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Reconstruction of Evolutionary History of Pleurostomatid Ciliates (Ciliophora, Litostomatea, Haptoria): Interplay of Morphology and Molecules

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Abstract. Pleurostomatids are raptorial ciliates that form a very distinct group within the Haptoria. Traditionally, the order Pleurostomatida was divided into two families: the Amphileptidae with two perioral kineties and a suture formed by the right side ciliary rows, and the Litonotidae with three perioral kineties and without suture. However, molecular phylogenies depicted the "traditional" Amphileptidae as a paraphyletic assemblage nesting also the Litonotidae. To overcome this problem we have analyzed genealogy of pleurostomatids using morphological data and 18S rRNA gene sequences, including newly sequenced genera *Acineria* and *Kentrophyllum*. Specifically, we have combined a morphological and molecular approach and have used also some other phylogenetic tools such as phylogenetic networks, split spectrum analysis, quartet mapping as well as the likelihood method of tracing history of morphological characters. These analyses show that: (1) there are not two but three distinct pleurostomatid lineages – Epiphyllidae fam. nov., Amphileptidae and Litonotidae; (2) epiphyllids (*Epiphyllum* + *Kentrophyllum*) represent a basal pleurostomatid group which is defined by two perioral kineties, by the presence of a suture on both the right and the left side of the body, by the loss of the oral bulge extrusomes, and by the extrusome fringe extending all around the body except for the oral region; (3) the families Amphileptidae and Litonotidae are monophyletic each, and represent sister groups; (4) *Acineria* belongs to the Litonotidae, as already indicated by morphological data; (5) *Loxophyllum* is a monophyletic and crown genus of the Litonotidae; and (6) *Litonotus* is paraphyletic, which could be very likely caused by a rapid radiation event that did not allow primary nucleotide homologies to be fixed.

Key words: 18S rRNA gene, *Acineria*, Epiphyllidae fam. nov., *Kentrophyllum*, Korea.

INTRODUCTION

Pleurostomatids are raptorial ciliates living, especially, in the periphyton of fresh and salt waters all around the globe. The first pleurostomatid genus, *Amphileptus*, was validly described almost 200 years ago by the prominent German protozoologist, Christian Gottfried Ehrenberg, in 1830. Since then over 100 nominal species grouped in more than 10 genera have been discovered (Jankowski 2007, Lynn 2008). The first monographic studies on pleurostomatids were provided by Schewiakoff (1896) and Kahl (1931). More recent treaties came from Foissner *et al.* (1995) and Lin

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et al. (2009). The former authorities reviewed saprobiologically important taxa, and the latter assembled morphological and ecological data on pleurostomatids occurring in the Yellow and Bohai Seas.

Within the subclass Haptoria, pleurostomatids represent a morphologically well-defined monophylum. Their body is lanceolate to leaf-like and distinctly laterally flattened whereby its narrow ventral side caries a slit-like cytostome, the so-called pleurostome, that is typically studded with toxicysts. The flat right side bears ordinary cilia, while the vaulted left side is either barren or exhibits only short bristles (e.g., Foissner *et al.* 1995, Lynn 2008, Vďačný *et al.* 2011a). Furthermore, pleurostomatids share a unique monotelokinetal stomatogenetic mode unlike all other haptorians which have a holotelokinetal formation of oral ciliature (Fryd-Versavel *et al.* 1975, Foissner 1996). The monophyletic origin of pleurostomatids is also undoubtedly corroborated by genes coding for the 18S and 28S rRNA molecules as well as for α-tubulin (e.g., Strüder-Kypke *et al.* 2006; Gao *et al.* 2008; Pan *et al.* 2010, 2013; Zhang *et al.* 2012).

The order Pleurostomatida was traditionally classified into two families: Amphileptidae and Litonotidae. The family Amphileptidae possesses a single left and a single right perioral kinety as well as a suture formed by the right side ciliary rows. According to Lynn (2008), this family includes seven genera: *Amphileptiscus*, *Amphileptus*, *Apoamphileptus*, *Epiphyllum*, *Kentrophyllum*, *Opisthodon*, and *Pseudoamphileptus*. By contrast to amphileptids, the family Litonotidae displays three perioral kineties, i.e., one kinety is right of and two kineties are left of the pleurostome. There is no suture present in litonotids (Foissner and Foissner 1988, Foissner and Leipe 1995, Lynn and Small 2002, Jankowski 2007, Lynn 2008, Vďačný *et al.* 2011a). The family Litonotidae collates four genera: *Acineria*, *Litonotus*, *Loxophyllym*, and *Siroloxophyllum* (Lynn 2008). In molecular studies, only the family Litonotidae is depicted as a monophyletic lineage, whereas the family Amphileptidae is shown as a paraphyletic assemblage that includes also litonotids (e.g., Gao *et al.* 2008; Pan *et al.* 2010, 2013; Zhang *et al.* 2012). Among all pleurostomatid genera, only monophyly of *Loxophyllum* is strongly statistically supported in the 18S rRNA gene phylogenies (e.g., Pan *et al.* 2010, 2013; Wu *et al.* 2013).

In spite of the recent progress in unraveling phylogenetic relationships among pleurostomatids, morphological evolution of this ciliate group has not been investigated by modern phylogenetic techniques, and several serious dilemmas about paraphyly of the family Amphileptidae and of the genus *Litonotus* have not been addressed yet. To overcome these problems and reconstruct the evolution of pleurostomatids, we have combined a morphological approach with a molecular approach, using sophisticated tools such as phylogenetic networks (Huson 1998, Bryant and Moulton 2004, Huson and Bryant 2006), split spectrum analyses (Wägele and Mayer 2007), quartet mapping (Schmidt *et al.* 2002) as well as the likelihood method of tracing history of morphological characters (Schulter *et al.* 1997, Pagel 1999, Maddison and Maddison 2007).

MATERIALS AND METHODS

Collection, sample processing and sequencing

For this study, two pleurostomatid species from not yet molecularly analyzed genera were collected from South Korea for sequencing. *Kentrophyllum verrucosum* was isolated from the salt Sorae wetland (salinity ~ 1.2%) in the surroundings of the metropolitan city of Incheon. *Acineria incurvata* was found in the Yeongsan river near the town of Damyang, Jeollabokdo Province. Species were identified morphologically based on live observation and protargol impregnation following Wilbert's protocol (1975). Determination of *K. verrucosum* followed Lin *et al.* (2005b), while that of *A. incurvata* followed Foissner *et al.* (1995). One or more cells from each species were transferred in sterile water and washed several times to remove contaminants. DNA extraction was performed using RED Extract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instruction and each reaction volume was reduced to one tenth (Gong *et al.* 2007). The 18S rRNA gene of these two species was amplified by polymerase chain reaction (PCR) using the 16S-like F (5'-AAC CTG GTT GAT CCT GCC AG-3') and 16S-like R (3'-TGA TCC TTC TGC AGG TTC AC-5') primers (Medlin *et al.* 1988). PCR amplifications were performed using a TaKaRa ExTaq DNA polymerase Kit (TaKaRa Bio-medicals, Otsu, Japan). PCR cycling parameters followed the protocol described in Chen and Song (2001). PCR products were directly used for sequencing on an ABI 3730XL automatic sequencer (Macrogen Inc., Seoul, Korea), with PCR primers serving as sequencing primers. The sequence fragments were checked and assembled into contigs using the Geneious ver. 6.1.6 program created by Biomatters (available from http://www.geneious.com/).

