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Molecular Phylogeny of Litostome Ciliates (Ciliophora, Litostomatea) with Emphasis on Free-Living Haptorian Genera

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The monophyly of the litostomes was tested using nine newly sequenced and four previously unpublished small subunit ribosomal RNA (SSrRNA) gene sequences from free-living Haptoria as well as from endosymbiotic Trichostomatia: the vestibuliferids *Balantidium coli* and *Isotricha prostoma*, the cyclotrichiid *Mesodinium pulex,* and the haptorids *Loxophyllum rostratum*, *Dileptus* sp., *Enchelyodon* sp., *Enchelys polynucleata*, *Epispathidium papilliferum* (isolates A and B), *Spathidium stammeri*, *Arcuospathidium muscorum*, *Arcuospathidium cultriforme*, and the unusual *Teuthophrys trisulca*. Phylogenetic analyses depicted the litostomes as a monophyletic group consisting of the trichostomes (subclass Trichostomatia) and the free-living haptorians (subclass Haptoria). The cyclotrichiids *Mesodinium* and *Myrionecta* (order Cyclotrichiida) branched either basally within or outside the Litostomatea. In most analyses, the haptorians did not receive support as a monophyletic group. Instead, *Dileptus* branched basally to all litostome taxa, and *Epispathidium papilliferum* grouped with the Subclass Trichostomatia. Some subgroupings, however, of haptorian genera corresponded to suggested superfamilial taxa (e.g., orders Spathidiida and Pleurostomatida). Within the monophyletic trichostomes, we can distinguish three clades: (1) an Australian clade; (2) the order Entodiniomorphida; and (3) the order Vestibuliferida. However, *Balantidium*, currently classified in the Vestibuliferida, did not group with the other vestibuliferids, suggesting that this order may be paraphyletic.

Key words: Cyclotrichiida; Haptoria; Litostomatea; phylogeny; SSrRNA gene; Vestibuliferida.

Introduction

The class Litostomatea Small and Lynn, 1981 is divided into two subclasses: the free-living Haptoria Corliss, 1974, also referred to as Gymnostomata (Foissner et al. 1999, 2002), and the

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endosymbiotic Trichostomatia Bütschli, 1889. The Trichostomatia has been confirmed as a monophyletic taxon (Cameron and O'Donogue 2004; Cameron et al. 2001, 2003; Wright and Lynn 1997a, b; Wright et al. 1997) and the relationships within this subclass are well resolved. However, monophyly of the subclass Haptoria and the relationships among genera therein have not been corroborated by gene sequencing (Cameron and O'Donogue 2004; Cameron et al. 2003; Wright and Lynn 1997a). In morphological studies, the Haptoria present a very diverse assemblage of loosely associated groups, comprising over 1000 species. Phylogenies based on morphological characters also did not produce a consistent topology (Foissner 2003c; Foissner and Foissner 1988; Lipscomb and Riordan 1990, 1992).

Four taxonomic schemes have been proposed for the litostome ciliates (Table 1). Foissner and Foissner (1988) used ultrastructure and details of oral and somatic infraciliature for their classification, and introduced the dorsal brush as a key character for the haptorian ciliates. However, they stated "A natural grouping of the orders and suborders within the subclass is at present almost impossible." The system proposed by Lipscomb and Riordan (1990) was based on their cladistic analysis of 46 ultrastructural and morphological characteristics. Both Grain (1994) and Lynn and Small (2002) based their differing systems on ultrastructural and morphological data.

Since the taxon sampling of haptorian litostomes stood at only six species, it may be that sampling additional genera for gene sequencing will reveal well-supported clades. Thus, we increased the taxon sampling by adding ten new sequences of the small subunit ribosomal RNA (SSrRNA) gene from the pleurostomatid *Loxophyllum rostratum*, the haptorids *Dileptus* sp., *Enchelyodon* sp., *Enchelys polynucleata*, *Epispathidium papilliferum*, *Spathidium stammeri*, *Arcuospathidium cultriforme*, *Arcuospathidium muscorum*, the unusual *Teuthophrys trisulca*, and the cyclotrichiid *Mesodinium pulex*. In addition, we included two new sequences of vestibuliferid trichostomes: *Balantidium coli* and *Isotricha prostoma*.

Based on the morphological studies, we started our molecular analysis with the following hypotheses: (1) the litostomes are a monophyletic clade subdivided into two monophyletic subclades, the haptorians and trichostomes (Foissner and Foissner 1988; Lynn and Small 2002); (2) *Loxophyllum* is a pleurostome because it shows the typical slit-like ventral oral area (Foissner et al. 1995); (3) *Balantidium* and *Isotricha* are trichostomes that have lost ancestral toxicysts, possibly as the oral cavity invaginated (Foissner and Foissner 1985; Grain 1966; Wright and Lynn 1997a); (4) spathidiids evolved from a *Dileptus*-like ancestor (Xu and Foissner 2005); (5) *Enchelyodon* is a haptorian, related to either the trachelophyllids or the spathidiids (Foissner 1984; Foissner and Foissner 1988); (6) *Enchelys* is a haptorian that lacks oral dikinetids and, therefore, branches basal to other litostome taxa (Foissner and Foissner 1985, 1988); (7) *Teuthophrys* is a specialized spathidiid since it shares its infraciliary and extrusome pattern with this group (Foissner et al. 1999); (8) *Arcuospathidium* and *Epispathidium* will form a clade with both *Spathidium* species, and be sufficiently different to be recognized as separate genera (Foissner 1984); and (9) *Mesodinium* will form a clade with *Myrionecta* within the litostomes, but be clearly distinguished from the other litostome clades (Johnson et al. 2004; Krainer and Foissner 1990).

Results

SSrRNA Gene Sequence/Primary Structure

The sequences of the partially or completely amplified SSrRNA gene were deposited in GenBank. Their length, GC-content (%), and GenBank accession number are as follows: *Loxophyllum rostratum* — 1623 bp, 41%, DQ411864; *Arcuospathidium cultriforme* — 1559 bp, 43%, DQ411860; *Arcuospathidium muscorum* — 1631 bp, 42%, DQ411859; *Epispathidium papilliferum* (isolate A) — 1629 bp, 43%, DQ411857; *Epispathidium papilliferum* (isolate B) — 1629 bp, 43%, DQ411858; *Spathidium stammeri* — 1642 bp, 43%, DQ411862; *Teuthophrys trisulca* — 1562 bp, 43%, DQ411863; *Enchelys polynucleata* — 1640 bp, 42%, DQ411861; *Dileptus* sp. — 1641 bp, 41%, AF029764; *Enchelyodon* sp. — 1637 bp, 41%, U80313; *Balantidium coli* — 1640 bp, 42%, AF029763; *Isotricha prostoma* — 1641 bp, 41%, AF029762; *Mesodinium pulex* — 1577 bp, 45%, DQ411865.

All complete sequences with the exception of *M. pulex* were approximately 1640 nucleotides long — the typical length for litostome SSrRNA. This is a result of several deletions across the SSrRNA gene (Leipe et al. 1994; Wright and Lynn 1997a, b; Wright et al. 1997). The cyclotrichiid sequence showed further deletions and had a length of 1577 nucleotides. A comparison of the secondary structure of the variable region 4 (V4) for *Mesodinium pulex* and *Spathidium stammeri*

Table 1. Four taxonomic schemes for classification of litostome ciliates. Genera previously sequenced are in italics and our new sequences are in bold italics.

