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Phylogeny and classification of the Litostomatea (Protista, Ciliophora), with emphasis on free-living taxa and the 18S rRNA gene

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ABSTRACT

The class Litostomatea is a highly diverse ciliate taxon comprising hundreds of species ranging from aerobic, free-living predators to anaerobic endocommensals. This is traditionally reflected by classifying the Litostomatea into the subclasses Haptoria and Trichostomatia. The morphological classifications of the Haptoria conflict with the molecular phylogenies, which indicate polyphyly and numerous homoplasies. Thus, we analyzed the genealogy of 53 in-group species with morphological and molecular methods, including 12 new sequences from free-living taxa. The phylogenetic analyses and some strong morphological traits show: (i) body polarization and simplification of the oral apparatus as main evolutionary trends in the Litostomatea and (ii) three distinct lineages (subclasses): the Rhynchostomatia comprising Tracheliida and Dileptida; the Haptoria comprising Lacrymariida, Haptorida, Didiniida, Pleurostomatida and Spathidiida; and the Trichostomatia. The curious *Homalozoon* cannot be assigned to any of the haptorian orders, but is basal to a clade containing the Didiniida and Pleurostomatida. The internal relationships of the Spathidiida remain obscure because many of them and some "traditional" haptorids form separate branches within the basal polytomy of the order, indicating one or several radiations and convergent evolution. Due to the high divergence in the 18S rRNA gene, the chaeneids and cyclotrichiids are classified *incertae sedis*.

1. Introduction

The ciliate class Litostomatea Small and Lynn, 1981 represents a very diverse taxon in terms of body size and shape, somatic ciliary patterns, oral structures, and life histories (Lynn, 2008). The size ranges from about 30×15 μm in *Pseudoholophrya minuta* to 2500×200 μm in *Homalozoon vermiculare*. Likewise, shape varies conspicuously (e.g., bursiform, cylindroidal, vermiform, clavate, axe-shaped, lanceolate, spatulate), as does the ratio of body length to body width spanning a range of about 1:1–30:1. Many litostomateans display bizarre morphologies, such as an extensible "neck" (e.g., *Lacrymaria*), an agile proboscis (e.g., *Dileptus*), toxicyst-bearing tentacles (e.g., *Actinobolina*), and a variety of body lobes and spines (e.g., *Ophryoscolex*). The somatic and oral ciliature are no less diverse. For instance, the ciliary rows extend meridionally to spirally and the cilia may form isolated bands and tufts. As concerns life history, many litostomateans are free-living, voracious predators of other protists or small metazoans such as rotifers and nematodes, while others are endocommensals or parasites in vertebrates, ranging from fish to reptiles and mammals. In spite of this morphological and ecological diversification, Litostomatea has been consistently viewed as a monophylum in both molecular and morphological phylogenies (Foissner and Foissner, 1988; Gao et al., 2008; Leipe et al., 1994; Lipscomb and Riordan, 1990, 1992; Small and Lynn, 1981; Strüder-Kypke et al., 2006, 2007; Vd'ačný et al., 2010, submitted for publication; Wright and Lynn, 1997a,b; Wright et al., 1997).

The monophyletic origin of the Litostomatea is supported by five strong apomorphies: (1) the somatic kinetids are single basal bodies with a convergent postciliary microtubule ribbon, a short kinetodesmal fiber, and two transverse microtubule ribbons (Leipe et al., 1992; Small and Lynn, 1981); (2) the cytopharynx is of the rhabdos type, i.e., it is lined by transverse microtubule ribbons (Foissner and Foissner, 1985, 1988; Grain, 1966a,b); (3) the cilia of at least one somatic kinety are differentiated to clavate bristles, forming the so-called dorsal brush or "clavate field" (Foissner, 1996; Lynn, 2008); (4) the stomatogenesis is holotelokinetal, except for pleurostomatids and trichostomatids where it is monotelokinetal and cryptotelokinetal, respectively (Cameron and O'Donoghue, 2001; Foissner, 1996; Fryd-Versavel et al., 1975);

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and (5) the micronucleus conspicuously increases in size during the first maturation division, and conjugation is heteropolar, except for the pleurostomatid haptorians in which it is homopolar (Raikov, 1972; Vd'ačný and Foissner, 2008; Xu and Foissner, 2004).

Recently, significant progress has been made in understanding the deep phylogeny of the litostomateans. The class Armophorea was found to be sister of the Litostomatea both forming the infraphylum Lamellicorticata (Vd'ačný et al., 2010). A resolution at the base of the Litostomatea recognized three main lineages: Rhynchostomatia, Haptoria, and Trichostomatia (Vd'ačný et al., submitted for publication). In contrast, the phylogeny of the haptorian orders and families remained a difficult enterprise for both morphologists and molecular taxonomists. The Haptoria always appeared polyphyletic and their relationships remained unclear (e.g., Gao et al., 2008; Pan et al., 2010; Strüder-Kypke et al., 2006, 2007; Vd'ačný et al., 2010, submitted for publication). Likewise, phylogenies based on morphological characteristics failed to produce a consistent topology (Corliss, 1974; Foissner and Foissner, 1988; Grain, 1994; Jankowski, 2007; Lipscomb and Riordan, 1990, 1992; Lynn and Small, 2002; Fig. 1).

For better understanding of the haptorian evolution, we: (i) sequenced the 18S rRNA gene of 12 free-living Litostomatea, (ii) de-

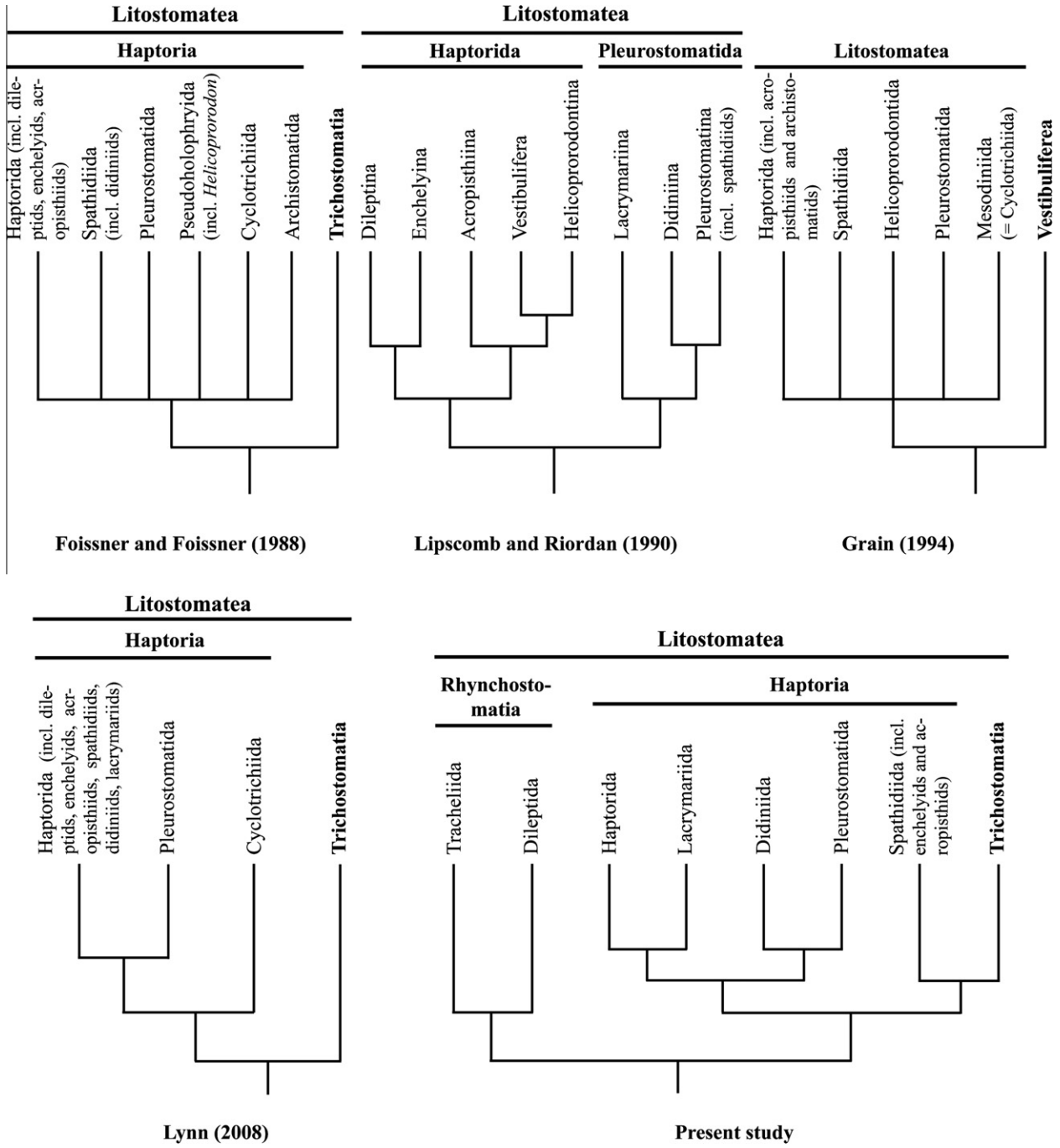

Fig. 1. Five classifications of the Litostomatea. Names of subclasses and classes in bold. Foissner and Foissner (1988) used ultrastructure and details of the oral and somatic infraciliature. The system of Lipscomb and Riordan (1990) was based on a cladistic analysis of 46 ultrastructural and morphological characters. Both Grain (1994) and Lynn (2008) based their systems on ultrastructural and morphological data. Grain (1994) raised the subclasses to classes, calling them Litostomatea (=Haptoria) and Vestibuliferea (=Trichostomatia). In the present study, we propose a refined system using 18S rRNA gene sequences and morphological characteristics.