Morphological phylogenetic methods

Morphological trees are based on 30 interphase characters whose states are summarized in Table 1, and their distribution in 26 taxa (18 pleurostomatid and 8 out-group species) is given in Table 2. These 30 characters cover all diagnostic and phylogenetic informative features used in natural classification of pleurostomatid ciliates and other free-living haptorians. In order to compare morphological and molecular trees as well as to compute combined morphological-molecular trees, we included into phylogenetic analyses only those species which have 18S rRNA gene sequences deposited in GenBank. This criterion was applied also for selection of out-group taxa which included all sequenced free-living haptorians that are considered to be closely related to pleurostomatids (Vďačný *et al.* 2011a, Vďačný and Foissner 2013). Morphological data were obtained either from our own observations, original descriptions or authoritative redescriptions: Berger *et al.* (1983), Foissner (1983, 1984a), Foissner and Leipe (1995), Foissner *et al.* (1995, 1999, 2002), Song *et al.* (2003, 2004), Lin and Song (2004), Lin *et al.* (2005a, b; 2007a; 2008a), Kim and Min (2009), Pan *et al.* (2010, 2013), and Wu *et al.* (2013).

The most parsimonious trees were computed in PAUP* ver. 4.0b8 (Swofford 2003) using a heuristic search and 10 random addition species replicates, the accelerated transformation (ACCTRAN) optimalization algorithm as well as the three bisection-reconnection (TBR) branch-swapping algorithm. All character states were unordered and unweighted except for characters 4 and 17 which were double-weighted, characters 12 and 13 which were triple-weighted, and character 25 which has weight 5. Weighting of characters reflected their taxonomic and phylogenetic importance according to the previous classification frameworks (Foissner and Leipe 1995, Lynn 2008) as well as according to the 18S rRNA gene phylogenies (Vďačný *et al.* 2011a). This approach resulted in a much better match between morphologic and molecular phylogenies than unweighted analyses did. A 50% majority-rule consensus tree was constructed from all most parsimonious trees found during the heuristic search. The reliability of its branching pattern was assessed by bootstrap method with 100 replicates.

Morphological evolution was also analyzed in the Bayesian inference framework. The Standard discrete evolutionary model and the symmetric Dirichlet distribution for state rate variation among sites were implemented in MrBayes ver. 3.2.1 (Ronquist and Huelsenbeck 2003). Two parallel runs with four chains were performed with the Markov Chain Monte Carlo simulations. Posterior probabilities of the branching pattern were estimated from one million generations and trees sampled every 100 generations. The first 25% of sampled trees were discarded before constructing the 50% majority-rule consensus tree and calculating its posterior probabilities.

Molecular phylogenetic methods

18S rRNA gene sequences of 18 pleurostomatid and 8 out-group taxa were aligned according to their secondary structure (Q-INS-i method) using Mafft ver. 6240 (Katoh and Toh 2008). The resulting alignment was masked with G-blocks ver. 0.91b allowing gap positions within the final blocks (Talavera and Castresana 2007). The General-Time-Reversible model for nucleotide substitution, considering invariable sites and a gamma distributed substitution rate among sites (GTR + I + Γ), was chosen by jModeltest ver. 0.1.1 under the Akaike Information Criterion (Posada 2008) as the best evolutionary model for the masked alignment. Three different approaches were employed to analyze this alignment: maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). MP trees were constructed in PAUP* using a heuristic search, 10 random sequence addition replicates, and the ACCTRAN and TBR algorithms in effect. ML analyses were performed in PhyML ver. 3.0 under the selected GTR + I + Γ model (Guindon and Gascuel 2003). Nodal support in the MP and ML consensus trees came from 1000 bootstrap replicates. BI analyses were computed in MrBayes on the CIPRES Portal ver. 1.15 (Miller *et al.* 2009). Markov Chain Monte Carlo simulations were run with two sets of four chains under the selected GTR + I + Γ model. The chain length was one million generations with trees saved every 100 generations. The first 25% of sampled trees were discarded as ‘burn-in’. The remaining trees were used to calculate branch lengths and posterior probabilities of nodes in the 50% majority-rule consensus tree.

To assess statistically significant differences in log likelihoods between best and alternative tree topologies, the approximately unbiased (AU), the weighted Shimodaira-Hasegawa (WSH), and the weighted Kishino-Hasegawa (WKH) tests were performed using CONSEL ver. 0.1j (Shimodaira and Hasegawa 2001; Shimodaira 2002, 2008). To this end, per-site log likelihoods of the best tree and constrained alternative trees were calculated in the ML framework with a heuristic search, the TBR algorithm, and 10 random sequence addition replicates using PAUP* under the selected GTR + I + Γ model. A *P*-value of < 0.05 was chosen for rejection of the null hypothesis that the log likelihoods of the constrained and best unconstrained trees are not significantly different.

18S rRNA gene phylogenetic networks were constructed with SplitsTree ver. 4 (Huson 1998, Huson and Bryant 2006), using the neighbornet algorithm with uncorrected distances (Bryant and Moulton 2004). To assess the reliability of the split graphs, bootstrap analyses with 1000 replicates were carried out. Number of split-supporting nucleotide positions in the analyzed 18S rRNA gene alignment was found using the computer program SAMS (Wägele and Mayer 2007). Results of the split spectrum analysis were visualized as described in Wägele *et al.* (2009). To unravel relationships among three pleurostomatid lineages (families), likelihood quartet mapping was performed under the GTR model and parameters estimated with Tree-Puzzle ver. 5.2 (Schmidt *et al.* 2002). The quartet mapping analyses included sampling of neighbor-joining trees with all possible quartets.

Combined phylogenetic analyses and reconstruction of ancestral morphologies

Combined morphological-molecular analyses were performed on a matrix containing 30 interphase morphological characters and 18S rRNA gene sequences of 26 taxa. The MP analyses, including 1000 bootstrap replicates, were carried out as described above. In the Bayesian inference, the standard discrete model and the Dirichlet distribution with its alpha parameter fixed to infinity was fitted to the morphological partition, while the GTR + I + Γ evolutionary model was fitted to the molecular partition. The 50% majority-rule consensus tree and its posterior probabilities were calculated as described above.

Evolution of morphological characters in pleurostomatids was studied with the computer program Mesquite ver. 2.5 (Maddison and Maddison 2007). History of character states compiled in Tables 1 and 2 was traced on the combined phylogenetic tree shown in Fig. 3. Directionality of morphological evolution was defined by rooting the combined tree with eight out-group taxa. Proportional likelihoods of character states at ancestral nodes were calculated using the Markov k-state 1-parameter evolutionary model in which the single parameter is the rate of change and any particular change from one state to another is equally probable (Schulter *et al.* 1997, Pagel 1999).

Table 1. Characters, character states and coding used for the cladistic analyses.