Foissner and Foissner (1988)	Lipscomb and Riordan (1990)	Grain (1994)	Lynn and Small (2002)
C. Litostomatea	C. Litostomatea	C. Litostomatea	C. Litostomatea
SC. Haptoria	SC. Haptoria		SC. Haptoria
O. Haptorida	O. Haptorida	O. Haptorida	O. Haptorida
SO. Acropisthiina	SO. Acropisthiina	SO. Acropisthiina	*Didinium*, ***Dileptus, Enchelyodon,***
SO. Dileptina	SO. Dileptina	SO. Archistomatina	***Spathidium, Teuthophrys***
Dileptus	***Dileptus***		***Enchelys, Epispathidium***
SO. Enchelyina	SO. Enchelyina	SO. Belonophryina	***Arcuospathidium,*** *Homalozoon*
Enchelys	***Enchelys***		
O. Spathidiida	SO. Helicoprorodontina	O. Spathidiida	
SO. Belonophryina	SO. Vestibuliferina	SO. Dileptina	
	Balantidium, Isotricha	***Dileptus, Teuthophrys***	
SO. Didiniina	O. Pleurostomatida	SO. Didiniina	
Didinium		*Didinium*	
SO. Spathidiina	SO. Didiniina	SO. Enchelyina	
Spathidium, Arcuospathidium,	*Didinium*	***Enchelys***	
Epispathidium, Enchelyodon			
O. Pleurostomatida	SO. Lacrymariina	SO. Lacrymariina	
SO. Amphileptina	SO. Pleurostomatina	SO. Spathidiina	
Loxophyllum, *Siroloxophyllum*,	***Loxophyllum, Spathidium***	***Enchelyodon, Epispathidium,***	
Amphileptus, Pseudoamphileptus	*Amphileptus, Pseudoamphileptus*	***Arcuospathidium, Spathidium***	
SO. Litonotina		SO. Trachelophyllina	
O. Pseudoholophryida		O. Helicoprorodontida	
SO. Pseudoholophryida		O. Pleurostomatida	O. Pleurostomatida
		Loxophyllum, *Siroloxophyllum,*	***Loxophyllum,*** *Siroloxophyllum,*
		Amphileptus, Pseudoamphileptus	*Amphileptus, Pseudoamphileptus*
SO. Helicoprorodontida		O. Mesodiniida	
O. Archistomatida		***Mesodinium,*** *Myrionecta*	
O. Cyclotrichiida			O. Cyclotrichiida
Mesodinium, *Myrionecta*			***Mesodinium,*** *Myrionecta*
SC. Trichostomatia		*C. Vestibuliferea*	SC. Trichostomatia
Balantidium, Isotricha		***Balantidium, Isotricha***	***Balantidium, Isotricha***

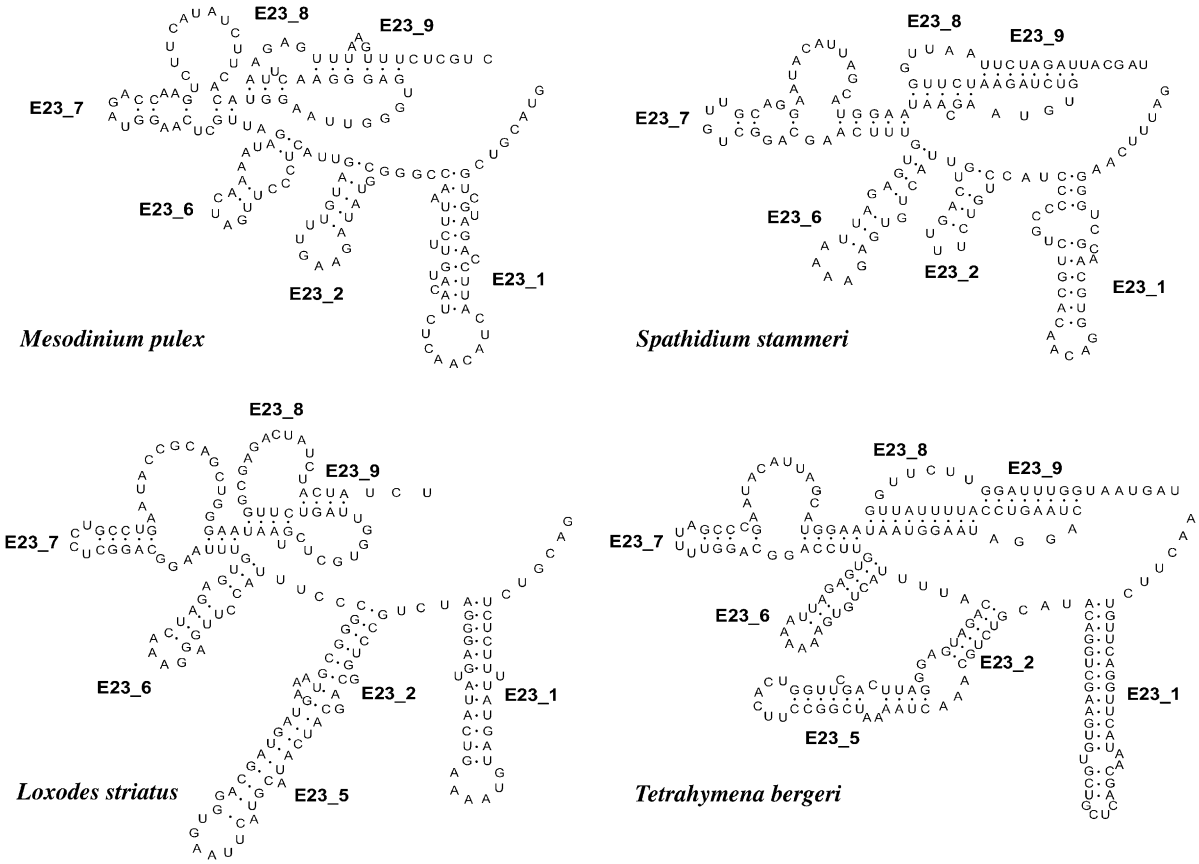

Figure 1. Models of the secondary structure of variable region 4 (V4) of the small subunit rRNA molecule, comprising helices 23_1, 23_2, 23_5, 23_6, 23_7, 23_8, and 23_9 for the ciliate species *Mesodinium pulex*, *Spathidium stammeri* (Class Litostomatea), *Loxodes striatus* (Class Karyorelictea), and *Tetrahymena bergeri* (Class Oligohymenophorea).

(Class Litostomatea), *Loxodes striatus* (Class Karyorelictea), and *Tetrahymena bergeri* (Class Oligohymenophorea) showed that *Mesodinium* as well as *Myrionecta rubra* (data not shown) had the typical deletions common to all litostome ciliates: deletions in helices 23_1, 23_8, 23_9, and deletion of the entire helix 23_5 (Fig. 1) (Wright et al. 1997). Additionally, in both *Mesodinium* and *Myrionecta*, helix 10 was missing entirely (data not shown).

Our sequence of *M. pulex*, collected from Puget Sound was 34 nucleotides longer (1577 vs. 1543 bp) than the sequence of *M. pulex* collected from the Choptank River, Maryland, by Johnson et al. (2004) due to the use of different PCR primers. Over the common length, the sequences were 98% identical. Differences were mainly located in the primer regions and in the first part of the sequence. Only a few differences were present in the 3′-region of the sequence. Alignment to sequences of other litostomes and the cyclotrichiid *M. rubra* showed that many of the observed differences mentioned were insertions of single nucleotides in the published *M. pulex* sequence of Johnson et al. (2004). No differences between the two *Mesodinium* sequences were observed in the hypervariable regions of helix E23 (V4), except in helix 23_6, or in V9 (helix 49). Our *M. pulex* and the published *M. rubra* SSrRNA gene sequence shared 95% similarity, while the published *M. pulex* shared only 94% similarity with *M. rubra*.