Table 1
Characterization of new 18S rRNA gene sequences of 12 litostomatean ciliates (arranged alphabetically).

Taxa	Collection site	Culture conditions[a]	No. of cells picked	Sequence length (nt)	No. of clones sequenced	Average pairwise distance between clones (%)	GC content (%)
Apobryophyllum schmidingeri Foissner and Al-Rasheid, 2007	Germany, terrestrial mosses	NFP	30	1640	22	0.21 (0.00–0.50)	43.0
Arcuospathidium namibiense tristicha Foissner et al., 2002	Germany, terrestrial mosses	NFP	15	1639	3	0.40 (0.30–0.50)	43.1
Arcuospathidium sp.[b]	Australia, leaf litter	NFP	70	1639	21	0.30 (0.00–0.60)	43.1
Balantidion pellucidum Eberhard, 1862[c]	USA, Idaho, Boise, garden water tank	ES	20	1634	–	–	43.3
Cultellothrix lionotiformis (Kahl, 1930) Foissner, 2003[c]	Finland, Pyhä-Luosto NP, forest soil	NFP	20	1638	–	–	43.3
Enchelyodon sp. 2[b]	USA, Idaho, Boise, floodplain soil	NFP	50	1641	18	0.33 (0.10–0.70)	43.4
Enchelys gasterosteus Kahl, 1926	Jamaica, bromeliad tank	ES	10	1636	21	0.25 (0.00–0.50)	43.3
Fuscheria sp.[b,c]	USA, Idaho, Boise, ephemeral puddle	ES	20	1638	–	–	41.9
Protospathidium muscicola Dragesco and Dragesco-Kernéis, 1979	Botswana, floodplain soil	NFP	35	1639	13	0.22 (0.00–0.40)	43.3
Semispathidium sp.[b]	South Africa, Krüger NP, floodplain soil	NFP	18	1641	20	0.24 (0.00–0.50)	43.4
Spathidium sp.[b]	USA, Idaho, Boise, floodplain soil	NFP	8	1641	20	0.20 (0.00–0.40)	43.3
Trachelophyllum sp.[b]	USA, Idaho, Boise, floodplain soil	NFP	20	1641	23	0.28 (0.00–0.60)	43.3

[a] ES – environmental sample, NFP – non-flooded Petri dish culture.
[b] These species are new and their descriptions are in preparation.
[c] PCR products sequenced directly.

scribe the haptorian lineages and homoplasies, and (iii) discuss the morphological apomorphies of the Litostomatea and Haptoria. This resulted in a refined classification of the Litostomatea.

2. Material and methods

2.1. Collection and sample processing

Twelve free-living litostomateans from the orders Haptorida and Spathidiida were sampled mainly in terrestrial and semi-terrestrial habitats around the world (Table 1). Most species sequenced were cultivated, using the non-flooded Petri dish method described in Foissner et al. (2002). There are three exceptions: *Balantidion pellucidum* was isolated in a garden water tank in the town of Boise, Idaho, USA; *Fuscheria* sp. was found in a temporary rainwater puddle from the surroundings of Boise; and *Enchelys gasterosteus* occurred in a bromeliad tank from Jamaica. Species were identified using live observation, protargol impregnation, and SEM (Foissner, 1991). Half of the species studied represent new taxa whose descriptions are in preparation. Eight to seventy cells were picked with a micropipette, washed at least twice in sterile spring water to remove contaminants, and transferred into 180 μl ATL buffer (Qiagen, Hildesheim, Germany). Samples were stored at +1 to +3 °C pending DNA extraction.

2.2. DNA extraction, PCR amplification, and molecular cloning

Prior to DNA extraction, Proteinase K 20 μl (20 mg/ml; Qiagen, Valencia, CA, USA) was added and the samples were incubated at 56 °C for 1 h. Genomic DNA of nine species was extracted using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA), while that of *B. pellucidum*, *Cultellothrix lionotiformis*, and *Fuscheria* sp. was isolated with the modified chelex protocol (Strüder-Kypke and Lynn, 2003). The 18S rRNA gene was PCR-amplified using the universal forward and reverse eukaryotic primers EukA and EukB (Medlin et al., 1988). The amplification reaction contained 10–20 ng of DNA template, 2.5 U HotStar Taq DNA polymerase (Qiagen, Valencia, CA, USA), 200 μM of dNTP, and 0.5 μM of each oligonucleotide primer. The final volume was adjusted to 50 μl with sterile distilled water. PCR conditions were as follows: initial hot start denaturation at 95 °C for 15 min, 30 identical amplification cycles (denaturing at 95 °C for 45 s, annealing at 55 °C for 1 min, and extension at 72 °C for 2.5 min), and final extension at 72 °C for 10 min. To check the quality of the amplified DNA, PCR products were run on a 1% agarose gel. The resulting PCR products were cloned into the vector plasmid pCR 2.1 using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA, USA). Plasmids were sequenced bi-directionally using M13 forward and reverse primers supplied with the kit and the internal primer Euk528F (Elwood et al., 1985) at Beckman Coulter Genomics (Danvers, MA, USA) to obtain the full-length 18S rRNA gene sequences. The 18S rRNA gene PCR products for *B. pellucidum*, *C. lionotiformis*, and *Fuscheria* sp. were directly sequenced at Sequetech Corporation (Mountainview, CA, USA), using the amplification primers and two internal primers.

2.3. Sequence processing and alignments

The sequence fragments were imported into Chromas ver. 2.33 (Technelysium Pty Ltd.) to check for data quality and trim the 5′ and 3′ ends. Trimmed sequences were assembled into contigs using BioEdit (Hall, 1999). The consensus sequences, based on sequences from 3 to 23 clones (Table 1), were created in BioEdit with an inclusion threshold frequency of 90% identity. These consensus sequences were subsequently aligned to litostomatean 18S rRNA sequences available in the ARB-package (Ludwig et al., 2004). The alignment was manually corrected according to the secondary structural features of the 18S rRNA molecule. Ambiguously aligned and hyper variable regions were masked, using a sequence alignment filter created for the alignment in ARB.

2.4. Phylogenetic analyses

To determine the phylogenetic positions of the twelve newly sequenced haptorids and spathidiids, we analyzed an 18S rRNA gene sequence alignment containing 1408 unambiguously aligned nucleotide characters of 49 representative rhynchostomatian, haptorian, and trichostomatian taxa (Table 2). The program Modeltest (Posada and Crandall, 1998) was used to determine the best fit model of nucleotide substitution under the Akaike Information Criterion (AIC). The best fit model was the Second Transition Model with invariable sites and gamma distribution (TIM2 + I + Γ) with the following parameter values: gamma distribution shape param-

Table 2
List of ciliate species with GenBank accession numbers of corresponding 18S rRNA gene sequences included in the phylogenetic analyses. Sequences obtained during this study are in bold.