No.	Character	Character states	
		Plesiomorphic	Apomorphic
1.	Body shape	Bursiform (coded 0)	Lanceolate (coded 1)
2.	Differentiation of anterior body end	None (coded 0)	Head-like structure (coded 1) Proboscis-like structure (coded 2)
3.	Body distinctly laterally flattened	No (coded 0)	Yes (coded 1)
4.	Dorsal warts	Absent (coded 0)	Present (coded 1)
5.	Needle-like spines	Absent (coded 0)	Present (coded 1)
6.	Number of contractile vacuoles	1 (coded 0)	2 (coded 1) ≥ 3 (coded 2)
7.	Localization of contractile vacuoles	Terminal (coded 0)	Subterminal (coded 1) Diagonal (coded 2) In a ventral row (coded 3) In a dorsal row (coded 4) In a dorsal and a ventral row (coded 5)
8.	Course of somatic kineties	Meridional (coded 0)	Helical (coded 1)
9.	Somatic kineties curved anteriorly	No (coded 0)	Yes (coded 1)
10.	Somatic ciliature differentiated into girdles	No (coded 0)	Yes (coded 1)
11.	Number of ciliary girdles	1 (coded 0)	2 (coded 1)
12.	Right side somatic kineties form a suture	No (coded 0)	Yes (coded 1)
13.	Left side somatic kineties form a suture	No (coded 0)	Yes (coded 1)
14.	Left side kineties unciliated or bearing stumps	No (coded 0)	Yes (coded 1)
15.	Subapical condensation of cilia in one somatic kinety right of dorsal brush	Absent (coded 0)	Present (coded 1)
16.	Head kineties	Absent (coded 0)	Present (coded 1)
17.	Number of dorsal brush rows	3 (coded 0)	1 (coded 1) 2 (coded 2) ≥ 4 (coded 3)
18.	Localization of dorsal brush	Dorsal side (coded 0)	Around the whole body (coded 1) Near ventral side (coded 2)
19.	Dorsal brush distinctly separated from anterior body end	No (coded 0)	Yes (coded 1)
20.	Oral apparatus	Restricted to anterior body pole (coded 0)	Extending over ventral side (coded 1)
21.	Shape of oral bulge	Circular to elliptical (coded 0)	Conical (coded 1) Slit-like (coded 2)
22.	Anterior end of oral bulge curved	No (coded 0)	Yes (coded 1)
23.	Circumoral kinety	Not modified (coded 0)	Transformed into perioral kinety 1 and 2 (coded 1)
24.	Oralized somatic monokinetids	Absent (coded 0)	Present (coded 1)
25.	Perioral kinety 3	Absent (coded 0)	Present (coded 1)
26.	Oral extrusome localization	In apical bulge region (coded 0)	Extending also over ventral bulge region (coded 1) Not attached to bulge (coded 2)
27.	Extrusomes attached to somatic cortex	No (coded 0)	Yes (coded 1)
28.	Extrusome shape	Basically rod-shaped (coded 0)	Needle-shaped (coded 1) Ovate (coded 2)
29.	Macronuclear pattern	Mononucleate (coded 0)	Binucleate (coded 1) Moniliform (coded 2) Multinucleate, scattered (coded 3)
30.	Number of micronuclei	1 (coded 0)	≥ 2 (coded 1)

RESULTS

Characters and character states

Morphological analyses are based on five groups of characters that are considered to be diagnostic and phylogenetically informative in haptorians: cell morphology (characters 1–5), contractile vacuole pattern (characters 6 and 7), somatic ciliature (characters 8–19), oral structures (characters 20–28), and nuclear apparatus (characters 29 and 30). The characters and character states are summarized in Table 1, and their distribution in taxa is shown in Table 2.

Character 1: Body shape. Haptorians basically exhibit two body forms: bursiform (sac-shaped) and lanceolate (leaf-shaped). The former shape is typically

Table 2. Distribution of character states in the taxa analyzed with the computer programs PAUP* and MrBayes. For characters and character states, see Table 1. A question mark (?) indicates missing data while a dash (–) indicates an inapplicable character.

Taxon	Character states					
	1–5	6–10	11–15	16–20	21–25	26–30
Haptorida						
Enchelyodon sp.	00000	??000	–0000	00000	0–00–	00???
Fuscheria sp.	00000	00000	–0001	02000	0–01–	0010?
Fuscheria terricola	00000	00000	–0001	02000	0–01–	00100
Lacrymariida						
Lacrymaria marina	01000	00100	–0000	13110	0–00–	00000
Phialina salinarum	01000	00100	–0000	13110	0–00–	00000
Didiniida						
Didinium nasutum	02000	00011	10000	03010	1–?0–	00001
Monodinium sp.	02000	00011	00000	03010	1–?0–	0000?
Pleurostomatida						
Epiphyllidae fam. nov.						
Epiphyllum shenzhenense	10100	24000	–1110	01001	20100	2102?
Kentrophyllum verrucosum	10101	25000	–1110	01001	20100	21021
Amphileptidae						
Amphileptus aeschtae	10100	23000	–1010	01001	20100	1003?
Amphileptus procerus	10100	25000	–1010	01001	20100	00010
Pseudoamphileptus macrostoma	10100	25000	–?010	01201	20100	00–10
Litonotidae						
Acineria incurvata	10100	01000	–0010	01001	21101	10010
Litonotus paracygnus	10100	01000	–0010	01001	20101	10010
Litonotus pictus	10100	25000	–0010	01001	20101	1002?
Loxophyllum caudatum	10110	01000	–0010	01001	20101	1101?
Loxophyllum chinense	10110	23000	–0010	01001	20101	1101?
Loxophyllum jini	10110	24000	–0010	01001	20101	11021
Loxophyllum meridionale	10110	21000	–0010	01001	20101	11010
Loxophyllum planum	10110	01000	–0010	01001	20101	11010
Loxophyllum rostratum	10110	01000	–0010	01001	20101	11010
Loxophyllum rugosum	10110	01000	–0010	01001	20101	1101?
Loxophyllum salinum	10110	23000	–0010	01001	20101	11010
Loxophyllum spirellum	10110	01000	–0010	01001	20101	1101?
Siroloxophyllum utriculariae	10100	12000	–0010	01001	20101	11010
Incertae sedis						
Homalozoon vermiculare	00100	24000	–0010	01000	0–00–	00021

found in species from the orders Didiniida, Haptorida, Lacrymariida, and in *Homalozoon*. Therefore, we consider this body organization as plesiomorphic. Among all haptorians, only pleurostomatids display a distinctly lanceolate or leaf-like body, which is thus considered as one of their apomorphies. However, some contractile species, such as *Amphileptus marinus* or *Litonotus paracygnus*, become fusiform when fully extended but they assume again the typical lanceolate shape when contracted (Song 1994, Song *et al.* 2004, Kim and Min 2009). In *Epiphyllum* and *Kentrophyllum*, the body is sometimes almost ellipsoidal but its general organization strongly indicates that this morphology is derived from lanceolate or *vice versa* (Petz *et al.* 1995, Lin *et al.* 2005b, Pan *et al.* 2010).

Character 2: Differentiation of anterior body end. Unlike didiniids and lacrymariids, the anterior body end of pleurostomatids and haptorids is not differentiated to any special structure. We consider this state as plesiomorphic. A proboscis-like structure evolved in didiniids (Wessenberg and Antipa 1968; Foissner *et al.* 1995, 1999), while a head-like structure was formed in lacrymariids (Foissner *et al.* 1995, 1999; Foissner 1997; Song *et al.* 2003). Both these peculiarities were recognized as apomorphies by Vďačný *et al.* (2011a).

Character 3: Body flattening. Pleurostomatids live, typically, in the periphyton and benthos of fresh and salt waters. Their right body side, which bears ordinary cilia, is used to glide on the surface, while the vaulted left side with shortened cilia faces the free water (Foissner *et al.* 1995). Pleurostomatids and *Homalozoon* share this apomorphic body organization, whereas didiniids, lacrymariids, and haptorids maintained the plesiomorphic laterally not flattened cell (Vďačný *et al.* 2011a).

Character 4: Dorsal warts. These are typical structures of *Loxophyllum* (Foissner and Leipe 1995, Foissner *et al.* 1995). Warts are arranged in a various number along the dorsal margin of the body, and they contain extrusomes forming distinct clusters. All species of the genus *Loxophyllum* (except for *L. jini*; for details see Lin *et al.* 2005a) share this unique apomorphy that has not been found in any other pleurostomatid genus as well as in any other ciliates from the subclass Haptoria.