Phylogenetic Analyses

In the first subset of phylogenetic analyses, species of the postciliodesmatophoran subclasses Karyorelictea (*Loxodes striatus*) and Heterotrichea (*Blepharisma americanum*) were used as out-group and all available haptorid as well as many trichostome species were included to determine the phylogenetic position of the newly sequenced litostomes. Due to the extremely long branches, the cyclotrichiid species *M. pulex* and

M. rubra were not included in the final phylogenetic analyses. Moreover, due to the long-branch separating the litostomes from other ciliate taxa, we performed a second subset of analyses, including only the in-group taxa. Although the overall topology did not differ in the resulting trees, Bayesian support and bootstrap support did differ. In general, support values of the second subset (Fig. 3) were higher and are used in the text.

The tree constructed using Bayesian Inference depicted the litostomes as a monophyletic group with high posterior probability (1.0, Fig. 2). The distance (NJ) and parsimony (MP) analyses also confirmed this monophyly with bootstrap support of 100% (Fig. 2). It is important to note that the Bayesian posterior probability (support if >0.95) is different from bootstrap support values (Huelsenbeck et al. 2002). While the BI and MP trees (Fig. 2) placed species of the Class Armophorea as sister-group to the litostome ciliates, in the NJ analysis the classes Armophorea and Spirotrichea were clustered together (data not shown). However, neither topology was supported (0.55 BI, 6% MP, 46% NJ).

The subclass Trichostomatia was strongly supported as a monophyletic group in all analyses (1.0 BI, 99% MP, 100% NJ; Figs 2, 3), whereas the genera of the subclass Haptoria did not form a monophyletic clade (Figs 2, 3).

Subclass Haptoria: *Dileptus* was not grouped by strong support with any other taxon, supporting its placement in a suprafamilial taxon (Figs 2, 3). *Loxophyllum rostratum* clustered as sister taxon to *Siroloxophyllum utriculariae* and these two genera grouped with *Amphileptus* and *Pseudoamphileptus*, forming a strongly supported pleurostomatid clade (1.0 BI, 100% MP, NJ; Figs 2, 3). *Enchelyodon* grouped with *Homalozoon vermiculare* and *Didinium nasutum* as sister clade to the pleurostomes (Figs 2, 3). However, there was no Bayesian support for this topology (0.71 posterior probability; Fig. 3) nor was there any bootstrap support from distance or parsimony methods (27% MP, 16% NJ; Fig. 3). The separation of the subclass Haptoria into two orders, Pleurostomatida and Haptorida, was not supported by any analysis, all of which depicted the order Haptorida as paraphyletic (i.e., the spathidiids rather grouping with the subclass Trichostomatia), while the order Pleurostomatida was supported as monophyletic.

Family Spathidiidae (Figs 2, 3): The family Spathidiidae currently includes 20 genera based on morphological criteria, among them are *Arcuospathidium, Epispathidium, Protospathidium, Spathidium, Supraspathidium*, and *Perispira* (Foissner et al. 1999, 2002; Lynn and Small 2002). In our molecular phylogenies, *Spathidium stammeri* was sister species to the previously sequenced *Spathidium* sp., and they both formed a highly supported clade together with *Arcuospathidium cultriforme* and *Teuthophrys trisulca* (1.0 BI, 93% MP, 100% NJ), corresponding to the family Spathidiidae. The second *Arcuospathidium* species, *A. muscorum*, as well as *Enchelys polynucleata* branched basally to them (Figs 2, 3). Therefore, the genus *Arcuospathidium* is paraphyletic. The two isolates of *Epispathidium papilliferum* were identical in their SSrRNA gene sequence, but their relationship to the other spathidiid genera was not close. In the BI and MP analyses, *E. papilliferum* branched basal to the subclass Trichostomatia (1.0 BI, 32% MP, Figs 2, 3). However, they were grouped basal within the spathidiid clade in NJ, although bootstrap support for this topology was only poor (72%).

Order Vestibuliferida: The vestibuliferids were paraphyletic in all analyses with the balantidiid *Balantidium coli* separated from the *Dasytricha* isolates, which clustered with *Isotricha prostoma* and its sister species *I. intestinalis* (Figs 2, 3). The BI analysis depicted *B. coli* as basal to all other trichostome species (0.55 BI), whereas MP and NJ grouped it as sister group to the Australian clade (41% MP, 44% NJ, data not shown).

Order Cyclotrichiida: Our isolate of *M. pulex* was 98% similar to the previously published *M. pulex* sequence (Johnson et al. 2004). Both sequences showed no differences in hypervariable regions. Therefore, we considered the two isolates conspecific and only the new sequence was used in our phylogenetic study. *Mesodinium* and *Myrionecta* were closely related and formed a well-supported clade that diverged considerably from the other litostome taxa (data not shown). However, they still shared three molecular synapomorphies with all litostome taxa: (1) shorter/missing helix 10; (2) shorter helix 23_1; and (3) entirely deleted helix 23_5 (Fig. 1). Additional insertions and deletions were present in *Myrionecta* and *Mesodinium*, especially in the regions of helices 10_1, in helices of the hypervariable region V4, and in helix 43 (variable region V7).

Discussion

Monophyly of the Class Litostomatea and its Included Subclasses

Consistent with other molecular studies (Cameron and O'Donoghue 2004; Cameron et al. 2001,