Species name	GB number	Species name	GB number	Species name	GB number
Amphileptus aeschtae	EU242510	*Enchelyodon* sp. 1	U80313	*Ophryoscolex purkynjei*	U57768
Amphileptus procerus	AY102175	***Enchelyodon* sp. 2**	JF263446	*Pelagodileptus trachelioides*	AB558117
Amylovorax dehorityi	AF298817	***Enchelys gasterosteus***	JF263447	*Phialina salinarum*	EU242508
Apobryophyllum schmidingeri	JF263441	*Enchelys polynucleata*	DQ411861	***Protospathidium muscicola***	JF263449
Arcuospathidium cultriforme	DQ411860	*Entodinium caudatum*	U57765	*Pseudoamphileptus macrostoma*	AY102173
Arcuospathidium muscorum	DQ411859	*Epispathidium papilliferum*	DQ411857	*Pseudomonilicaryon fraterculum*	HM581677
Arcuospathidium namibiense tristicha	JF263442	*Eudiplodinium maggii*	U57766	*Rimaleptus mucronatus*	HM581675
***Arcuospathidium* sp.**	JF263443	***Fuscheria* sp.**	JF263448	***Semispathidium* sp.**	JF263450
Balantidion pellucidum	JF263444	*Homalozoon vermiculare*	L26447	*Siroloxophyllum utricullariae*	L26448
Balantidium coli	AF029763	*Isotricha intestinalis*	U57770	*Spathidium* sp. 1	Z22931
Bandia cribbi	AF298824	*Lacrymaria marina*	DQ777746	***Spathidium* sp. 2**	JF263451
Bitricha tasmaniensis	AF298821	*Litonotus paracygnus*	EU242509	*Spathidium stammeri*	DQ411862
Cultellothrix lionotiformis	JF263445	*Loxophyllum jinni*	EF123708	*Teuthophrys trisulca africana*	DQ411863
Dasytricha ruminantium	U57769	*Loxophyllum rostratum*	DQ190465	*Trachelius ovum*	HM581673
Didinium nasutum	U57771	*Macropodinium yalabense*	AF042486	***Trachelophyllum* sp.**	JF263452
Dileptus sp.	DQ487195	*Monodinium* sp.	DQ487196		
Diplodinium dentatum	U57764	*Monomacrocaryon terrenus*	HM581674		

Table 3
Log likelihoods and P-values of AU (approximately unbiased), SH (Shimodaira–Hasegawa), and WKH (weighted Kishino–Hasegawa) tests for tree comparisons considering different topological scenarios. Significant differences (P-value < 0.05) between the best unconstrained and constrained topologies are in bold.

Topology	Log likelihood (–ln L)	Difference to best tree (–ln L)	AU	SH	WKH	Conclusion
Best maximum likelihood tree (unconstrained)	5906.2455	–	0.724	0.948	0.578	–
Monophyly of *Homalozoon*, didiniids and pleurostomatids	5923.7895	17.54	0.196	0.593	0.145	Not rejected
Sister relationship of *Homalozoon* and pleurostomatids	5914.1958	7.95	0.227	0.825	0.106	Not rejected
Sister relationship of didiniids and pleurostomatids	5918.3176	12.07	0.338	0.738	0.221	Not rejected
Didiniids belong to the order Haptorida	5934.4408	28.19	**0.010**	0.312	**0.015**	Rejected
Didiniids belong to the order Spathidiida	5969.6132	63.37	**1e–004**	**0.021**	**0.008**	Rejected
Homalozoon belongs to the order Spathidiida	5972.5596	66.31	**0.003**	**0.024**	**0.015**	Rejected
Monophyly of lacrymariids and haptorids *sensu stricto*	5929.6651	23.42	**0.028**	0.428	**0.039**	Rejected
Monophyly of lacrymariids and pleurostomatids	5914.7489	8.50	0.160	0.805	0.106	Not rejected
Monophyly of haptorids *sensu stricto* and "traditional" haptorids	5975.5102	69.26	**3e–009**	**0.007**	**0.002**	Rejected
Monophyly of spathidiids, "traditional" haptorids and trichostomatians	5907.0875	0.84	0.618	0.932	0.422	Not rejected
Monophyly of spathidiids and "traditional" haptorids	5915.0885	8.84	0.385	0.901	0.201	Not rejected
Monophyly of spathidiids and haptorids *sensu stricto*	5971.5885	65.34	**0.003**	**0.012**	**0.007**	Rejected
Sister relationship of spathidiids and trichostomatids	5959.2886	53.04	**0.002**	**0.029**	**0.012**	Rejected
Monophyly of trichostomatids and free-living haptorians with oralized somatic monokinetids	6049.5997	143.35	**0.001**	**0.000**	**0.000**	Rejected
Monophyly of the genus *Arcuospathidium*	5925.9314	19.69	0.149	0.559	0.115	Not rejected
Monophyly of the genus *Spathidium*	5931.3853	25.14	**0.021**	0.384	**0.040**	Rejected
Monophyly of the genus *Enchelys*	5958.6282	52.38	**0.009**	**0.019**	**0.005**	Rejected
Monophyly of the genus *Enchelyodon*	6024.2720	118.03	**6e–041**	**0.000**	**0.000**	Rejected

eter $\Gamma = 0.6200$; proportion of invariable sites I = 0.6380; base frequencies A = 0.3008, C = 0.1660, G = 0.2492, T = 0.2840; and rate matrix for the substitution model [AC] = 2.1350, [AG] = 4.9465, [AT] = 2.1350, [CG] = 1.000, [CT] = 6.1471, and [GT] = 1.0000. A Bayesian inference (BI) tree was computed in MrBayes (Ronquist and Huelsenbeck, 2003), using the model suggested by Modeltest and the Markov Chain Monte Carlo (MCMC) algorithm. The chain length was 5000,000 generations with trees sampled every 1000 generations. The first 25% of trees were considered burn-in trees and were discarded prior tree reconstruction. A 50% majority rule consensus of the remaining trees was used to calculate posterior probabilities (PP) of the branching pattern. The maximum likelihood (ML) analysis was computed on the CIPRES Portal V 1.15 (http://www.phylo.org), using RAxML with settings as described in Stamatakis et al. (2008). The neighbor-joining (NJ) tree was constructed using PAUP* ver. 4.0b8 with randomly added species and tree bisection-reconnection (TBR) branch-swapping algorithm in effect (Swofford, 2003) under ML distance with settings as suggested by Modeltest. The reliability of the ML and NJ trees was tested by the bootstrap approach, using 1000 pseudoreplicates and a heuristic search algorithm. Support values from all tree building methods were annotated onto the tree with the best log-likelihood score which was chosen for presentation.

2.5. Test of hypotheses

In addition to the best ML tree, 16 unrooted trees with enforced topological constraints (Table 3) were built in PAUP*, using ML criterion and heuristic search with TBR and 10 random sequence addition replicates. The site-wise likelihoods for the best unconstrained ML tree and all constrained trees were calculated in PAUP* under the TIM2 + I + Γ model with parameters as suggested by Modeltest (see above). The reliability of the constrained trees was analyzed in likelihood frameworks through the approximately unbiased test (AU), the Shimodaira–Hasegawa test (SH), and the weighted Kishino–Hasegawa test (WKH) implemented in the CONSEL software package (Shimodaira and Hasegawa, 2001).

3. Results

3.1. Small subunit rRNA gene sequences

The complete 18S rRNA gene of twelve free-living litostomatean ciliates is on average only 1639 (1634–1641) nucleotides long and has a GC content of about 43% (Table 1), as usual for litostomatean sequences. Further, all sequences show the litostomatean deletions in the helices 23–1, 23–8, 23–9, and deletion of the entire helix 23–5 (Leipe et al., 1994; Strüder-Kypke et al., 2006; Vd'ačný et al., submitted for publication; Wright and Lynn, 1997a,b; Wright et al., 1997). The level of intraspecies sequence variation is relatively low with an average of 0.27% (Table 1). We assume that it results from intraspecific variation, with a possible addition of sequencing errors.

3.2. Molecular phylogenetics

To determine the phylogenetic positions of the twelve newly sequenced litostomateans and to reconstruct the evolutionary history of the class Litostomatea, we carried out three phylogenetic analyses based on 1408 unambiguously aligned nucleotide characters of 53 in-group species. Due to mutational saturation, cyclotrichiid (*Mesodinium pulex* and *Myrionecta rubra*) and some haptorid (*Chaenea teres* and *C. vorax*) sequences were not included in the final analyses, as they caused long branch artefacts and poor