Character 5: Spines. The genus *Kentrophyllum* is characterized by presence of 8–12 µm-long, needle-shaped, rigid and immobile spines that are arranged around the ventral and dorsal cell margin except for the oral area (Petz *et al.* 1995, Lin *et al.* 2005b). No other ciliates from the subclass Haptoria exhibit such a spine pattern and, therefore, we consider this as an autapomorphy of *Kentrophyllum*.

Character 6: Number of contractile vacuoles. A single contractile vacuole is found as an ancestral state for the subclass Haptoria (Vďačný *et al.* 2010, 2011a, b). Two or more vacuoles are considered as an apomorphic state that occurs, especially, within the family Amphileptidae but also in some other ciliates from the subclass Haptoria (e.g., *Homalozoon vermiculare*). Although the higher number of contractile vacuoles is apomorphic, this character state evolved, according to the molecular phylogenetic analyses, several times independently in more or less genetically fairly distant lineages of haptorians (Vďačný *et al.* 2011a). However, the likelihood method of tracing of character history show that the one-vacuole state of the last common ancestor of the family Litonotidae evolved from a multi-vacuole state of the last common ancestor of the order Pleurostomatida. Thus, very likely, it is not an ancestral plesiomorphy inherited from the last common ancestor of the subclass Haptoria, but an apomorphy of the family Litonotidae (see Chapter Reconstruction of pleurostomatid morphological evolution).

Character 7: Localization of contractile vacuoles. A terminally situated contractile vacuole is characteristic for the majority of species from the subclass Haptoria as well as for the sister group of the class Litostomatea, i.e., Armophorea (Vďačný *et al.* 2010). Therefore, this character state is considered as plesiomorphic. Several different locations, all considered as apomorphies, are found in pleurostomatids: a single subterminal vacuole, two diagonally arranged vacuoles, a dorsal or a ventral row of vacuoles, and many scattered vacuoles (e.g., Kahl 1931, Foissner *et al.* 1995, Lin *et al.* 2009).

Character 8: Course of somatic kineties. Somatic kineties are arranged meridionally in all haptorians except for contractile lacrymariid species in which they form a helical pattern (Foissner 1997, Song *et al.* 2003). This state of helical arrangement is thus considered to be apomorphic.

Character 9: Anterior end of somatic kineties. The course of somatic kineties remains unchanged, i.e. meridional, in the anterior body region of pleurostomatids, haptorids, and lacrymariids (Foissner *et al.* 1995, Lin *et al.* 2009, Vďačný *et al.* 2011a). However, anterior ends of ciliary rows curve distinctly leftwards in didiniids (Wessenberg and Antipa 1968; Foissner *et al.* 1995, 1999). This apomorphic state evolved very likely independently also in the last common ancestor of the order Spathidiida (Vďačný and Foissner 2013).

Character 10: Presence of ciliary girdles. In didiniids, somatic ciliature is differentiated into one or two conspicuous ciliary girdles (Wessenberg and Antipa 1968). This unique feature is considered as an important apomorphy of the order Didiniida (Vďačný *et al.* 2011a).

Character 11: Number of ciliary girdles. Within the order Didiniida, there are genera with one or two ciliary girdles (Jankowski 2007, Lynn 2008). The majority of genera including *Monodinium* exhibit a single girdle in the anterior body half. The sole exception is *Didinium* which displays two girdles, one in the anterior and one in the posterior body half (Foissner *et al.* 1995, 1999). This pattern is considered as apomorphic.

Character 12: Suture on the right body side. The right side ciliary rows form a suture in the anterior portion of the cell in the families Amphileptidae and Epiphyllidae fam. nov. However, no suture-like pattern can be recognized in the family Litonotidae and other haptorians (Foissner and Leipe 1995, Jankowski 2007, Lynn 2008). Therefore, we find the right side suture as an apomorphic feature of amphileptids and epiphyllids.

Character 13: Suture on the left body side. Two only recently discovered genera, *Epiphyllum* and *Kentrophyllum*, are outstanding in having a suture also on the left side of the body (Lin *et al.* 2005b, Pan *et al.* 2010). This unique synapomorphy separates *Epiphyllum* and *Kentrophyllum* from all other pleurostomatid genera and indicates their close relationship.

Character 14: Cilia of the left body side. Among all haptorians, only pleurostomatids and *Homalozoon* have cilia on the left side of the body shortened to stump-like bristles (Foissner and Leipe 1995, Foissner *et al.* 1995). According to the cladistic and molecular analyses, this modification of the left side cilia is considered as an important synapomorphy of *Homalozoon* and pleurostomatids, separating them from all other haptorians (Vďačný *et al.* 2011a).

Character 15: Subapical ciliary condensation. In *Fuscheria*, there is a subapical condensation of cilia in one somatic kinety that is situated right of the dorsal brush (Berger *et al.* 1983, Foissner and Foissner 1988, Foissner *et al.* 2002, Gabilondo and Foissner 2009). This unique feature represents an autapomorphy of *Fuscheria*.

Character 16: Head kineties. In the head-like region of the cell, members of the order Lacrymariida have helically arranged kineties made of narrowly spaced basal bodies (Foissner *et al.* 1995, 1999; Foissner 1997, Song *et al.* 2003). These head kineties are their unique and specific trait, thus representing one of their most important synapomorphies (Vďačný *et al.* 2011a).

Character 17: Number of dorsal brush rows. Three-rowed dorsal brush is argued to be the ancestral state for the class Litostomatea (Xu and Foissner 2005; Gabilondo and Foissner 2009; Vďačný *et al.* 2010, 2011a, b). A number higher or lower than three brush rows is considered as an apomorphic state. Pleurostomatids and *Homalozoon* typically have only a single brush row that is often anteriorly fragmented; *Fuscheria* displays two rows; didiniids exhibit four to five rows; and in lacrymariids all ciliary rows are anteriorly differentiated into a brush (Foissner 1984b; Foissner and Foissner 1988; Foissner *et al.* 1995, 1999, 2002; Foissner 1997). Among all analyzed genera, only *Enchelyodon* maintained the plesiomorphic three-rowed brush (Vďačný and Foissner 2013).

Character 18: Localization of dorsal brush. Brush is usually situated on the dorsal side in free-living ciliates of the class Litostomatea (Lynn 2008). This structure is only rarely localized in a different region of the body. In the order Lacrymariida, all somatic kineties form a brush at their anterior end, i.e., the brush is arranged around the whole cell perimeter (Foissner 1984b; Foissner *et al.* 1995, 1999, 2002; Vďačný *et al.* 2011a). In *Pseudoamphileptus*, dorsal brush was dislocated near the ventral side of the body (Foissner 1983), which is a unique localization not only within the order Pleurostomatida but also within the whole class Litostomatea.

Character 19: Separation of dorsal brush from anterior body end. Beginning of the dorsal brush is typically situated in the anterior portion of the cell, almost touching circumoral or perioral kinety. Vďačný *et al.* (2011a) considered this pattern as a plesiomorphic state within the class Litostomatea. There are only two exceptions, didiniids and lacrymariids in which the brush beginning is distinctly separated from the circumoral kinety by files of narrowly spaced monokinetids. In didiniids, these are the distinctly curved anterior ends of somatic kineties producing an anterior ciliary girdle (Wessenberg and Antipa 1968; Foissner *et al.* 1995, 1999), while in lacrymariids these are the head kineties (Foissner *et al.* 1995, 1999, 2002; Vďačný *et al.* 2011a). Such a separation of the dorsal brush from the anterior body end is found as an apomorphic state.

Character 20: Localization of oral apparatus. Apically situated oral apparatus is an ancestral condition for the subclass Haptoria (Vďačný *et al.* 2010, 2011a, b, 2012). An apomorphic state occurs in pleurostomatids, where the slit-like oral apparatus (pleurostome) extends over the narrow ventral side reaching at least the mid-body. This is an important synapomorphy of pleurostomatids, strongly supporting their monophyletic origin (Vďačný *et al.* 2011a).