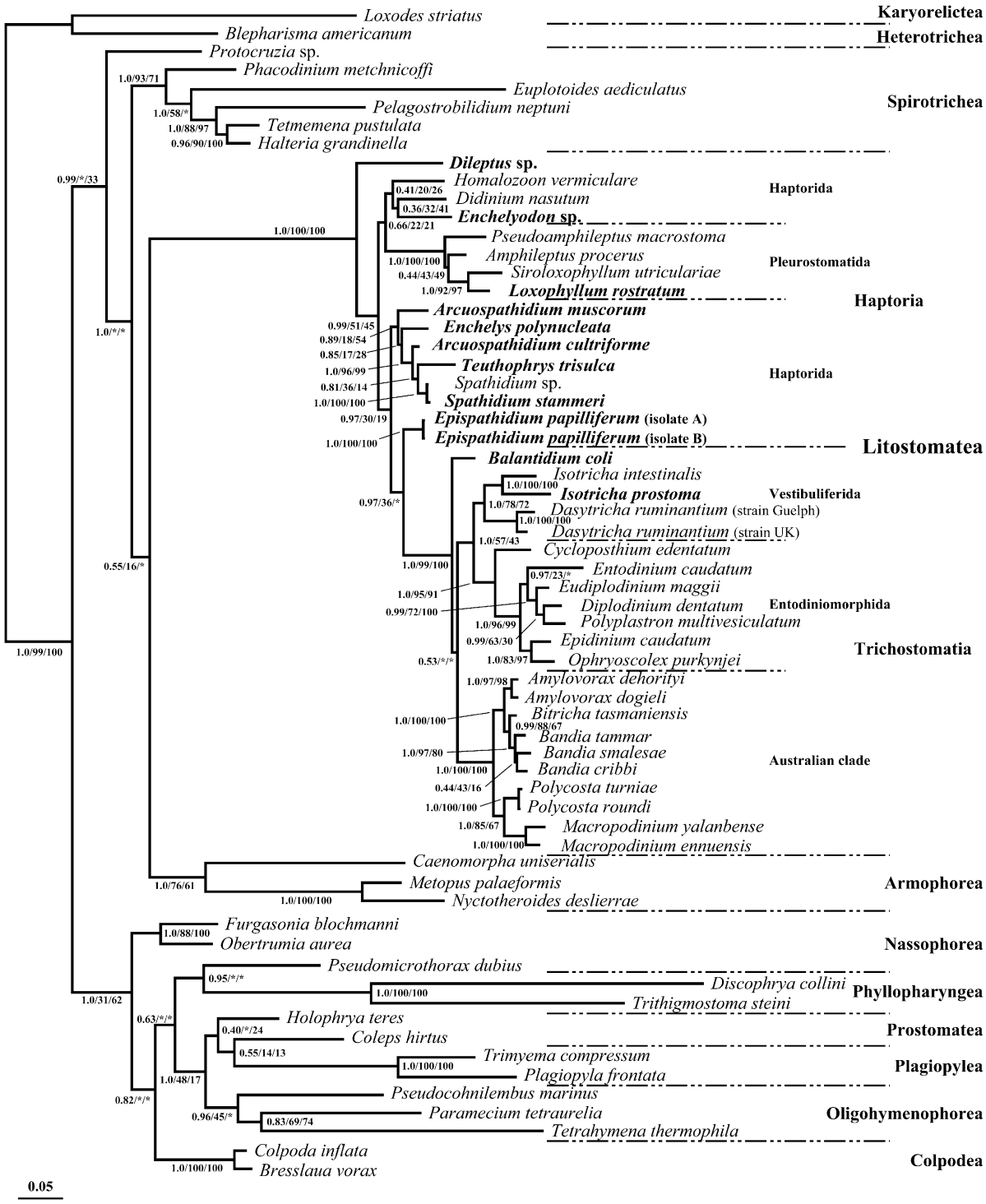

Figure 2. Maximum likelihood tree computed with MrBayes ver. 3.1.1 (Ronquist and Huelsenbeck 2003), based on the General Time-reversible (GTR) model with gamma-distribution and an estimate of invariable sites, determined by Modeltest (Posada and Crandall 1998). The first numbers at the nodes represent the posterior probability values of the Bayesian analysis, and the second and third numbers represent bootstrap values (percent out of 1000 replicates) for maximum parsimony (Swofford 2002) and neighbor joining (Saitou and Nei 1987), respectively. An asterisk indicates bootstrap values of less than 10%. The scale bar represents 5 changes per 100 positions. New sequences appear in bold face.

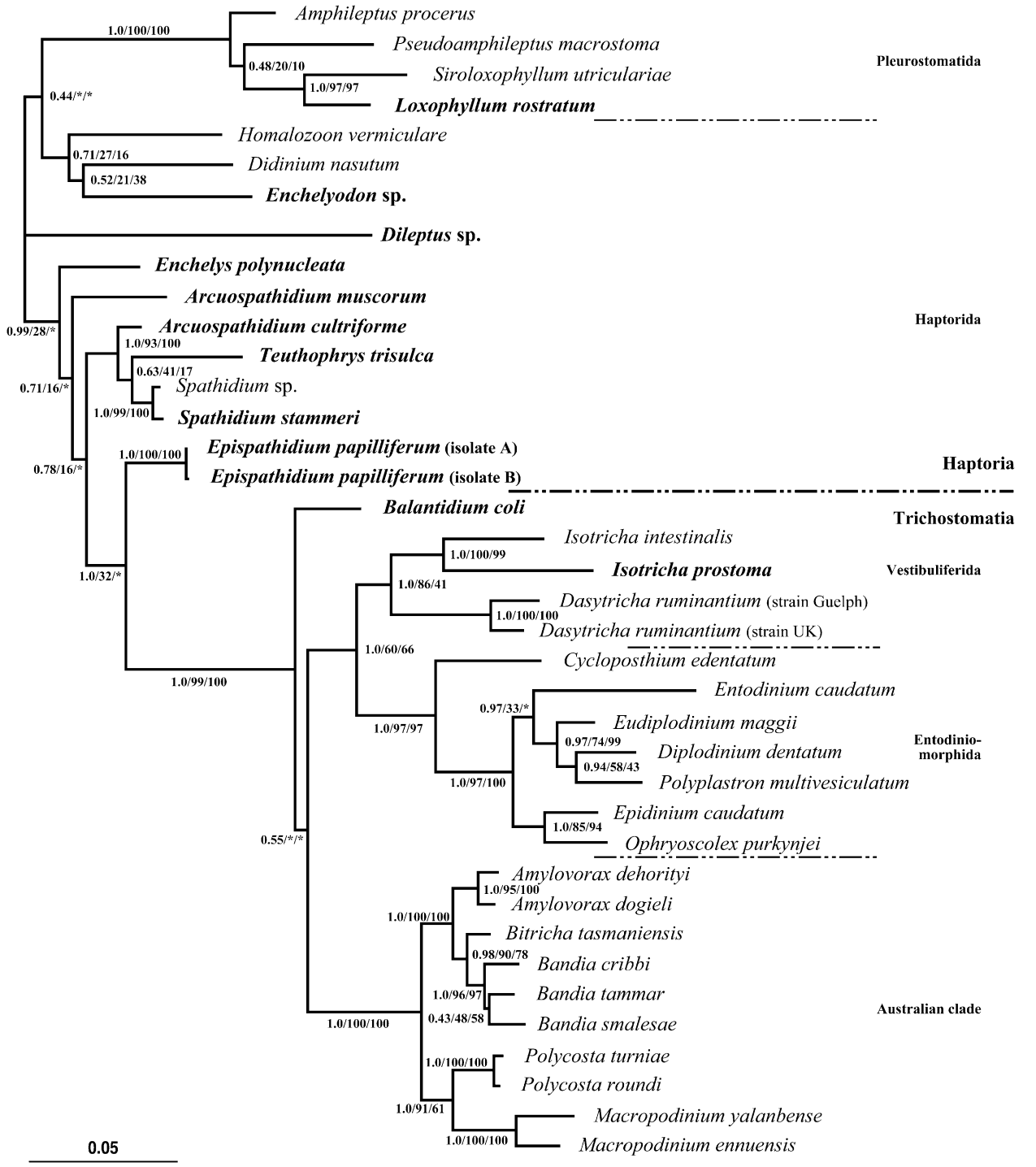

Figure 3. Maximum likelihood tree based on in-group (i.e., litostome) sequences, computed in the same way as described in Fig. 2. The first numbers at the nodes represent the posterior probability values of the Bayesian analysis, and the second and third numbers represent bootstrap values (percent out of 1000 replicates) for maximum parsimony (Swofford 2002) and neighbor joining (Saitou and Nei 1987), respectively. An asterisk indicates bootstrap values of less than 10%. The scale bar represents 5 changes per 100 positions. New sequences appear in bold face.

