AY392816) with an *E* value 10^{-103}. The sequences differed markedly in length. Whereas *Chilomastix wenrichi* had a short SSU rDNA sequence (1488 bp with primers; GC content 63%), the corresponding sequence from *C. mesnili* was rather long (ca 2500 bp; GC content 56%). There were considerable differences among particular *C. mesnili* SSU rDNA clones (uncorrected *p*-distance up to 1.6%), including up to 14 bp long indels, suggesting that several different paralogs of the SSU rRNA gene exist in the *C. mesnili* genome. Only minor differences were found between *C. wenrichi* SSU rDNA clones (up to 0.5%; no indels) and were probably due to Taq-polymerase errors.

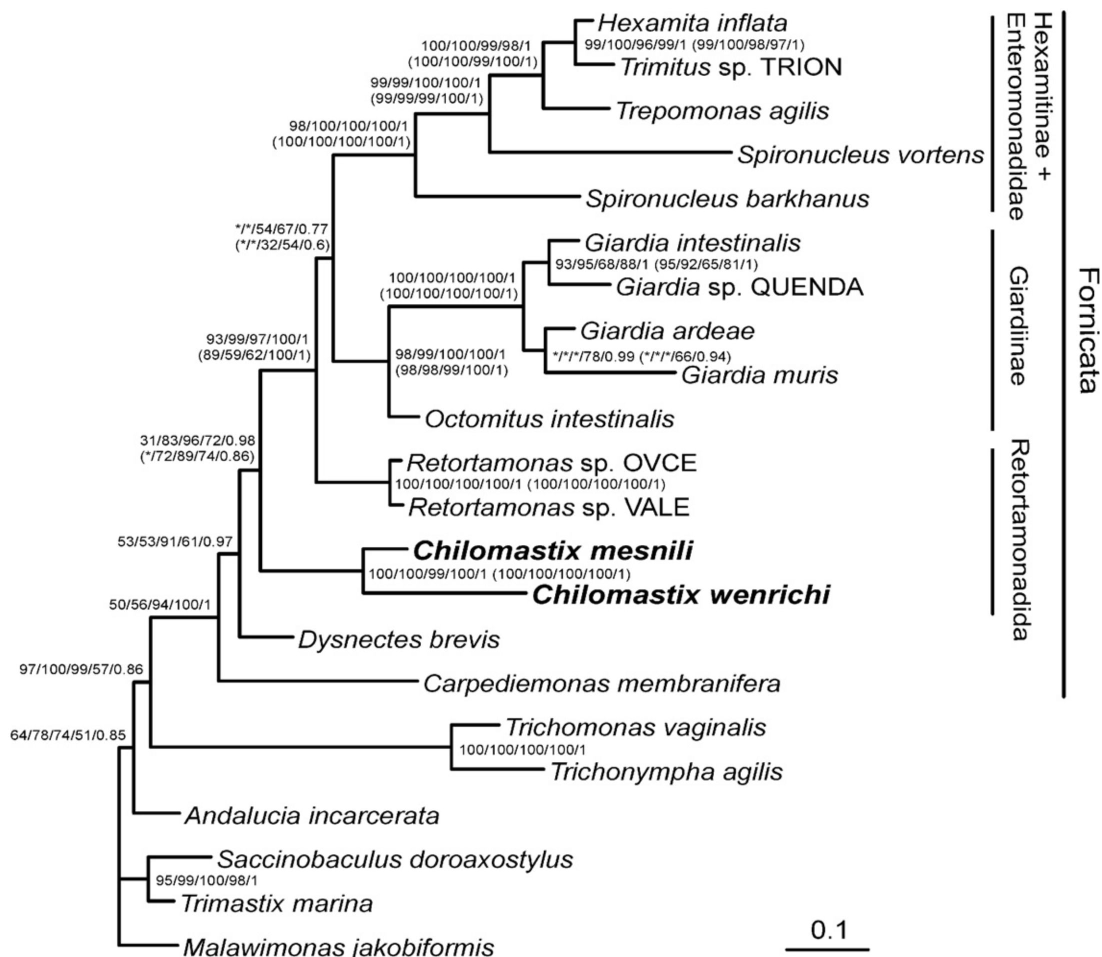

Fig. 1. Phylogenetic tree of Fornicata, rooted by other excavates, based on the SSU rRNA gene sequences. The tree was constructed from the second data set by the maximum likelihood method under TrNef + I + Γ model. Bootstrap values from maximum parsimony, Fitch-Margoliash method with Log Det distances, maximum likelihood distances, maximum likelihood, and Bayesian posterior probabilities are shown at the nodes, respectively. The corresponding values obtained by analyses of the third data set are shown in parentheses at the important nodes. Asterisks indicate nodes with a different topology resolved by the respective method.