Character 21: Shape of oral bulge. In frontal view, the oral bulge was ancestrally circular or elliptical in haptorians (Vďačný *et al.* 2011a, 2012). This plesiomorphic condition was maintained in the majority of taxa, except for didiniids where the oral bulge became distinctly conical and for pleurostomatids where the oral bulge was transformed into a slit-like structure. Both these deviations are considered as apomorphic states.

Character 22: Curving of anterior end of oral bulge. Only in two pleurostomatid genera, *Acineria* and *Amphileptiscus*, the anterior end of the oral bulge is hook-like curved (Augustin *et al.* 1987, Foissner *et al.* 1995, Song and Bradbury 1998). This state is found to be apomorphic, but very likely evolved convergently, as shown by different somatic and oral ciliary patterns of *Acineria* and *Amphileptiscus* (Song and Bradbury 1998).

Character 23: Circumoral kinety. This kinety encircles the proximal margin of the oral bulge, forming a ring-like pattern in the majority of haptorians (Foissner and Xu 2007, Lynn 2008). Pleurostomatids represent an exception in that their circumoral kinety was transformed into perioral kinety 1 and 2 (Vďačný *et al.* 2011a). Both perioral kineties display a meridional course and continue posteriorly as somatic ciliary rows, i.e., they do not form the ancestral ring-like or closed pattern (Foissner and Leipe 1995).

Character 24: Oralized somatic monokinetids. These kinetids, which are localized at the anterior end of somatic kineties, bear nematodesmata that form an outer cytopharyngeal basket (Foissner and Foissner 1985, 1988). According to the molecular analyses and statistical tree topology tests, oralized somatic monokinetids evolved at least four times independently within the class Litostomatea (Vďačný *et al.* 2011a).

Character 25: Perioral kinety 3. In pleurostomatids, the circumoral kinety was transformed into a perioral kinety 1 and 2 (see Character 24). Ancestral state is, thus, two perioral kineties. This number remained unchanged in the families Amphileptidae and Epiphyllidae fam. nov., while an additional third perioral kinety evolved in the family Litonotidae (Vďačný *et al.* 2011a). Perioral kinety 3 is composed of narrowly spaced monokinetids, unlike perioral kineties 1 and 2 which are made of dikinetids (Foissner and Leipe 1995). The presence of perioral kinety 3 is considered as one of the most important apomorphies of litonotids (Vďačný *et al.* 2011a). However, perioral kinety 3 was also found in two species, *Amphileptus sikorai* and *A. yuianus*, which Lin *et al.* (2005b, c) assigned to the family Amphileptidae on the basis of several typical "amphileptid" characters such as suture on the right side of the body in both species and anteriorly localized extrusomes in *A. yuianus*. Thus, perioral kinety 3 very likely evolved convergently in these two taxa, but this needs to be proven by 18S rRNA gene phylogenies.

Character 26: Oral extrusome localization. In polar haptorians, extrusomes are typically attached to the oral bulge forming an anterior cluster (e.g., Foissner 1984b; Foissner *et al.* 1995, 1999, 2002). As concerns pleurostomatids, such an apical concentration of extrusomes is found only within some species of the genus *Amphileptus* (Lin *et al.* 2005c), while extrusomes are attached along the whole length of the oral bulge in the majority of pleurostomatid taxa (Lin *et al.* 2009). We consider this state as apomorphic. However, there is also another apomorphic state in two pleurostomatid genera, *Epiphyllum* and *Kentrophyllum*, which completely lost oral extrusomes. This state evolved independently also in several fairly distant haptorian taxa, for instance, in the family Coriplitidae (Oertel *et al.* 2008) or in some species from the family Arcuospathidiidae (Foissner *et al.* 2002, Foissner and Xu 2007).

Character 27: Somatic extrusomes. Extrusomes are usually attached only to the oral cortex (Lynn 2008) and, thus, lack of extrusomes anchored in the somatic cortex is considered as ancestral. Several apomorphic states can be found in some pleurostomatids. For instance, some *Litonotus* species have extrusomes attached to the rear body end (Song and Wilbert 1989; Foissner *et al.* 1995; Lin *et al.* 2008b, 2009). In the genus *Loxophyllum*, extrusomes are grouped in clusters on the dorsal side of the cell and they can be also evenly distributed along almost the whole ventral body margin in some species (e.g., Foissner *et al.* 1995; Lin *et al.* 2005a, 2007b, 2008a; Pan *et al.* 2013). Moreover, *Epiphyllum* and *Kentrophyllum* have extrusomes anchored around the whole body except for the oral region (Petz *et al.* 1995, Lin *et al.* 2005b, Pan *et al.* 2010).

Character 28: Extrusome shape. In the subclasses Haptoria and Rhynchostomatia, the most common type of extrusomes is basically rod-shaped (e.g., Foissner 1984a, b; Foissner *et al.* 2002; Foissner and Xu 2007), which is thus considered as a plesiomorphic condition (Vďačný and Foissner 2013). There are many deviations from this simple ancestral morphology, all considered as apomorphies. For instance, *Amphileptus yuianus* possesses ovate extrusomes (Lin *et al.* 2005c) or *Fuscheria* displays needle-shaped extrusomes (Berger *et al.* 1983, Foissner *et al.* 2002).

Character 29: Macronuclear pattern. Basically, there are four macronuclear patterns in ciliates. The most widespread is a compact unsegmented macronucleus, which is considered by Raikov (1996) as plesiomorphic. This state is maintained in many species from the orders Haptorida, Lacrymariida and Didiniida (e.g., Foissner 1984b; Foissner *et al.* 1995, 1999, 2002). However, in the majority of species from the order Pleurostomatida, the macronucleus is fragmented into two nodules or many nodules that either form a moniliform strand or are scattered throughout the cytoplasm. All deviations from the mononucleate pattern are considered as apomorphic states.

Character 30: Number of micronuclei. A single micronucleus is considered as a plesiomorphic state (Raikov 1996). This condition is usually maintained in species with unsegmented macronucleus or with macronucleus segmented into two nodules. On the other hand, species with moniliform macronucleus or many scattered macronuclear nodules, typically, have a higher number of micronuclei. However, the likelihood method of tracing of character history shows that the single-micronucleus state of pleurostomatids evolved from a multi-micronucleate state of the last common ancestor of the order Pleurostomatida. This analysis also hints at highly homoplastic nature of this character.

Morphological trees

Morphological trees were constructed in a maximum parsimony framework as well as with the aid of the Bayesian inference. Unweighted MP approach resulted in statistically very poorly supported cladograms very likely due to the homoplastic nature and/or conflicting signal of several morphological characters. Weighted MP analyses, as described in the 'Material and Methods' section, found 1185 equally parsimonious trees with a length of 66 steps. A 50% majority-rule consensus tree of those trees is shown in Fig. 1. Results from MP bootstrap analyses and Bayesian inference were mapped onto that tree to show support of its branching pattern.

Monophylies of the orders Didiniida, Lacrymariida, Haptorida and Pleurostomatida are strongly supported in all morphological analyses. The Pleurostomatida cluster is founded on the following combination of morphological apomorphies: lanceolate body with slit-like oral apparatus extending over the ventral body side and with circumoral kinety transformed into the perioral kinety 1 and 2.