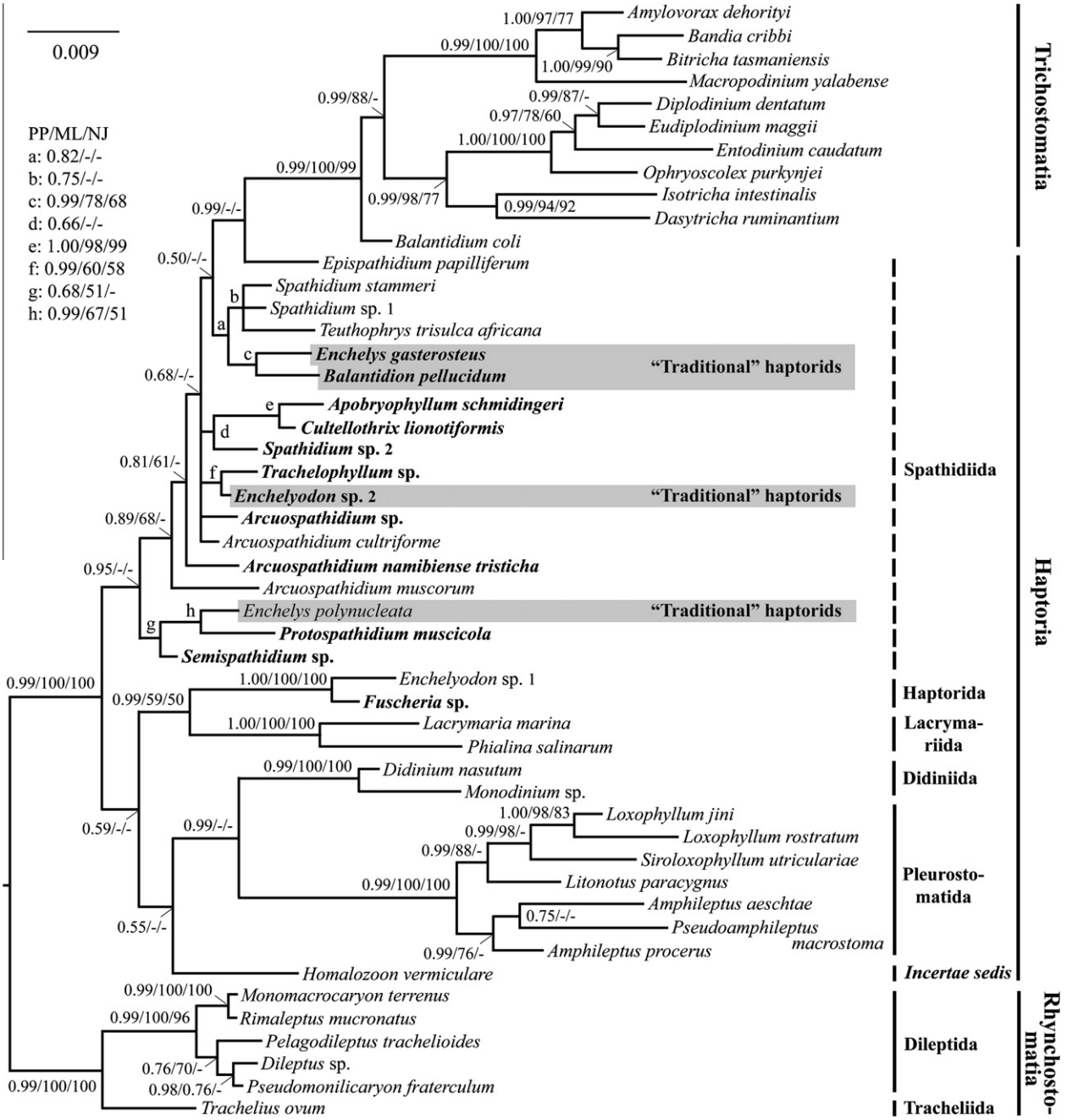

Fig. 2. Small subunit rRNA gene phylogeny based on 1408 unambiguously aligned nucleotide characters of 49 taxa from the class Litostomatea. Three methods (Bayesian inference, maximum likelihood, and neighbor-joining) were used for tree construction. Posterior probabilities (PP) for the Bayesian inference and bootstrap values for the maximum likelihood (ML) and neighbor-joining (NJ) analyses are shown at nodes (a dash indicates values below 0.50% or 50%, respectively). Sequences in bold were obtained during this study. The paraphyletic spathidiids are indicated by a dashed line. The scale bar indicates the fraction of substitutions per site.

resolution of the other haptorian sequences. Cyclotrichiids and chaeneids formed well-supported clades that diverged by approximately 38% and 18%, respectively, from other litostomateans (data not shown). On the other hand, their affiliation with haptorians is indicated by the typical litostomatean deletions in the 18S rRNA gene (see above).

Within the Litostomatea, all phylogenetic analyses consistently strongly support two lineages designated as Rhynchostomatia and Haptoria including the endocommensal Trichostomatia (Fig. 2). The subclass Rhynchostomatia receives strong support from all three methods (0.99 PP, 100% ML and 100% NJ) and its internal relationships are rather well resolved. In all analyses, *Trachelius ovum* is placed basal to the other rhynchostomatians representing a separate branch, the order Tracheliida. All other rhynchostomatians form a monophylum, the order Dileptida, supported by a posterior probability of 0.99 as well as by 100% ML and 96% NJ bootstrap values. Within the order Dileptida, there are two distinct clades designated as family Dimacrocaryonidae (0.99 PP, 100% ML, 100% NJ) and family Dileptidae (0.76 PP, 70% ML).

Although the subclass Haptoria gains very strong to full support from the three phylogenetic analyses (0.99 PP, 100% ML, 100% NJ), the relationships at the base of the subclass can hardly be reconstructed. Bayesian inference and maximum likelihood analyses depict two super-clades both receiving, however, very low nodal support (Fig. 2).

The first super-clade (0.59 PP, 16% ML) comprises "classical" haptorians which form two clades (Fig. 2): the Haptorida–Lacrymariida clade and the *Homalozoon*–Didiniida–Pleurostomatida clade. The sister relationship between the order Lacrymariida and two members of the order Haptorida is strongly supported by a posterior probability of 0.99, but only poorly supported by 59% ML and 50% NJ bootstrap values (Fig. 2). Nevertheless, this sister relationship is consistently rejected by the AU, SH and WKH tests at the conservative significance level of 0.01 (Table 3). The polyphyly of the order Haptorida *sensu* Foissner and Foissner (1988) is clearly shown in all molecular trees and sustained by all topology tests (Table 3). Specifically, only two species (*Enchelyodon* sp. 1 and *Fuscheria* sp.), out of six morphologically classified as haptorids, cluster close to other "classical haptorids", while four species (*B. pellucidum*, *Enchelyodon* sp. 2, *Enchelys gasterosteus*, and *E. polynucleata*) branch within the spathidiid clade, indicating convergent evolution of haptorids and spathidiids or retention of the plesiomorphic condition of the subclass Haptoria. The order Lacrymariida is fully supported by all methods.

The second haptorian clade unites *Homalozoon vermiculare*, didiniids and pleurostomatids (Fig. 2). There is strong Bayesian support for this clade (0.99 PP), but only very poor support from the maximum likelihood analysis (18% ML). On the other hand, the monophyletic origin of this clade is not rejected by the AU, SH, and WKH tests (Table 3). *Homalozoon* cannot be assigned to either didiniids or pleurostomatids, forming a separate branch basal to both orders. The traditional classification of *Homalozoon* within the order Spathidiida is rejected by the three topology tests even at the less conservative significance level of 0.05 (Table 3). Thus, our molecular phylogenies suggest that *Homalozoon* represents a monotypic order with long, independent evolution. The order Didiniida clusters as sister to the order Pleurostomatida with strong support from Bayesian inference (0.99 PP), but with very poor support from maximum likelihood analysis (39% ML). This relationship is not rejected by any of the statistical tests applied, whereas the previous classifications of didiniids among haptorids or spathidiids are firmly excluded (Table 3).

The monophyly of the order Didiniida is strongly supported by a posterior probability of 0.99 as well as by 100% ML and 100% NJ bootstrap values. Likewise, the order Pleurostomatida is depicted as a monophyletic group with high posterior probability (0.99 PP) and full support from maximum likelihood and distance methods (100% ML, 100% NJ). Two distinct clades can be recognized within the order Pleurostomatida (Fig. 2). The first clade represents the family Litonotidae and includes *Litonotus paracygnus* which is placed basal to *Siroloxophyllum utriculariae* and the cluster of two *Loxophyllum* species. This family is strongly to moderately supported by a posterior probability of 0.99 and by 88% ML bootstrap. The second pleurostomatid clade is the family Amphileptidae uniting *Amphileptus procerus*, *A. aeschtae*, and *Pseudoamphileptus macrostoma*. Although the monophyly of this family is strongly (0.99 PP) to moderately (76% ML) supported by two methods, the phylogenetic relationships among amphileptids are rather poorly resolved (Fig. 2).

The second haptorian super-clade includes spathidiids, several "traditional" haptorids, and all trichostomatians (Fig. 2). This super-clade obtains strong support from Bayesian inference (0.95 PP), but only poor support from maximum likelihood analysis (29% ML). The monophyly is not rejected by any of the topology tests (Table 3). The order Spathidiida is paraphyletic in all analyses and comprises spathidiids and several "traditional" haptorids, whose relationships are poorly resolved, as there are many polytomies. Short internodes and low support between the spathidiid lineages indicate a rapid radiation event at the base of the order. The genera *Spathidium*, *Enchelys* and *Enchelyodon* are also polyphyletic in both molecular trees and topology tests (Table 3 and Fig. 2); the genus *Arcuospathidium* is polyphyletic in all trees (Fig. 2), but monophyly is not rejected by any statistical test (Table 3). The genus *Trachelophyllum*, which is outstanding in having the body covered with epicortical scales, falls within the spathidiid radiation, clustering together with the "traditional" haptorid *Enchelyodon* sp. 2 (0.99 PP, 60% ML, 58% NJ).