Phylogenetic analysis of the first data set with a broad sampling of eukaryotic diversity showed monophyletic Fornicata (including *Chilomastix*) with low to high statistical support (bootstrap values for MP, LD, MLdist and ML were 51, 65, 88, and 100, respectively; Bayesian posterior probability 1; not shown), always with *Carpediemonas* forming basal branch. Therefore, detailed analysis of Fornicata was performed. A maximum likelihood tree based on the second data set is given in Fig. 1. Monophyletic Fornicata were recovered by all methods and were statistically strongly supported by MLdist and ML (bootstrap values 91 and 100, respectively) and the Bayesian analysis (posterior probability 1), but only weakly supported by the MP and LD analyses (bootstrap values 50 and 56, respectively). *Carpediemonas membranifera* (rather than *Dysnectes brevis*) formed the basal branch of Fornicata, though only the MLdist analysis supported the position well (bootstrap value 91), the support from the other methods being weak (bootstrap values 53–61, Bayesian posterior probability 0.97). A monophyletic Eopharyngia grouping was recovered by all methods and was well-supported by some of them (bootstrap values for MP, LD, MLdist, and ML were 31, 83, 96, and 72, respectively; Bayesian posterior probability 0.98).

Within Eopharyngia, the genus *Chilomastix* formed a clade that was strongly supported by all methods (bootstrap values 99–100, Bayesian posterior probability 1). Surprisingly, *Chilomastix* fell in a basal position within Eopharyngia, and support for this placement was strong with all methods (bootstrap values 93–100, Bayesian posterior probability 1). The rest of Eopharyngia formed three robust clades (bootstrap values always 98–100, Bayesian posterior probability 1), (i) *Retortamonas*, (ii) a Hexamitinae clade including enteromonads and (iii) Giardiinae. The relationships between the three clades, however, remained unclear and were not well supported by any method. In MP and LD analyses, *Retortamonas* and Giardiinae were sister groups (bootstrap values 42 and 80, respectively). By contrast, Giardiinae and Hexamitinae formed a common branch in MLdist, ML and Bayesian analyses (bootstrap values 54 and 67, respectively; Bayesian posterior probability 0.77).

Phylogenetic trees obtained from analyses of the third data set had the same topology as the trees from the second data set, except for the MP analysis, where a monophyletic Eopharyngia were not recovered (*Chilomastix* branched with *Dysnectes* instead of other Eopharyngia). In the LD, MLdist, ML and Bayesian analyses Eopharyngia were recovered with similar support (bootstrap values 72, 89 and 74, respectively; Bayesian posterior probability 0.86) as in the analyses of the first data set. The monophyly of the genus *Chilomastix* was again highly supported (bootstrap values 100, Bayesian posterior probability 1), and *Chilomastix* always formed the deepest branch within Eopharyngia (bootstrap values for the clade of *Retortamonas* and diplomonads were for MP, LD, MLdist and ML, 89, 59, 62 and 100, respectively; Bayesian posterior probability 1). Giardiinae, Hexamitinae and *Retortamonas* were always recovered as clades and were well supported (bootstrap values 98–100, Bayesian posterior probabilities 1). As in the case of the second data set, *Retortamonas* and Giardiinae clades formed a common branch in the MP and LD analyses (bootstrap values 55 and 35, respectively), while Giardiinae and Hexamitinae formed a common branch in the MLdist, ML and Bayesian analyses (bootstrap values 32 and 54, respectively; Bayesian posterior probability 0.6).

In a likelihood framework, three alternative hypotheses concerning the placement of the genus *Chilomastix* were evaluated using AU tests (the first hypothesis was tested using both second and third data set, while the remaining two hypotheses were tested using only the second data set). The first hypothesis was that Retortamonadida is monophyletic, i.e. *Retortamonas* and *Chilomastix* are clade. This hypothesis was rejected at the 1% confidence level for both data sets (p-values 0.004 and 2×10^{-7}, respectively). The second hypothesis was that *Chilomastix* forms the basal branch of Fornicata. This hypothesis could not be rejected ($p = 0.738$). The third hypothesis was that *Chilomastix* branches more basally than Parabasala which are sister to remaining fornicates. This hypothesis was rejected at the 5% confidence level ($p = 0.02$).

Topologies of trees constructed from the fourth data set were ill-resolved due to a low amount of data (the alignment consisted of 177 characters) and differed according to the particular method. However, *Chilomastix mesnili* always formed a robust clade (bootstrap values 98–100, Bayesian posterior probability 1) with the eukaryotes obtained from fin whale feces (not shown).