The internal relationships of the order Pleurostomatida are very poorly supported by the MP bootstrap and BI analyses (Fig. 1). Species with suture on the right side of the body, i.e. members of the genera *Amphileptus*, *Epiphyllum* and *Kentrophyllum*, form a clade that was found in all most parsimonious trees and was also supported by a posterior probability of 0.95, but was not recovered in the MP bootstrap analyses. Within this clade, *Epiphyllum* and *Kentrophyllum* are grouped together with strong statistical support (93% MP bootstrap and 0.99 PP). Their close phylogenetic relationship is corroborated by a unique combination of three morphological apomorphies: (1) a suture formed both on the left and the right side of the body, (2) a loss of the oral bulge extrusomes, and (3) extrusomes attached to the somatic cortex all around the body except for the oral region. Based on these features and results from the present molecular and combined analyses (see below), we establish a new family, Epiphyllidae, to unite the genera *Epiphyllum* and *Kentrophyllum*.

The monophyly of the family Litonotidae was depicted only in the 50% majority-rule consensus tree and is also supported by three morphological apomorphies: (1) three perioral kineties, (2) oral extrusomes attached along the whole length of the oral bulge, and (3) gradually shortened right side somatic kineties along the pleurostome, i.e., right side kineties do not form a suture. Relationships among litonotids are very poorly resolved and supported in all morphological analyses. Only monophyly of the genus *Loxophyllum* is shown in the 50% majority-rule consensus MP tree, which is also corroborated by the presence of dorsal warts carrying extrusomes.

Molecular trees and statistical topology tests

All phylogenetic (BI, ML and MP) analyses of the 18S rRNA gene alignment resulted in identical tree topologies and very similar nodal supports (Fig. 2). Monophylies of all analyzed orders were recognized with full support by all three algorithms used. Within the order Pleurostomatida, there are three distinct lineages each considered here as a family. The first clade is represented by two genera, *Epiphyllum* and *Kentrophyllum*, which we classify into a new family, Epiphyllidae (1.00 PP, 100% ML, 100% MP). The families Amphileptidae and Litonotidae are depicted

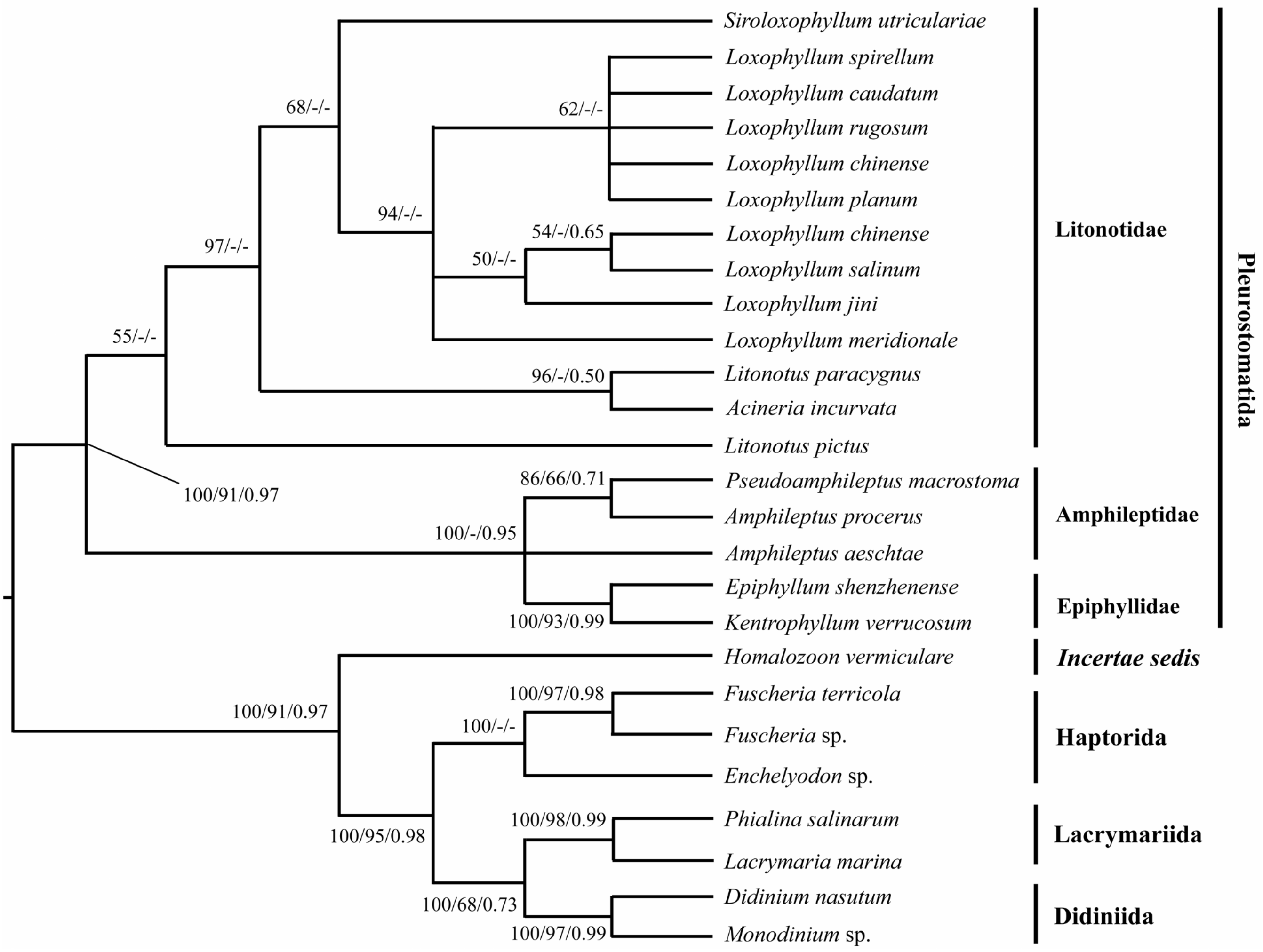


Fig. 1. Weighted 50% majority-rule consensus tree (length = 66, consistency index = 0.83, retention index = 0.93) inferred from 30 characters of 26 haptorian taxa. Tree was computed with the maximum parsimony algorithm using PAUP*. Nodal supports are indicated as follows: % of occurrence in the 1185 equally most parsimonious trees / bootstrap values in % for maximum parsimony / posterior probabilities for the Bayesian inference. A dash indicates support below 50% in the maximum parsimony analyses or 0.50 for the Bayesian inference.

as sister groups, forming a strongly supported monophylum (1.00 PP, 94% ML, 87% MP). Monophyly of the family Amphileptidae is strongly supported (0.99 PP, 91% ML, 80% MP), while monophyly of its name-bearing genus, *Amphileptus*, receives only a weak support (0.64 PP, 50% ML, 57% MP). Monophyletic origin of the family Litonotidae is strongly sustained with a posterior probability of 1.00 as well as with bootstrap values of 99% and 97% for ML and MP analyses, respectively. Within this family, only monophyly of the genus *Loxophyllum* is recovered in the phylogenetic trees (1.00 PP, 96% ML, 88% MP), while the genus *Litonotus* is depicted as paraphyletic with medium to poor support (0.92 PP, 65% ML, 74% MP). However, according to the statistical tree topology tests, its monophyly cannot be rejected ($P > 0.05$; Table 3). The genera *Acineria* and *Siroloxophyllum* form a fully (1.00 PP) to moderately (89% ML and 80% MP) supported clade, but a sister relationship of *Siroloxophyllum* and *Loxophyllum* is not rejected by the statistical AU, WSH and WKH tests ($P > 0.05$). On the other hand, a sister relationship of *Acineria* and *Litonotus* spp. is rejected at the least conservative significance level ($0.01 > P < 0.05$; Table 3).