The subclass Trichostomatia is monophyletic with very strong to full support from all three methods (0.99 PP, 100% ML, 99% NJ). The trichostomatians branch rather deeply within the order Spathidiida, where they cluster together with the aerobic, free-living *Epispathidium papilliferum* in the Bayesian (0.99 PP) and maximum likelihood (48% ML) analyses (Fig. 2). However, they form a separate lineage within the basal polytomy of the Haptoria in the NJ analysis (data not shown). All topology tests consistently sustain the paraphyletic placement of the trichostomatians within the order Spathidiida and consistently reject placement outside the spathidiid clade (i.e., as sister to the spathidiids) at a conservative significance level of 0.01 (Table 3). Further, all topology tests firmly exclude a monophyly of trichostomatians and haptorians with oralized somatic monokinetids (Table 3). *Balantidium coli* is placed basal to all other trichostomatians which are classified into three distinct groups, viz., the order Macropodiniida (e.g., *Amylovorax dehorityi*, *Bandia cribbi*, *Bitricha tasmaniensis*, and *Macropodinium yalabense*), the order Entodiniomorphida (e.g., *Diplodinium dentatum*, *Entodinium caudatum*, *Eudiplodinium maggii*, and *Ophryoscolex purkynjei*), and the order Vestibuliferida (e.g., *Isotricha intestinalis* and *Dasytricha ruminantium*).

3.3. Homoplasies in the class Litostomatea

Our molecular and comparative analyses show that the evolutionary history of the litostomateans is full of homoplasies, making reconstruction of phylogeny extremely difficult. We recognized at least six features, some considered as phylogenetically highly informative in the past, which evolved or were lost convergently in genetically fairly distant taxa.

3.3.1. Oralized somatic monokinetids

By definition, oralized somatic monokinetids are at the anterior end of the somatic kineties and possess nematodesmata contributing to the oral basket (Foissner and Foissner, 1985, 1988). Accord-

ing to the molecular data, this special kind of somatic monokinetids evolved at least four times independently in fairly distant taxa, viz., in the subclass Trichostomatia, in the order Dileptida, in the *E. gasterosteus–B. pellucidum* clade, in *E. polynucleata*, and in *Fuscheria* (Table 3 and Fig. 2).

3.3.2. Loss of the dikinetidal circumoral kinety

According to our molecular trees and topology tests, the circumoral kinety was lost at least three times independently in *E. gasterosteus*, *E. polynucleata*, and the entire subclass Trichostomatia (Table 3 and Fig. 2). We believe that the loss of this important kinety occurred after the evolution of oralized somatic monokinetids that, like oral dikinetids, contribute nematodesmata and transverse microtubules to the oral basket maintaining its functionality.

3.3.3. Loss of toxicysts

Toxicysts, slender tubular prey-immobilizing extrusomes, are one of the most important apomorphies of the class Litostomatea. However, they were obviously independently lost in several relatively distant free-living species and genera, such as *Apertospathula inermis*, *Arcuospathidium cooperi*, the coriplitids, and the endocommensal trichostomatians (e.g., Foissner and Xu, 2007; Lynn, 2008; Oertel et al., 2008). Based on molecular phylogenies, it is evident that the toxicysts were lost in the stemline of the subclass Trichostomatia.

3.3.4. Perioral kinety

The perioral kinety, which is composed of narrowly spaced monokinetids, accompanies the right branch of the circumoral kinety. Typically, it occurs in the rhynchostomatians (e.g., Golińska, 1995; Grain and Golińska, 1969; Vd'ačný et al., submitted for publication) and in the litonotine pleurostomatids, where it is designated as "third perioral kinety" (Foissner, 1984b; Foissner et al., 1995). Looking at molecular and ontogenetic data, it becomes clear that the perioral kineties of the rhynchostomatians and the litonotids are not homologous. In the former, the perioral kinety is generated during the second round of basal body proliferation by alignment of the densely ciliated anterior region of at least two right side somatic ciliary rows (Golińska, 1995; Vd'ačný and Foissner, 2009). By contrast, the third perioral kinety of the litonotids is generated within the parental kinety during the first round of basal body proliferation (Foissner, 1996; Fryd-Versavel et al., 1975).

3.3.5. Multi-rowed dorsal brush

There is evidence that the dorsal brush of the last common ancestor of the Litostomatea was three-rowed (for details, see Foissner and Xu, 2007; Gabilondo and Foissner, 2009; Vd'ačný et al., submitted for publication). Species with a multi-rowed dorsal brush (e.g., *Monomacrocaryon terrenus*, *Apobryophyllum schmidingeri*, *Lacrymaria marina*) are scattered throughout the molecular trees, suggestive of convergent evolution.

3.3.6. Multiple contractile vacuoles

A single terminal contractile vacuole seems to be an old plesiomorphy for the infraphylum Lamellicorticata because this pattern occurs in the majority of haptorians as well as in the class Armophorea, the sister group of the Litostomatea (Vd'ačný et al., 2010). The cladistic approach and molecular trees demonstrate that the bi- or multi-vacuolate state is one of the apomorphies of the subclass Rhynchostomatia (Vd'ačný et al., submitted for publication). Further, this feature evolved convergently in *Homalozoon*, in several spathidiids, e.g., in the genus *Supraspathidium* and in *Arcuospathidium bulli*, as well as in several pleurostomatids, e.g., in *Amphileptus pleurosigma* (Foissner and Xu, 2007; Foissner et al., 1995, 2002).

4. Discussion

4.1. Ground pattern and deep evolution of the class Litostomatea

The ground pattern of a monophyletic taxon is a combination of apomorphies and younger plesiomorphies present in the stem species (last common ancestor) from which the monophylum evolved (Ax, 1995). According to the molecular clock analysis by Wright and Lynn (1997c), the last common ancestor of the litostomateans lived in the Neoproterozoic about 650 million years ago. Based on morphology and ontogeny of basal litostomateans (i.e., rhynchostomatians) and armophoreans (i.e., the sister group of Litostomatea), Vd'ačný et al., (2010) hypothesized that the last common ancestor of the Litostomatea possessed the following more recent plesiomorphies: (1) an oblong body with ventrally located oral apparatus, (2) plate-like arranged postciliary microtubules to the right of and between the ciliary rows, and (3) a telokinetal stomatogenesis commencing in the dorsal or dorsolateral kineties and with migrating oral kinetofragments. Further, Vd'ačný et al. (2010, submitted for publication) argued that the last common ancestor of the Litostomatea evolved the following apomorphies: (1) monokinetidal somatic kineties anteriorly differentiated to a three-rowed dikinetidal dorsal brush, (2) a complex oral ciliary pattern comprising a dikinetidal circumoral kinety and several preoral kineties, (3) toxicysts fostering a predatory way of life, (4) a cytopharynx of the rhabdos type, and (5) a heteropolar conjugation mode (Fig. 3). Thus, the ground oral ciliary pattern of the ancient Litostomatea was morphologically more complex than that of extant haptorians and trichostomatians, which lost the preoral kineties and sometimes also the circumoral kinety, e.g., the free-living Enchelyidae and the endocommensal Trichostomatia. According to our analyses, rhynchostomatians are morphologically nearest to the last common progenitor of the Litostomatea, as they are the only litostomateans that maintained the ancestral oral apparatus (ventrally located oral opening, presence of many preoral kineties).

Our molecular data show a deep bifurcation of the Litostomatea (Fig. 2). The first lineage is named Rhynchostomatia and comprises tracheliids and dileptids, both characterized by a ventrally located oral opening at the base of a proboscis that carries a complex oral ciliature (for details on phylogeny, see Vd'ačný et al., submitted for publication). The second lineage includes the free-living haptorians and the endocommensal trichostomatians. It is very strongly to fully supported by all phylogenetic analyses as well as by two morphological synapomorphies: body polarization and simplification of the oral ciliature (Fig. 3). As suggested by the comparative and molecular studies of Xu and Foissner (2005) and Vd'ačný et al. (2010, submitted for publication), the last common ancestor of the Haptoria and Trichostomatia very likely evolved by shortening of the proboscis-like anterior body portion, i.e., by body polarization. This process caused the apicalization of the oral opening and the simplification of the oral ciliature, i.e., the loss of the preoral kineties whose vestiges ("adesmokineties") are rarely found in some spathidiids (Foissner, 2003; Xu and Foissner, 2005) and possibly also in entodiniomorphid trichostomatians, where some extra kinetofragments around the oral opening might be homologous to adesmokineties or preoral kineties, as they possibly originate via migrating basal bodies (Furness and Butler, 1986). As explained in Fig. 3, the polar position of the oral opening is not correlated with the length and shape of the oral bulge, thus representing a strong apomorphy of the Haptoria and Trichostomatia (Fig. 3). However, according to the molecular phylogenies, the position of the oral opening was modified in some of the more derived trichostomatians. Specifically, the opening sunk into an anterior vestibulum in *Balantidium*, and was displaced posteriorly in the isotrichids (e.g., *Dasytricha* and *Isotricha*).