2003; Hammerschmidt et al. 1996; Leipe et al. 1994; Stechmann et al. 1998; Wright and Lynn 1997b), our analyses confirmed the class Litostomatea, excluding the cyclotrichiids for the moment, as a monophyletic taxon. As with previous studies using SSrRNA (Embley et al. 1995; Hammerschmidt et al. 1996; Hirt et al. 1995; Leipe et al. 1994; Wright and Lynn 1997a, b; Wright et al. 1997) and the large subunit ribosomal RNA gene (LSrRNA, Baroin-Tourancheau et al. 1992, 1998), the sister group to the litostomes could not be unambiguously resolved.

The subclass Haptoria was always paraphyletic. The branching pattern within the haptorians was not well resolved, which may still be due to undersampling of haptorian genera. However, it may also be due to the choice of molecule. Recently, Foissner et al. (2004) concluded that the SSrRNA gene might not be providing a signal that reflects the true phylogeny of stichotrich genera. The same might be the case for the haptorian genera. While still many major taxa of litostome ciliates are not represented in the current data set (e.g. the haptorian families Actinobolinidae and Lacrymariidae, and the trichostome families Buetschliidae and Blepharocorythidae) and addition of representatives of those taxa may increase phylogenetic resolution, we cannot rule out that the SSrRNA does not hold sufficient signal to infer litostome phylogeny. Therefore, sequencing of additional genes (e.g. LSrRNA) may be necessary to resolve the phylogenetic relationships within the Class Litostomatea.

Within the haptorian radiation, the pleurostomatid ciliates have been generally accepted as an order in all taxonomic schemes (Table 1) and the molecular data supported a monophyletic cluster, comprising the genera *Amphileptus*, *Pseudoamphileptus*, *Siroloxophyllum*, and *Loxophyllum.* Thus, the hypothesis that *Loxophyllum* is a pleurostome is confirmed: *L. rostratum* is highly supported as sister taxon to *S. utriculariae*, and consistent with a morphological study (Foissner and Leipe 1995), both species are genetically divergent enough to justify placement within separate genera.

The trichostome ciliates formed a monophyletic assemblage in all analyses. However, within the trichostomes the order Vestibuliferida is paraphyletic in both our and previous analyses (Cameron et al. 2003). *Balantidium* branched outside the clade formed by the vestibuliferids *Isotricha* and *Dasytricha*, and instead it branched basally within the trichostomes or with the 'Australian clade'. Neither of the topologies was supported by bootstrap values (MP, NJ) and the posterior probability (BI) was also very low. Therefore, this topology may be the result of unequal sampling or a long-branch attraction artifact, and further analyses are necessary to confidently resolve this matter. Thus, we can conclude that *Balantidium* and *Isotricha* are trichostomes that have lost toxicysts and have an invaginated oral cavity. However, *Balantidium* is not closely related genetically to the vestibuliferids *Isotricha* and *Dasytricha*.

Phylogenetic Position of *Dileptus*

Morphologically, *Dileptus* differs from other haptorids in having a proboscis with ciliated oral dikinetids and a cytostome-cytopharynx-complex with non-ciliated oralized somatic monokinetids (Foissner and Foissner 1988). Based on these characteristics, Foissner and Foissner (1988) suggested a distinct taxon for the family Trachelliidae (including *Dileptus*, *Paradileptus*, and *Trachelius*) — the suborder Dileptina. Our molecular phylogenies did place *Dileptus* basal among the haptorids, outside the spathidiid clade. No sister-group relationship could be confirmed. *Dileptus* is morphologically as well as genetically separated from other haptorians. Therefore, it may deserve placement in a suprafamilial taxon, the suborder Dileptina as suggested by Foissner and Foissner (1988). The hypothesis that the spathidiids evolved from a *Dileptus*-like ancestor (Xu and Foissner 2005) is partly supported by the BI phylogeny, which groups *Dileptus* basal to the spathidiid branch (Fig. 3). However, support values are low and, therefore, the topology is not conclusive at this point.

Phylogenetic Positions of *Enchelys* and *Enchelyodon*

The genus *Enchelys* differs from other haptorid ciliates. It does not possess a dikinetidal circumoral kinety — a main feature of most haptorids. Instead, it has oralized somatic monokinetids, which are defined as somatic kinetids that bear nematodesmal bundles for the oral rhabdos (Foissner and Foissner 1985, 1988). Therefore, the genus was placed in a separate group, the suborder Enchelyina, within the order Haptorida and regarded as 'simple', and therefore ancestral to spathidiid genera. The suborder Enchelyina is a well-defined morphological subgroup of the haptorids, and *E. polynucleata* should cluster outside

the spathidiids. However, our molecular analysis did not support the suborder Enchelyina but placed *Enchelys* near *Arcuospathidium*. Such a position is unlikely if morphology is a strong indicator of relationships, suggesting that this may be due to undersampling and/or unequal sampling of the free-living haptorians. Thus, our hypothesis that *E. polynucleata* is a haptorian, different from other haptorids, and grouping within the morphologically well-defined enchelyids is not confirmed. Instead, *Enchelys* groups within the spathidiid clade, although morphological characteristics do not support this placement. However, due to the low number of litostome genera sequenced, we do not wish to reject this hypothesis at this time.

The genus *Enchelyodon* is a typical haptorid ciliate with dorsal brush, oral dikinetids, nematodesmata, and needle- to pin-shaped toxicysts. Foissner and Foissner (1988) and Grain (1994) placed it in the order Spathidiida, suborder SPATHIDIINA, family Trachelophyllidae. In our molecular analyses, *Enchelyodon* does not group within the spathidiid clade but groups basal among the haptorians, either with *Didinium* as sister-group, or basal to the order Pleurostomatida. However, neither placement has strong support. Thus, while we conclude that *Enchelyodon* is a haptorian, it is not possible to relate it to the spathidiids. Since there are currently no sequences of trachelophyllid litostomes, we cannot definitively reject this hypothesis.

Monophyly of the Family Spathidiidae

The family Spathidiidae Kahl in Doflein and Reichenow, 1929 now comprises 20 genera. Many of the different genera were previously lumped into the genus '*Spathidium*' (over 200 species have been described in this genus), since cultivation and staining of these ciliates is not easy and the morphological characters are not very distinct (Foissner 2003c). Only recently, valuable morphological and life cycle studies have started to shed light into the biology of this large group of ciliates (Dragesco and Dragesco-Kernéis 1979; Foissner 1981, 1983, 1984, 1996, 2000, 2003a—c; Foissner and Foissner 1985, 1988; Foissner et al. 1999, 2002; Xu and Foissner 2004, 2005). Meanwhile, species originally assigned to the genus *Spathidium* have been transferred to as many as eight different genera (Foissner 2003a). Xu and Foissner (2005) proposed that the spathidiids evolved from a *Dileptus*-like ancestor because some of them have adesmokinety-like fragments on the left side and the cytostomial opening is near the dorsal end of the oral bulge. Further, Xu and Foissner (2005) tried to construct an evolutionary line within the family Spathidiidae based on morphological and ontogenetic features. However, this is difficult, since the morphological boundaries between the spathidiid genera are rather slight (Foissner 1984, 2003a; Foissner et al. 2002).