Table 3. Log likelihoods and *P*-values of AU (approximately unbiased), WSH (weighted 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)	Δ (–ln L)[a]	AU	WSH	WKH	Conclusion
Best maximum likelihood tree (unconstrained)	6011.0031	–	0.896	0.991	0.815	–
Sister relationship of the families Epiphyllidae and Amphileptidae	6018.3007	7.30	0.223	0.311	0.186	Not rejected
Monophyly of the genus *Litonotus*	6015.8011	4.80	0.275	0.185	0.451	Not rejected
Sister relationship of the genera *Acineria* and *Litonotus*	6024.7103	13.71	**0.031**	0.133	**0.048**	Rejected
Sister relationship of the genera *Loxophyllum* and *Siroloxophyllum*	6020.3525	9.35	0.057	0.165	0.067	Not rejected

[a] Difference between log likelihoods of constrained and best (unconstrained) tree.

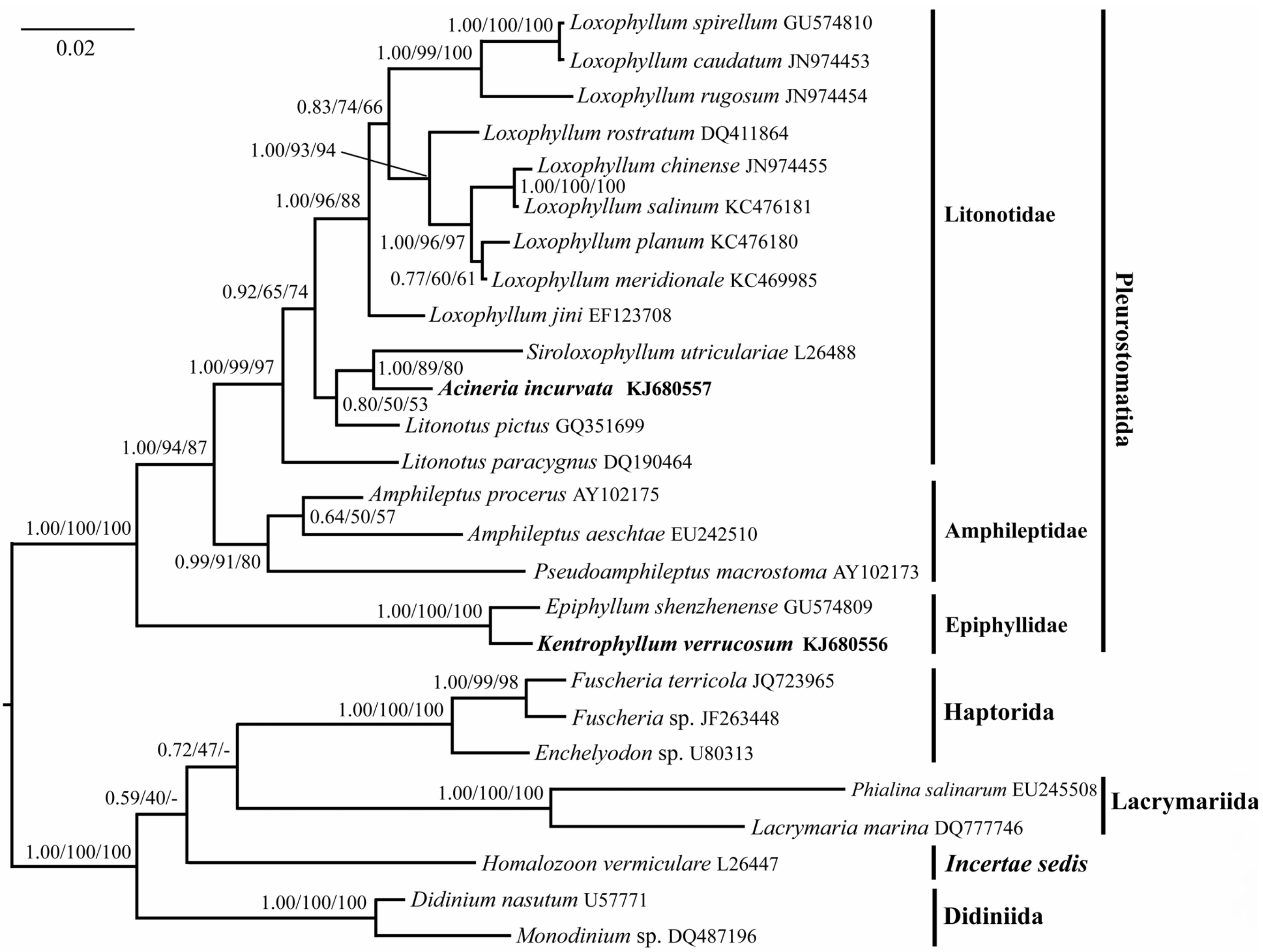


Fig. 2. Small subunit rRNA gene phylogeny based on 1462 nucleotide characters from 26 haptorian taxa. The tree was constructed using three methods (Bayesian inference, maximum likelihood, and maximum parsimony) with the GTR + I + Γ evolutionary model and the gamma shape parameter at 0.4970, the proportion of invariable sites at 0.6150, and a rate matrix for the model as suggested by jModeltest. Nodal supports are indicated as follows: posterior probabilities for the Bayesian inference / bootstrap values for maximum likelihood / bootstrap values for maximum parsimony. A dash indicates MP bootstraps below 50%. The scale bar indicates two substitutions per one hundred nucleotide positions. Sequences in bold were obtained during this study.

Combined trees

The Bayesian and MP analyses of the combined dataset of the 18S rRNA gene sequences and 30 morphological characters were almost completely consistent with the molecular phylogenies (cp. Fig. 2 and 3). Specifically, the combined analyses strongly supported monophylies of the four studied haptorian orders (PP 1.00, 100% MP) and of all three pleurostomatid families (PP 0.98–1.00, 80–100% MP). The family Epiphyllidae was positioned basally within the order Pleurostomatida, i.e., as a sister group of the families Amphileptidae and Litonotidae (1.00 PP, 91% MP). As concerns the family Amphileptidae, *Pseudoamphileptus macrostoma* and the two *Amphileptus* species formed a basal polytomy. Branching pattern within the family Litonotidae remained unchanged, that is, the genus *Litonotus* was depicted as paraphyletic (PP 0.67, 71% MP), *Acineria* and *Siroloxophyllum* formed a clade (PP 1.00, 79% MP), and the genus *Loxophyllum* was shown as monophyletic (PP 1.00, 95% MP).

Phylogenetic networks

Network analyses of the 18S rRNA gene dataset are qualitatively very similar to molecular and combined