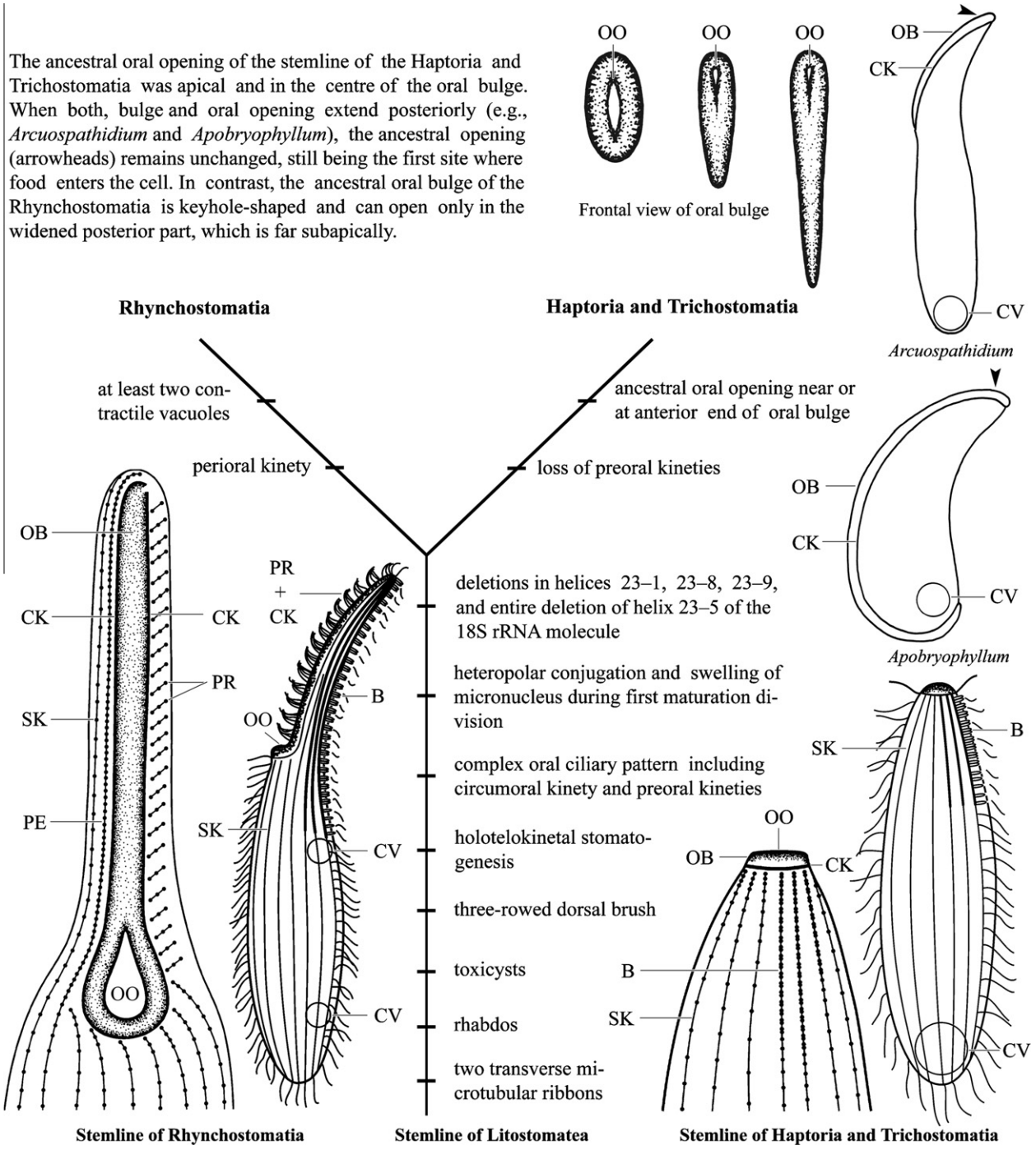

Fig. 3. An evolutionary scenario for the morphological evolution of two main litostomatean lineages. Only apomorphies are shown. B, dorsal brush; CK, circumoral kinety; CV, contractile vacuoles; OB, oral bulge; OO, oral opening; PE, perioral kinety; PR, preoral kineties; SK, somatic kineties.

4.2. Well-supported litostomatean clades

Within the litostomateans, there are five clades entirely supported by both molecular analyses and morphological apomorphies (Figs. 2 and 4): Rhynchostomatia, Trichostomatia, Lacrymariida, Didiniida, and Pleurostomatida. Based on the comparatively high morphological and genetic divergence (average pairwise distance between clades approximately 7.50%), we consider each clade as a subclass or order with a long, independent evolution. The phylogenetic relationships between the haptorian orders are poorly resolved, as strong posterior probabilities from Bayesian inference are weakly supported by bootstrap values from maximum likelihood and distance analyses. Thus, the molecular relationships of haptorids *sensu stricto* and lacrymariids and of didiniids and pleurostomatids remain questionable. An increase of taxon sampling, sequencing of additional genes, and intensified morphological research are needed to unravel their relationships unambiguously.

4.2.1. Rhynchostomatians and trichostomatians

Both taxa have been extensively discussed by Vd'ačný et al. (submitted for publication) and Lynn (2008). Although the Trichostomatia nest in the haptorian clade, we agree that their morphological and molecular distinctess is sufficient to rank them as a

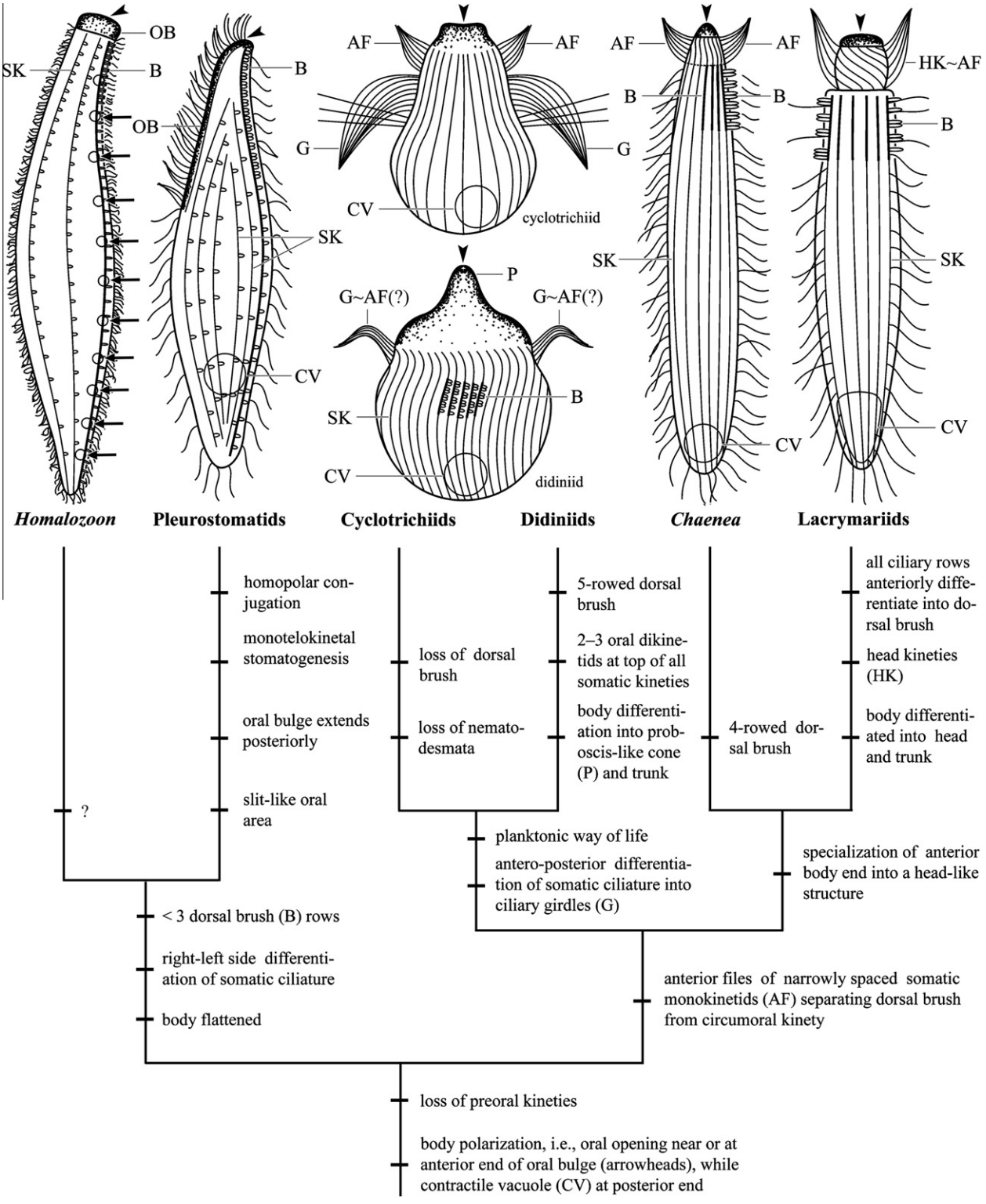

Fig. 4. Morphology-based evolutionary scenarios for some curious free-living litostomatean lineages. Only apomorphies are shown. Arrowheads denote the localization of the oral opening near or at the anterior end of the oral bulge, and arrows in *Homalozoon* mark the multiple contractile vacuoles. AF, anterior files of narrowly spaced somatic monokinetids; B, dorsal brush; CV, contractile vacuole; G, ciliary girdle; HK, head kineties; OB, oral bulge; SK, somatic kineties.

distinct subclass, especially when a Darwinian classification is used, as recommended by Mayr and Bock (2002).