Within the family Spathidiidae, the molecular phylogenetic relationships do partly corroborate the morphological characteristics. The genus *Arcuospathidium* is paraphyletic. *Arcuospathidium cultriforme* grouped unambiguously with the spathidiid genera *Spathidium* and *Teuthophrys*, while *A. muscorum* branched basally to this cluster, and as sister species to *E. polynucleata*. A comparison of the two sequenced species, *A. cultriforme* and *A. muscorum* showed considerable morphological differences in characteristic features (Foissner 1981; Xu and Foissner 2005). Xu and Foissner (2005), therefore, suggested a possible split of species in this genus. *Teuthophrys* was originally classified as a dileptid haptorid (Clément-Iftode and Versavel 1967). However, the reinvestigation by Foissner et al. (1999) suggested a spathidiid relationship based on its infraciliature and the arrangement of the extrusomes, which is supported by our molecular data. Xu and Foissner (2005) developed an evolutionary scenario for the family Spathidiidae based on morphological and morphogenetic features. It depicts *Protospathidium* as the most basal genus, with the ciliary pattern of *Spathidium* derived from it; *Arcuospathidium* and *Epispathidium* then are suggested to have derived from a *Spathidium*-like ancestor. The molecular data did not confirm this. Rather, *Epispathidium* was well separated from the other spathidiid genera, while the genus *Arcuospathidium* was paraphyletic.

Thus, in relation to our hypotheses, *Teuthophrys* is confirmed as a specialized spathidiid since it shares its infraciliary and extrusome pattern with this group: *Teuthophrys* groups on a highly supported branch with *Spathidium* and *A. cultriforme*. The hypothesis that *Arcuospathidium* and *Epispathidium* form a clade with *Spathidium* but are sufficiently different to be recognized as separate genera is partly confirmed. All three genera are well separated by genetic distances (calculated with DNADIST) that justify the establishment of different genera ($d = 0.01—0.03$). However, the two *Arcuospathidium* species are not congeneric and *Epispathidium* is considerably diverged genetically from the other spathidiid genera ($d = 0.027—0.036$).

Monophyly of the Order Cyclotrichiida and its Phylogenetic Position

The order Cyclotrichiida Jankowski, 1980 was reviewed by Krainer and Foissner (1990), and includes the genera *Askenasia, Rhabdoaskenasia, Mesodinium*, *Myrionecta,* and *Pelagovasicola.* Corliss (1979) placed these genera with the didiniid gymnostomes, order Haptorida, because of their apical mouth surrounded by inconspicuous oral ciliature. However, several studies suggested that morphological and ultrastructural differences to other litostome genera (i.e., lack of nematodesmata and dorsal brush, pattern of silverline system, infraciliature) might be sufficient for their placement in a different subclass or even class (Foissner and Foissner 1988; Foissner et al. 1999; Krainer and Foissner 1990; Lynn 1991). Johnson et al. (2004) analyzed SSrRNA gene sequences of *M. rubra* and *M. pulex* and found them to be highly divergent from other ciliate sequences. However, they were not able to unambiguously assign them to any ciliate class.

Myrionecta rubra and *M. pulex* formed a highly supported clade in our molecular phylogenies. Depending on the analysis, the cyclotrichiids clustered either basal within the litostomes or basal to all Intramacronucleata. The SSrRNA gene sequences of *Mesodinium* and *Myrionecta* were very divergent from all other ciliate sequences. Many nucleotide substitutions were found in otherwise conserved regions. The cyclotrichiids shared a shorter SSrRNA gene with species of the classes Litostomatea and Karyorelictea — compared to an average of 1750 nucleotides for other ciliate classes. When the secondary structure of the V4 region was constructed for a litostome ciliate (*Spathidium*), for the karyorelictid *Loxodes*, and for *Tetrahymena* as out-group, *Mesodinium* showed the typical litostome features: reduction of helices 23_1, 23_8 and 23_9, as well as absence of helix 23_5 (Fig. 1).

Based on these molecular peculiarities and considering morphological data as well, we suggest two hypotheses for the placement of the cyclotrichiids. (1) The shorter SSrRNA gene sequence and the typical 'litostome deletions' that we find in *M. pulex*, as well as the extrusomes, the girdle ciliary pattern, and the inconspicuous oral ciliature support a placement with the litostomes. However, the genetic divergence of this clade is so high that some phylogenetic analyses failed to group the cyclotrichiids with the litostomes. (2) Kinetid structure, the absence of a dorsal brush, and the amount and location of nucleotide substitutions support a cyclotrichiid placement outside the litostomes in a class of its own as suggested in previous studies (Foissner and Foissner 1988; Krainer and Foissner 1990). Neither of the hypotheses has unambiguous molecular or morphological support. However, we are confident that *Mesodinium* and *Myrionecta* are intramacronucleate ciliates. They do not have postciliodesmata along the somatic kineties like the karyorelicteans and heterotrichs. Therefore, a placement basal to all Intramacronucleata, as inferred in some analyses (data not shown) may be possible. Currently, we classify the cyclotrichiid ciliates as Intramacronucleata *sedis mutabilis*. Further morphological and molecular data (extrusome structure, other molecules) are needed to confirm the placement.

Comparison with Previously Published Schemes

Generally, all four taxonomic schemes proposed a class Litostomatea with a subclass Haptoria of free-living, usually holotrichous species, and a subclass Trichostomatia, consisting of endosymbiotic species (Table 1). Grain (1994) is the exception — he elevated the subclasses to class rank, naming them Litostomatea and Vestibulifera respectively.

The order Haptorida exists in all four models, but while Lynn and Small (2002) only list families, the other three schemes have established various suborders within this group (e.g., Dileptina, Didiniina, Spathidiina). While the subclass Haptoria or gymnostomes, according to Foissner et al. (1999, 2002), comprising all free-living litostomes, is not supported by the phylogenetic analyses (Cameron and O'Donoghue 2004; Cameron et al. 2003; Wright and Lynn 1997a), the molecular data provide some support for the haptorid suborders/orders Dileptina, Spathidiida, Pleurostomatida, and Cyclotrichiida, as diagnosed by Foissner and Foissner (1988).

Lipscomb and Riordan (1990) suggested a similar classification. However, our data do not support their suggestion that *Spathidium* belongs to the order Pleurostomatida. Indeed, besides molecular evidence, the morphological evidence for such a relationship is also meager (Foissner and Foissner 1988). In addition, Lipscomb and Riordan's (1992) cladistic analysis of 21 litostomes using morphological and ultrastructural characters showed that the vestibuliferids (*Balantidium*, *Isotricha*) grouped within the haptorian clade.

However, the molecular data show similarities of the vestibuliferids to the trichostome ciliates and the BI tree gives some support for the hypothesis that the endosymbiotic Trichostomatia evolved from free-living haptorids sensu stricto by reduction of the toxicysts and invagination of their oral structures (Foissner and Foissner 1985; Grain 1966; Wright and Lynn 1997a).

Conclusions

Although SSrRNA gene sequences of several new genera of free-living litostome ciliates were added and we now have sequenced 15 genera representing almost all major taxa, the relationships among haptorians remain largely unresolved. *Enchelyodon* sp., *D. nasutum*, and *H. vermiculare* cannot be assigned to any higher taxon. Additionally, the molecular tree topologies are not congruent with morphological classifications. Similar discrepancies between morphological and molecular data have been shown before (Foissner et al. 2004; Strüder-Kypke and Lynn 2003). The diversity within haptorid litostomes is very large, and the comparison and analysis of sequence data of even this many representative genera may not be sufficient to infer their phylogeny unambiguously. The molecular data reflect the problems of morphological studies: the haptorians are an extremely diverse group and the state of many characters (plesiomorph or apomorph) is not known. Thus, any subdivisions of the class Litostomatea must still be considered as preliminary.