4. Discussion

The evolution of Fornicata, one of recently recognized major eukaryotic groups (Simpson et al., 2002; Simpson, 2003; Adl et al., 2005), is poorly understood and the relationships among fornicate taxa are still poorly resolved. Previous hypotheses concerning the evolution of retortamonads and diplomonads have been based solely on ultrastructural data (Brugerolle, 1973, 1977, 1991; Kulda and Nohýnková, 1978; Simpson and Patterson, 1999) or, when including molecular data, consider only a single retortamonad genus, *Retortamonas* (Silberman et al., 2002; Kolisko et al., 2005; Keeling and Brugerolle, 2006; Yubuki et al., 2007). These studies have always assumed (or recovered) the monophyly of Retortamonadida on the basis of the strikingly similar cell structure of *Retortamonas* and *Chilomastix*. Simpson and Patterson (1999) defined exclusive retortamonadid morphological synapomorphies not shared with other eukaryotes including, most importantly, diplomonads. Results of the present study are, however, in considerable disagreement with the anticipated models of evolution of Fornicata.

In our analyses, Fornicata is split into four branches: *Carpediemonas membranifera*, *Dysnectes brevis*, *Chilomastix* spp., and *Retortamonas* spp. + Diplomonadida. The close relationship of *Retortamonas* and diplomonads is consistent with previous studies, as is the weak resolution of the position of *Retortamonas* within this clade (Silberman et al., 2002; Simpson et al., 2002; Kolisko et al., 2005; Keeling and Brugerolle, 2006; Yubuki et al., 2007). The hypothesis of a monophyletic Diplomonadida forming the sister branch to *Retortamonas* is favored by morphological data and by analyses of HSP90 gene (Kolisko et al., in press). Interestingly, *Chilomastix* forms a sister branch to the clade of *Retortamonas* + diplomonads instead of branching with *Retortamonas* only. The basal position of the genus *Chilomastix* in Eopharyngia was robustly supported by all examined methods of tree reconstruction, and a monophyletic Retortamonadida was rejected by AU tests. Although we could not rule out the possibility that *Chilomastix* branches even more basally than *Carpediemonas* and *Dysnectes*, we support the hypothesis of a monophyletic Eopharyngia as it was preferred by most phylogenetic methods (only the maximum parsimony analysis of one of two full-length datasets recovered an alternative optimal tree) and conforms to previous hypotheses. However, morphological synapomorphies of Eopharyngia have not been defined so far (see Simpson, 2003). As independent evolution of the distinctive retortamonadid morphology in ancestors of *Retortamonas* and *Chilomastix* would seem rather improbable, we interpret our topology as suggesting that Retortamonadida are paraphyletic rather than polyphyletic.

Our results allow us to propose a new scenario of the evolution of Eopharyngia. If Diplomonadida truly form an internal branch of Retortamonadida, they must have once possessed complete set of retortamonadid (and also excavate) features. These characters would have been lost during the early evolution of Diplomonadida, and diplozoic cells appeared. The groove-like cytostomes of Retortamonadida changed to tube-shaped ones in some

phagotrophic Hexamitinae or have been lost altogether in the pinocytotic Giardiinae. Two microtubular fibres surrounding the nuclei, the infra- and supranuclear fibres, were formed either *de novo* or by modification of existing retortamonadid microtubular structures, possibly in connection to the loss of the microtubular corset. The supranuclear fibre was putatively homologized by Simpson (2003) with the R4 (or anterior) root of some excavate taxa, including the fornicates *Carpediemonas* and *Dysnectes*. Interestingly, neither *Retortamonas* nor *Chilomastix* possesses an R4. We therefore assume that the supranuclear fibre could be either a novel structure or a remnant of the subpelicular corset supporting the dorsal side of each nucleus, rather than a homolog of R4.

Our new hypothesis on the phylogeny and evolution of Eopharyngia is based on single-gene analyses. To rule out the possibility that the paraphyly of Retortamonadida is an artifact, perhaps caused by the divergent nature of their SSU rDNA sequences, analyses of more genes must be performed in the future. However, only SSU rDNA sequences have been published so far for *Dysnectes* and *Chilomastix* (Yubuki et al., 2007; this paper). The taxon sampling of the molecular-phylogenetic analyses is also still poor, in particular the sampling of Retortamonadida. Sequences for only two apparently closely related *Retortamonas* species are available to date. The situation is further complicated by the fact that previous TEM studies were performed on *Retortamonas* species from insects (Brugerolle, 1977, 2006) while molecular studies have examined only *Retortamonas* spp. from vertebrates (Silberman et al., 2002). According to our TEM studies (Kulda et al., unpublished), *Retortamonas* spp. from vertebrates differ considerably from those from insects by the absence of the subpellicular microtubular corset (an extended version of the dorsal fan of other typical excavates), which we regard as a very important structure in evolution of Eopharyngia. The possibility that they represent, in fact, two different evolutionary lineages should be investigated and new phylogenetic studies based both on morphological and phylogenetic data should be performed. A convincing reconstruction of eopharyngian phylogeny can be obtained only by a multi-gene study. It is clear, however, that before such a study can be conducted, it will be of crucial importance to improve the taxonomic sampling of this still enigmatic taxon.

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