4.2.2. Phylogenetic scenario and classification of lacrymariids

Lacrymariids were classified as a family in the order Haptorida by Foissner and Foissner (1988). Based on a cladistic analysis of 46 ultrastructural and morphological characteristics, Lipscomb and Riordan (1990) raised the lacrymariids to subordinal rank and assigned them to the order Pleurostomatida.

Our molecular phylogenies show that the lacrymariids represent a distinct haptorian lineage separated from both, haptorids *sensu stricto* and pleurostomatids by long internodes. A close rela-

tionship between lacrymariids and haptorids *s.s.* is very strongly supported in the Bayesian tree, while poorly recovered in maximum likelihood and distance analyses (Fig. 2), and rejected by all topology tests which cannot exclude a sister relationship with the pleurostomatids (Table 3). However, such a relationship is neither indicated in the molecular trees nor supported by morphological data (Figs. 2 and 4).

Foissner (1984a) suggested an evolutionary scenario in which the lacrymariids evolved from a chaeneid intermediate by "cephalization" (see also discussion on phylogenetic position of *Chaenea*). In lacrymariids, the anterior body portion was differentiated into a "head" with spirally arranged kineties composed of narrowly spaced cilia. Further, all ciliary rows specialized anteriorly to form the dorsal brush, which is thus multi-rowed (vs. typically three-rowed in haptorids and four-rowed in chaeneids) and separated from the circumoral dikinetids by the head kineties (vs. typically abutting on the circumoral kinety in haptorids; Fig. 4). We suggest ordinal rank for the lacrymariids based on the following features: (i) a comparatively high genetic distance (7.55%) from the haptorids *s.s.* and pleurostomatids; (ii) full support for a distinct clade by all phylogenetic analyses (Fig. 2); and (iii) some strong morphological apomorphies, such as the "head" and multi-rowed brush (Fig. 4).

4.2.3. Phylogenetic scenario and classification of Homalozoon, didiniids and pleurostomatids

A monophyletic origin of the *Homalozoon*–didiniid–pleurostomatid clade is indicated by the molecular phylogenies (strongly supported by Bayesian inference, but poorly recovered in maximum likelihood analysis) and is not rejected by the topology tests (Table 3). Their monophyly is corroborated by the lifestyle, as these ciliates typically occur in limnetic habitats, while many other free-living haptorians inhabit terrestrial and semi-terrestrial environments. Further, *Homalozoon*, didiniids and pleurostomatids share a differentiation of the somatic ciliature into ciliated and non-ciliated regions. However, this differentiation is right-left sided in *Homalozoon* and pleurostomatids, while antero-posterior in didiniids.

Homalozoon cannot be assigned to any of the haptorian orders and consistently represents a separate branch in molecular trees, suggestive of a monotypic order within the subclass Haptoria (Fig. 2). According to the Bayesian analysis, *Homalozoon* might be basal to the didiniids and pleurostomatids (Fig. 2). This position is partially sustained by morphological data (Foissner et al., 1995; Leipe et al., 1992), as *Homalozoon* displays features typical of the pleurostomatids (e.g., flattened body and the same pattern of right-left side differentiation of the somatic ciliature) and the didiniids (e.g., oral bulge restricted to anterior body end). On the other hand, morphological data also suggest a rather close relationship of *Homalozoon* and the pleurostomatids, which is not rejected by topology tests (Table 3 and Fig. 4).

The sister relationship of didiniids and pleurostomatids recovered in Bayesian analysis is not rejected by topology tests, but is not supported either by maximum likelihood and distance analyses or by morphology (Table 3 and Figs. 2 and 4).

Based on the morphology of the stemline of the Haptoria, we hypothesize that the didiniids evolved the following apomorphies: (1) differentiation of body into an anterior cone or cone-like proboscis and a globular trunk, (2) specialization of the somatic ciliature into one or more ciliary girdles developed by polymerization of the anterior basal bodies of the somatic kineties, (3) separation of the five-rowed dorsal brush from the oral dikinetids by the ciliary girdle(s), and (4) by developing two to three oral dikinetids at the top of all somatic kineties (Fig. 4). These strong apomorphies and the comparatively high genetic distance from other haptorians sustain the ordinal rank ascribed to the didiniids by Jankowski (1978).

The pleurostomatids have been accepted as an ordinal-level taxon in both morphological (Foissner, 1984b; Foissner and Foissner, 1988; Foissner et al., 1995; Grain, 1994; Lipscomb and Riordan 1990, 1992; Lynn, 2008; Lynn and Small, 2002) and molecular studies (e.g., Gao et al., 2008; Pan et al., 2010; Strüder-Kypke et al., 2006; Vd'ačný et al., submitted for publication). The monophyly and ordinal status of the pleurostomatids is sustained by the following combination of apomorphies: a leaf-like flattened body with right–left differentiation of the ciliature (right side with ciliated kineties, left side with rows of short bristles), a slit-like oral area extending along the ventral cell margin, a monotelokinetal stomatogenesis, and a homopolar conjugation mode (Fig. 3). By contrast, all other litostomateans have a holotelokinetal or cryptotelokinetal stomatogenesis and conjugate in a heteropolar way (Foissner, 1996; Fryd-Versavel et al., 1975; Raikov, 1972; Vd'ačný and Foissner, 2008, 2009; Xu and Foissner, 2004).

In the molecular phylogenies, two distinct clades can be recognized within the Pleurostomatida (Fig. 2): the family Litonotidae which is characterized by having the so-called third perioral kinety in addition to the left and right branch of the circumoral kinety which are often designated as the first and second perioral kinety; and the family Amphileptidae which is defined by a suture or "spica" formed by the right side somatic ciliary rows (Foissner, 1984b; Foissner and Foissner, 1988).

4.3. Problematic free-living litostomatean clades

Most problematic clades belong to the vernacular "haptorids" or "haptorians" (see Fig. 1 for diverse classification attempts). The reasons range from very high genetic divergence (i.e., cylcotrichiids and chaeneids) to under-sampling (haptorids). Specifically, there are more than 150 haptorian genera of which we have sequences from only about 30, and very likely only a half of the morphological diversity has been described (Foissner and Xu, 2007; Foissner et al., 2002).

4.3.1. Polyphyly of the order Haptorida

All phylogenetic analyses and topology tests strongly suggest that the order Haptorida is polyphyletic, as species morphologically classified into this order branch off the trees at different sites and most of them are even placed within the order Spathidiida (Fig. 2). Indeed, in Bayesian analyses, *Enchelyodon* sp. 1 and *Fuscheria* sp. are the only ones that are classified outside the spathidiid cluster, forming a fully supported clade that is possibly more closely related to other "classical" haptorids (lacrymariids, didiniids and pleurostomatids). All other taxa (i.e., *B. pellucidum*, *Enchelyodon* sp. 2, *Enchelys gasterosteus* and *E. polynucleata*) fall into the spathidiid radiation and are here referred to as "traditional" haptorids.

4.3.2. Radiations within the order Spathidiida

The order Spathidiida was founded by Foissner and Foissner (1988) to unite spathidiids, didiniids, and belonophryids (including the well-known *Actinobolina* and *Belonophrya*). Later, Foissner et al. (2002) added the trachelophyllids which have epicortical scales, so-called lepidosomes. Bayesian analysis and topology tests show that didiniids do not belong to spathidiids, but represent a separate order defined by several strong apomorphies (see above). There are no sequences available from the belonophryids, which are outstanding in having toxicyst-bearing tentacles distributed over the body.

The Bayesian phylogeny corroborates that trachelophyllids belong to the order Spathidiida which is an extremely diverse assemblage comprising also several "traditional" haptorids (i.e., *B. pellucidum*, *Enchelyodon* sp. 2 or *Enchelys* spp.). The inclusion of haptorids among spathidiids suggests that traits used to define

the orders Spathidiida and Haptorida are either plesiomorphies inherited from the stemline of the subclass Haptoria or features that evolved convergently several times. Indeed, convergent evolution was revealed in our molecular phylogenies (see Section 3.3). The poor resolution among spathidiid lineages is very likely caused by one or several radiations that occurred at the base of the order, as indicated by short internodes and poor nodal support. Many more spathidiid sequences and sophisticated features, such as resting cyst morphology or ontogenetic peculiarities, are necessary to unravel the spathidiid evolution.