Methods

Origin of ciliates: *Loxophyllum rostratum* Cohn, 1866 was collected from mangrove mud at Harbor Branch, Fort Pierce, Florida, USA, in March 2002. Individual cells were picked and fixed in 80% ethanol for subsequent DNA extraction. Part of the sample was fixed in Bouin's and later stained with the Quantitative Protargol Stain (QPS, Montagnes and Lynn 1993). The species was identified after live observation and protargol impregnation by M. Strüder-Kypke.

Teuthophrys trisulca africana Dragesco and Dragesco-Kernéis, 1986 was isolated from a floodplain soil sample from the Murray River near the town of Albury, Australia, in soil collected by H. Blatterer (Linz, Austria) and R. Shiel (Australia) in August 1997. It was cultivated in Eau de Volvic with *Tetrahymena mobilis* and pieces of mealworm as food. It was identified by W. Foissner and has been described in detail by Foissner et al. (1999).

Spathidium stammeri Wenzel, 1959 was isolated from soil collected in 2002 from the margin of a mangrove swamp in the Dominican Republic. It basically matches the original description (Wenzel 1959), especially in the spiny cysts, but it has a distinctly lower number of somatic kineties and thus may represent a distinct taxon. Specimens, obtained with the non-flooded Petri dish method (Foissner et al. 2002) and identified by W. Foissner, were used for the molecular analysis.

Epispathidium papilliferum (Kahl, 1930) Foissner, 1984 was isolated from soil collected in 2002 from the floodplain ("Müllerboden") of a small river southeast of Vienna, Austria. For a detailed site description, see Foissner et al. (2005). Specimens, identified by W. Foissner, were obtained with the non-flooded Petri dish method and used for the molecular analysis. Both isolates A and B were derived from this population.

Arcuospathidium cultriforme cultriforme (Penard, 1922) Foissner, 1984, type of the genus, were isolated from mud and soil collected in 2002 from a shallow, ephemeral pond at the foot of the Pöstlingberg in the surroundings of the town of Linz, Austria. For a detailed description see Xu and Foissner (2005). Specimens, identified by W. Foissner, were obtained with the non-flooded Petri dish method and used for the molecular analysis.

Arcuospathidium muscorum (Dragesco and Dragesco-Kernéis, 1979) Foissner, 1984, identified by W. Foissner, was isolated from the same source as described for *Epispathidium*.

Enchelys polynucleata (Foissner, 1984) Foissner, Agatha, and Berger, 2002 was isolated from inland sand dune soil collected in 2004 from the Hoge Veluwe National Park in The Netherlands. Specimens, identified by W. Foissner, were obtained with the non-flooded Petri dish method and used for the molecular analysis.

Enchelyodon sp. was obtained from a freshwater pond near Tübingen, Germany, fixed in 70% ethanol, and was a gift from Prof. Dr. Christian Bardele (Zoologisches Institut der Universität Tübingen, Germany).

Dileptus sp., identified by M. Strüder-Kypke, was collected from a pond near Guelph, Ontario, Canada, cultured in Cerophyl with barley grain and flagellates. The obtained sequence was identical to that of *Dileptus* sp. obtained from Carolina Biological supplies (Burlington, NC) (unpubl. data).

Mesodinium pulex was collected from Puget Sound, Washington, USA and was a gift from

Dr. Hans Henrik Jakobsen (Western Washington University, Shannon Point Marine Center, Anacortes, WA). The cells were fixed in 70% ethanol prior to DNA extraction. Ethanol fixed material can be obtained from the corresponding author upon request.

Balantidium coli was isolated from the colon of a lowland gorilla (*Gorilla gorilla gorilla*). The ciliate's genomic DNA and SSrRNA gene were gifts from Dr. C. Graham Clark (Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, London, UK).

Isotricha prostoma was isolated from fresh rumen fluid samples collected at the University of Guelph's abattoir (Department of Animal and Poultry Science) from two cows that came from a herd in Elora, ON. The rumen fluid samples were filtered through two layers of cheese cloth to remove plant and feed material, and put into a 1 l beaker and incubated for 1 h at 39 °C to separate the protozoa. The micro-aerotolerant and motile *Isotricha prostoma* aggregated together on the surface of the rumen fluid and were easily removed using a hand-drawn Pasteur pipette. The cells were then filtered through 100-μm Nitex mesh to further remove any unwanted rumen digesta, examined under a dissecting microscope, and confirmed to be clean. Clean isolates of *Isotricha prostoma*, identified by A.-D. G. Wright, were then fixed in 70% ethanol until DNA extraction.

Due to the nature of sampling (isolated from the natural environment, enrichment cultures, or directly from the digestive tract of the host) and because many of the samples were amplified and sequenced several years ago, the authors cannot provide cultures, fixed material, or DNA samples, with the exception stated above (*Mesodinium pulex*).

DNA extraction and amplification: (a) *Loxophyllum rostratum*, *Teuthophrys trisulca*, *Spathidium stammeri*, *Arcuospathidium cultriforme*, *Enchelys polynucleata*, and *Mesodinium pulex*. DNA was extracted from the ethanol-fixed cells, following the modified Chelex extraction described by Strüder-Kypke and Lynn (2003). Between 3 and 20 μl of the supernatant were used in the subsequent PCR reactions. The PCR amplification was performed in a Perkin-Elmer GeneAmp 2400 thermocycler (PE Applied Biosystems, Mississauga, ON), using the forward primers 82F (5′-GAAACTGCGAATGGCTC-3′) for *Aruospalthidium cultriforme* and *Teuthopgrys trisulca* and Primer A (5'-AACCTGGTTGATCCTGCCAGT-3'; Medlin et al. 1988) for all other species; as well as the reverse Primer B (5'-TGATCCTTCTGCAGGTTCACCTAC-3′; Medlin et al. 1988). The PCR products of *Teuthophrys trisulca*, *Spathidium stammeri*, *Arcuospathidium cultriforme*, and *Enchelys polynucleata* were subsequently cloned (TOPO TA Cloning kit, Invitrogen, Carlsbad, CA). PCR products were purified with the GeneClean kit (Qbiogen, Carlsbad, CA); the cloned sequences were purified with either Qiaprep Spin (Qiagen, Mississauga, ON) or with the SNAP Miniprep kit (Invitrogen). Sequencing was performed in both directions in an ABI Prism 377 Automated DNA Sequencer (Applied Biosystems Inc., Foster City, CA), using dye terminator and Taq FS with two forward and two reverse internal SSrRNA primers (Elwood et al. 1985) and the amplification primers.

(b) *Isotricha prostoma, Dileptus* sp., and *Enchelyodon* sp. DNA was extracted and purified using cetyltrimethylammonium bromide (CTAB) following the protocol of Wright et al. (1997). Primer A and Primer B above (Medlin et al. 1988) were used in a PCR amplification using a PTC-100 thermal cycler (MJ Research Inc., Watertown, MA). The PCR product was excised under long-wave ultraviolet light ($<$ 60 s exposure) and purified using the GeneClean kit (Qbiogen). Sequencing was performed as described under a).