4.3.3. Classification of cyclotrichiids

The cyclotrichiids unite planktonic ciliates which have the cilia arranged in one or several girdles (Corliss, 1979; Jankowski, 1980; Lynn, 2008). Typical members are *Askenasia*, *Cyclotrichium*, *Mesodinium* and *Myrionecta*. Whether these and other cyclotrichiids are really related to each other is questionable because the morphological data are rather incomplete (Foissner et al., 1999; Krainer and Foissner, 1990) and sequences are available only from *Mesodinium* and *Myrionecta* (Johnson et al., 2004; Strüder-Kypke et al., 2006). This must be taken into account when we refer to "cyclotrichiids" in the following discussion.

The cyclotrichiids were traditionally placed among the didiniids due to the planktonic lifestyle and the arrangement of the somatic ciliature (Corliss, 1979). Later, Jankowski (1980) suggested a distinct order for the cyclotrichiids. This seems justified due to the lack of a dorsal brush and the extreme genetic divergence from all litostomateans (Foissner and Foissner, 1988; Foissner et al., 1999; Johnson et al., 2004; Krainer and Foissner, 1990; Strüder-Kypke et al., 2006). In molecular phylogenies, the cyclotrichiids represent a fully supported, but extremely long branch that is placed within the basal polytomy of the class Litostomatea (Strüder-Kypke et al., 2006) or sometimes even outside (Johnson et al., 2004). However, the basal position of the cyclotrichiids is very likely an artefact caused by many nucleotide substitutions and deletions in the conserved regions of the 18S rRNA gene. The very high mutational saturation of this gene makes it difficult or even impossible to unravel the phylogenetic position of the cyclotrichiids not only among the litostomateans, but also among the intramacronucleate ciliates (Johnson et al., 2004; Strüder-Kypke et al., 2006). Thus, we cannot exclude that Corliss' classification is, indeed, correct and the cyclotrichiids originated from the didiniids by losing the dorsal brush and in some genera also the nematodesmata. Further, it is important to note that simplification processes occurred several times in the evolution of the litostomateans not only at the morphological but also at the molecular level. The latter is well documented by the comparatively short 18S rRNA gene in all litostomateans, including the cyclotrichiids, which is caused by deletions in helices 23–1, 23–8, 23–9, and the absence of the entire helix 23–5 (Leipe et al., 1994; Strüder-Kypke et al., 2006; Vd'ačný et al., submitted for publication; Wright and Lynn, 1997a,b; Wright et al., 1997). On the other hand, we cannot exclude that the similar general morphology of cyclotrichiids and didiniids evolved convergently under the selective pressure of the planktonic lifestyle. Sequences from other, less mutation-saturated genes are needed to unravel the phylogenetic position of the cyclotrichiids unambiguously.

4.3.4. Classification of chaeneids

The classification history of the genus *Chaenea* is rather complex, possibly due to its simple morphology. Foissner (1984a) assigned *Chaenea* to the family Trachelophyllidae. Later, Foissner (1999) transferred *Chaenea* to the family Acropisthiidae due to the presence of oralized somatic monokinetids and the lack of epicortical scales, the most important apomorphy of the family Trachelophyllidae. The classification of *Chaenea* in the family Acropisthiidae was sustained by the cladistic analyses of Lipscomb and Riordan (1990). Based on the enchelyodonid general body organization and the oralized somatic monokinetids, Foissner et al. (2002) placed *Chaenea* into the family Fuscheriidae. However, Lynn (2008) considered this family as a junior synonym of the Acropisthiidae and retained *Chaenea* therein. The molecular phylogenies do not favor any of these classifications, as chaeneids do not cluster either with *Trachelophyllum* or with *Fuscheria*, but form a fully supported long-branch clade appearing at the basal polytomy of the Haptoria (Gao et al., 2008; Pan et al., 2010). As in the cyclotrichiids, this placement is very likely artificial and caused by a high nucleotide substitution rate in the chaeneid 18S rRNA gene, differing by about 18% from that of other haptorians. Accordingly, molecular support from other genes is necessary to unravel the phylogeny of this "simple" ciliate. Possibly, it is related to the lacrymariids, as originally proposed by Foissner (1984a), based on some remarkable morphological similarities (Fig. 4).

4.3.5. Pseudoholophryids and their phylogenetic position

The order Pseudoholophryida was established by Foissner and Foissner (1988) for pseudoholophryids (*Ovalorhabdos*, *Paraenchelys*, *Pseudoholophrya*, and *Songophrya*) and helicoprorodontids (*Helicoprorodon* and *Trachelotractus*). In absence of molecular data, their phylogeny remains obscure.

4.4. A refined classification of the Litostomatea

There are four morphology-based classifications of the Litostomatea (Fig. 1), all basically suggesting two distinct lineages (Foissner and Foissner, 1988; Grain, 1994; Lipscomb and Riordan, 1990; Lynn, 2008): the subclass Haptoria (=class Litostomatea *sensu* Grain, 1994) with aerobic, free-living predators and the subclass Trichostomatia (=class Vestibuliferea *sensu* Grain, 1994) with anaerobic endocommensals. The sole exception is the framework of Lipscomb and Riordan (1990), who recognized two orders within the Litostomatea: Haptorida (including the trichostomatids) with oralized somatic monokinetids and Pleurostomatida with oral dikinetids (including the spathidiids, lacrymariids, and didiniids). In the four models, there are two to six haptorian orders each with a number of suborders and/or families (Fig. 1). Generally, the subordinal classification of the Haptorida, Spathidiida and Pleurostomatida is very complex and highly dependent on authors. For instance, the dileptids were assigned to the order Haptorida by Foissner and Foissner (1988) and Lipscomb and Riordan (1990, 1992), to the order Spathidiida by Grain (1994), while Jankowski (1980) and Vd'ačný et al. (submitted for publication) raised the dileptids to subclass rank naming them Rhynchostomatia. Similarly, the didiniids were classified within the order Spathidiida by Foissner and Foissner (1988), within the order Pleurostomatida by Lipscomb and Riordan (1990), and within the order Haptorida by Lynn (2008). This indicates that some of the morphological characters used for the litostomatean classification are either plesiomorphies or unrecognized convergences, that is, traits inappropriate to create a natural system. This is sustained by the present investigations, which show many homoplasies and support previous classifications only partially, i.e., we recognize not two but rather, three monophyletic lineages: Rhynchostomatia, Haptoria, and Trichostomatia. Within the Haptoria, five lineages have sufficient support for ordinal rank: Lacrymariida, Haptorida, Didiniida, Pleurostomatida, and Spathidiida. We refine the classification of the Litostomatea as follows:

Class Litostomatea Small and Lynn, 1981

1. *Subclass Rhynchostomatia Jankowski, 1980*
   1. Order Tracheliida Vd'ačný et al., submitted for publication (Tracheliidae Ehrenberg, 1838)

2. Order Dileptida Jankowski, 1978 (Dimacrocaryonidae Vd'ačný et al., submitted for publication; Dileptidae Jankowski, 1980)

2. *Subclass Haptoria Corliss, 1974*
   1. Order Lacrymariida Lipscomb and Riordan, 1990 stat. nov. (Lacrymariidae de Fromentel, 1876)
   2. Order Haptorida Corliss, 1974 (Echelyodonidae Foissner et al., 2002; Fuscheriidae Foissner et al., 2002; Pleuroplitidae Foissner, 1996)
   3. Order Didiniida Jankowski, 1978 (Didiniidae Poche, 1913)
   4. Order Pleurostomatida Schewiakoff, 1896 (Amphileptidae Bütschli, 1889; Litonotidae Kent, 1882)
   5. Order Spathidiida Foissner and Foissner, 1988 (Acropisthiidae Foissner and Foissner,1988; Actinobolinidae Kahl, 1930; Apertospathulidae Foissner et al., 2005; Arcuospathidiidae Foissner and Xu, 2007; Bryophyllidae Foissner, 2004; Enchelyidae Ehrenberg, 1838; Myriokaryonidae Foissner, 2003; Protospathidiidae Foissner and Xu, 2007; Spathidiidae Kahl in Doflein and Reichenow, 1929; Teutophryidae Chatton and de Beauchamp, 1924; Trachelophyllidae Kent, 1882)

3. *Subclass Trichostomatia Bütschli, 1889*

Incertae sedis: order Cyclotrichiida Jankowski, 1980; order Pseudoholophryida Foissner and Foissner, 1988; genus *Chaenea* Quennerstedt, 1867; Homalozoon Stokes, 1890

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