(c) *Epispathidium papilliferum* and *Arcuospathidium muscorum*. DNA was extracted from ethanol-fixed cells using a Kavenoff Zimm procedure (Kavenoff and Zimm 1973) as modified by Steinbrück and Schlegel (Steinbrück and Schlegel 1983). Concentrated cells were lysed in 10 mM Tris—HCl, 0.5 M EDTA, 0.2% SDS, pH 9.5 at 65 °C for 20 min, followed by a proteinase K digestion. The PCR amplification of the SSrRNA genes was performed in a PTC-200 thermal cycler (MJ Research) using universally conserved forward (5'-CTGGTTGATCCTGCCAG-3') and reverse primers (5'-GTAGGTGAACCTGCAG-3'). The PCR products were purified on QIAquick PCR purification columns (Qiagen), ligated into the pGEM-T easy cloning vector (Promega), and used to transform *Escherichia coli* JM109 competent cells (Promega). Sequencing was performed in both directions in an ABI Prism 3100 Automated DNA Sequencer (Applied Biosystems) using internal SSrRNA and two vector-specific primers.

Sequence availability and phylogenetic analyses: The nucleotide sequences used are available from the GenBank/EMBL databases and are listed in Table 2.

The sequence fragments were imported into Sequencher ver. 4.0.5 (Gene Codes Corp.),

trimmed at the ends, assembled into contigs, and checked for sequencing errors. The new sequences were added to our existing DCSE (Dedicated Comparative Sequence Editor; De Rijk and De Wachter 1993) database and automatically aligned against previously deposited litostome sequences. Considering secondary structural features of the SSrRNA molecule, we further refined the alignment. Two files were prepared for phylogenetic analyses. In both files, all positions were used for the analyses with the exception of insertions of one or more nucleotides in single species (e.g. *Euplotes*). The first file contained the in-group and out-group species (Fig. 2) and had 1798 positions. A second file comprised only litostome species, thus containing only 1654 positions (Fig. 3). Additional analyses were performed with files that excluded hypervariable positions. However, the trees resulting from these analyses had a less resolved branching pattern and very low support values (data not shown). The alignment is available from the corresponding

Table 2. Nucleotide sequences used in this study, including their GenBank/EMBL database accession numbers and reference.

Species	GenBank/EMBL accession number	Reference
Amphileptus procerus (submitted as *Hemiophrys procera)*	AY102175	Zhu, Yu and Shen, unpubl.
Amylovorax dehorityi	AF298817	Cameron et al. (2001)
Amylovorax dogieli	AF298825	Cameron et al. (2001)
Bandia cribbi	AF298824	Cameron and O'Donoghue (2004)
Bandia smalesae	AF298822	Cameron and O'Donoghue (2004)
Bandia tammar	AF298823	Cameron and O'Donoghue (2004)
Bitricha tasmaniensis	AF298821	Cameron et al. (2001)
Blepharisma americanum	M97909	Greenwood et al. (1991)
Bresslaua vorax	AF060453	Lynn et al. (1999)
Caenomorpha uniserialis	U97108	Hirt et al., unpubl.
Coleps hirtus	X76646	Stechmann et al. (1998)
Colpoda inflata	M97908	Greenwood et al. (1991)
Cycloposthium edentatum	AF042485	Cameron et al. (2003)
Dasytricha ruminantium (strain Guelph)	U57769	Wright and Lynn (1997a)
Dasytricha ruminantium (strain UK)	U27814	Embley et al. (1995)
Didinium nasutum	U57771	Wright and Lynn (1997a)
Diplodinium dentatum	U57764	Wright and Lynn (1997b)
Discophrya collini	L26446	Leipe et al. (1994)
Entodinium caudatum	U57765	Wright et al. (1997)
Epidinium caudatum	U57763	Wright et al. (1997)
Eudiplodinium maggii	U57766	Wright and Lynn (1997b)
Euplotoides aediculatus	X03949	Sogin et al. (1986)
(submitted as *Euplotes aediculatus*)	M14590	
Furgasonia blochmanni	X65150	Bernhard et al. (1995)
Halteria grandinella	AF194410	Shin et al. (2000)
Holophrya teres (submitted as *Prorodon teres)*	X71140	Stechmann et al. (1998)
Homalozoon vermiculare	L26477	Leipe et al. (1994)
Isotricha intestinalis	U57770	Wright and Lynn (1997a)
Loxodes striatus	U24248	Hammerschmidt et al. (1996)
Macropodinium ennuensis	AF298820	Cameron et al. (2003)

Table 2. (*continued*)

Species	GenBank/EMBL accession number	Reference
Macropodinium yalanbense	AF042486	Cameron et al. (2003)
Metopus palaeformis	M86385	Embley et al. (1992)
Nyctotheroides deslierresae	AF145353	Affa'a et al. (2004)
Ophryoscolex purkynjei	U57768	Wright and Lynn (1997b)
Paramecium tetraurelia	X03772	Sogin and Elwood (1986)
Phacodinium metchnicoffi	AJ277877	Shin et al. (2000)
Plagiopyla frontata	Z29440	Embley et al. (1995)
Polycosta roundi	AF298819	Cameron and O'Donoghue (2004)
Polycosta turniae	AF298817	Cameron and O'Donoghue (2004)
Polyplastron multivesiculatum	U57767	Wright et al. (1997)
Protocruzia sp.	AF194409	Shin et al. (2000)
Pseudoamphileptus macrostoma (submitted as *Hemiophrys macrostoma)*	AY102173	Zhu, Yu and Shen, unpubl.
Pseudocohnilembus marinus	Z22880	Dyal et al., unpubl.
Pseudomicrothorax dubius	X65151	Bernhard et al. (1995)
Siroloxophyllum utriculariae (submitted as *Loxophyllum utriculariae*)	L26448	Leipe et al. (1994)
Spathidium sp.	Z22931	Hirt et al. (1995)
Strombidium purpureum	U97112	Hirt et al., unpubl.
Tetmemena pustulata (submitted as *Stylonychia pustulata*)	X03947 M14600	Elwood et al. (1985)
Tetrahymena thermophila	M10932	Spangler and Blackburn (1985)
Trimyema compressum	Z29438	Embley et al. (1995)
Trithigmostoma steini	X71134	Leipe et al. (1994)

author upon request. Missing nucleotides at the beginning or end of sequences were treated as missing by MrBayes and PAUP and gaps within the alignment were regarded as fifth character state.

For the Bayesian inference analysis, Modeltest (Posada and Crandall 1998) was employed to find the model of DNA substitution that best fits our data. For both files, the General-Time-Reversible (GTR) model with invariable sites and gamma distribution was depicted as best model. This model ($n = 6$, rates = invgamma) was used in MrBayes version 3.1.1, a phylogenetic program employing Bayesian Inference (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), which we used to infer a phylogenetic tree (BI). Two parallel runs were performed and the maximum posterior probability of a phylogeny out of 1,000,000 trees, approximating it with the Markov Chain Monte Carlo (MCMC) and sampling every 50th generation (tree), was computed, discarding the first 2000 trees as burn-in. A maximum parsimony (MP) analysis was performed with PAUP* version 4.0b10 (Swofford 2002). Overall, 713 and 282 parsimony-informative characters were analyzed respectively, with the tree bisection-reconnection (TBR) branch-swapping algorithm in effect. Species were added randomly ($n = 10$) and the data were bootstrap resampled 1000 times. PHYLIP version 3.6a2 (Felsenstein 2004) was employed to construct a distance matrix, using DNADIST to calculate genetic distances with the F84 model assuming gamma distribution (Felsenstein and Churchill 1996; Kishino and Hasegawa 1989). The distance trees, however, were constructed with PAUP, based on the GTR model and assumed gamma distribution of substitution rates, using the Neighbor Joining (NJ) algorithm (Saitou and Nei 1987). The data were bootstrap resampled 1000 times.

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