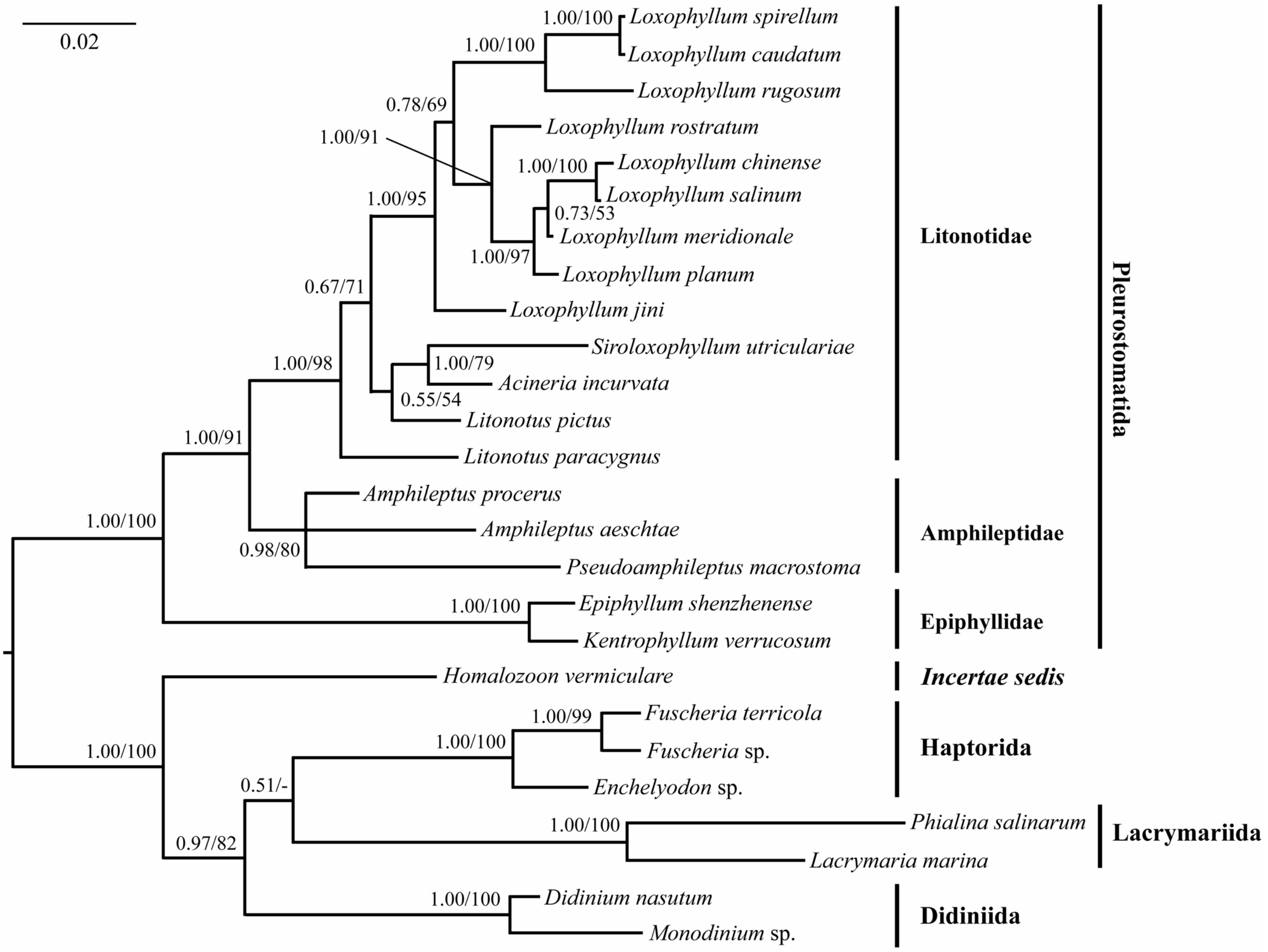


Fig. 3. Phylogenetic analysis of the combined molecular and morphological dataset comprising 26 haptorian taxa and 1492 characters. The tree was constructed with Bayesian inference using mixed models and with the maximum parsimony analysis implemented in PAUP*. Nodal support is indicated by posterior probabilities for Bayesian inference and the bootstrap values for the maximum parsimony. A dash indicates MP bootstraps below 50%. The scale bar indicates two changes per one hundred characters.

trees. The neighbornet graph shows several monophyletic lineages that are strongly supported with distinct sets of very long parallel edges and full bootstrap support: the orders Didiniida, Lacrymariida, Haptorida, and Pleurostomatida as well as the family Epiphyllidae. Further, the order Pleurostomatida is very distinctly separated (bootstrap BS = 100%) from all other haptorian orders whose phylogenetic relationships could not be unambiguously resolved due to some conflict in the 18S rRNA gene (Fig. 4).

As concerns the order Pleurostomatida, split graphs reveal three lineages each considered here as a family (see above). The most distinct group is represented by the family Epiphyllidae (BS 100%) which is separated by a distinct set of comparatively long parallel splits (BS 90%) from the families Amphileptidae and Litonotidae. Comparatively short sets of splits support monophylies of the former family and its name-bearing genus, *Amphileptus*, by the 60% and 84% bootstrap values, respectively. Monophyly of the family Litonotidae is, likewise, supported by five distinct but comparatively short parallel edges and the 92% bootstrap. On the other hand, the neighbornet graph shows some conflict in the phylogenetic signal among the litonotid genera, as indicated by several parallelograms connecting *Litonotus*, *Acineria*, *Siroloxophyllum*, and *Loxophyllum*. Basal position of *L. paracygnus* within the Litonotidae is supported by a set of eight edges with the 73% bootstrap. Monophyly of the genus *Loxophyllum* is only weakly supported by a few very short parallel edges and the 13% bootstrap. This indicates an explosive radiation of litonotid genera with *Loxophyllum* being the crown genus of the family Litonotidae (Fig. 4).

Split spectrum

Numbers of conserved clade-supporting positions in the 18S rRNA gene for the first 30 splits are shown in Fig. 5. The best split supports monophyly of the family Epiphyllidae with 13 binary and 43 asymmetric nucleotide positions. This is also the longest split found in the phylogenetic networks (Fig. 4). The second best split corroborates the monophyly of the order Pleurostomatida with 10 binary, 15 asymmetric and 28 noisy positions. The sister relationship of the families Amphileptidae and Litonotidae is sustained by split no. 11 (6 binary, 3 asymmetric and 5 noisy positions). On the other hand, there is no distinct nucleotide pattern supporting a sister relationship of the families Epiphyllidae and Amphileptidae within the 200 best splits. Conserved primary homologies supporting the monophyly of the family Amphileptidae are represented by split no. 91 with 3 asymmetric positions. Monophyly of the genus *Loxophyllum* (split no. 30) has a total support of 8, i.e., 1 binary, 5 asymmetric and 2 noisy positions. Monophyly of the genus *Amphileptus* (split no. 91) is sustained only with 2 asymmetric positions, while no split with primary homologies corroborating the monophyly of the genus *Litonotus* is present within the 200 best splits.

Quartet likelihood analyses

Four-cluster mapping clearly shows that there is a strong signal in the 18S rRNA gene to resolve phylogenetic relationships among the three pleurostomatid lineages, i.e., epiphyllids, amphileptids, and litonotids. Specifically, 98.7% of the quartets are fully resolved (tips of the triangle), only 1.3% of the quartets are partly resolved (rectangles on the sides of the triangle), and there are no unresolved quartets (central triangle). The basal position of epiphyllids and sister relationship of amphileptids and litonotids is the most favored topology supported by 92.1% of data points falling on the left tip of the triangle. Basal position of amphileptids is corroborated by 4.7% of data points, while sister relationship of epiphyllids and amphileptids by only 1.9% of data points (Fig. 6).

Reconstruction of pleurostomatid morphological evolution

Based on the likelihood method and the Markov evolutionary model, we reconstructed morphologies of the last common ancestors (LCA) of the order Pleurostomatida and the families Epiphyllidae, Amphileptidae and Litonotidae (Fig. 7). The LCA of the Pleurostomatida had the following basic pattern (proportional likelihoods for the following characters ranged from 0.9535 to 0.9997, if not stated otherwise): (1) a lanceolate and laterally flattened body, (2) a moniliform macronucleus (proportional likelihood 0.7274), (3) at least two contractile vacuoles, (4) an apical group of oral extrusomes (proportional likelihood 0.7863), (5) a suture formed by right side ciliary rows (proportional likelihood 0.5561), (6) a single-rowed dorsal brush situated on the dorsal side of the body, and (7) a slit-like oral apparatus lined by two perioral kineties.

According to the reconstruction of character state histories, the LCA of the family Epiphyllidae evolved a suture also on the left side of the body (proportional likelihood 0.9986), lost the apical group of extrusomes (proportional likelihood 0.9975), but formed an extru-

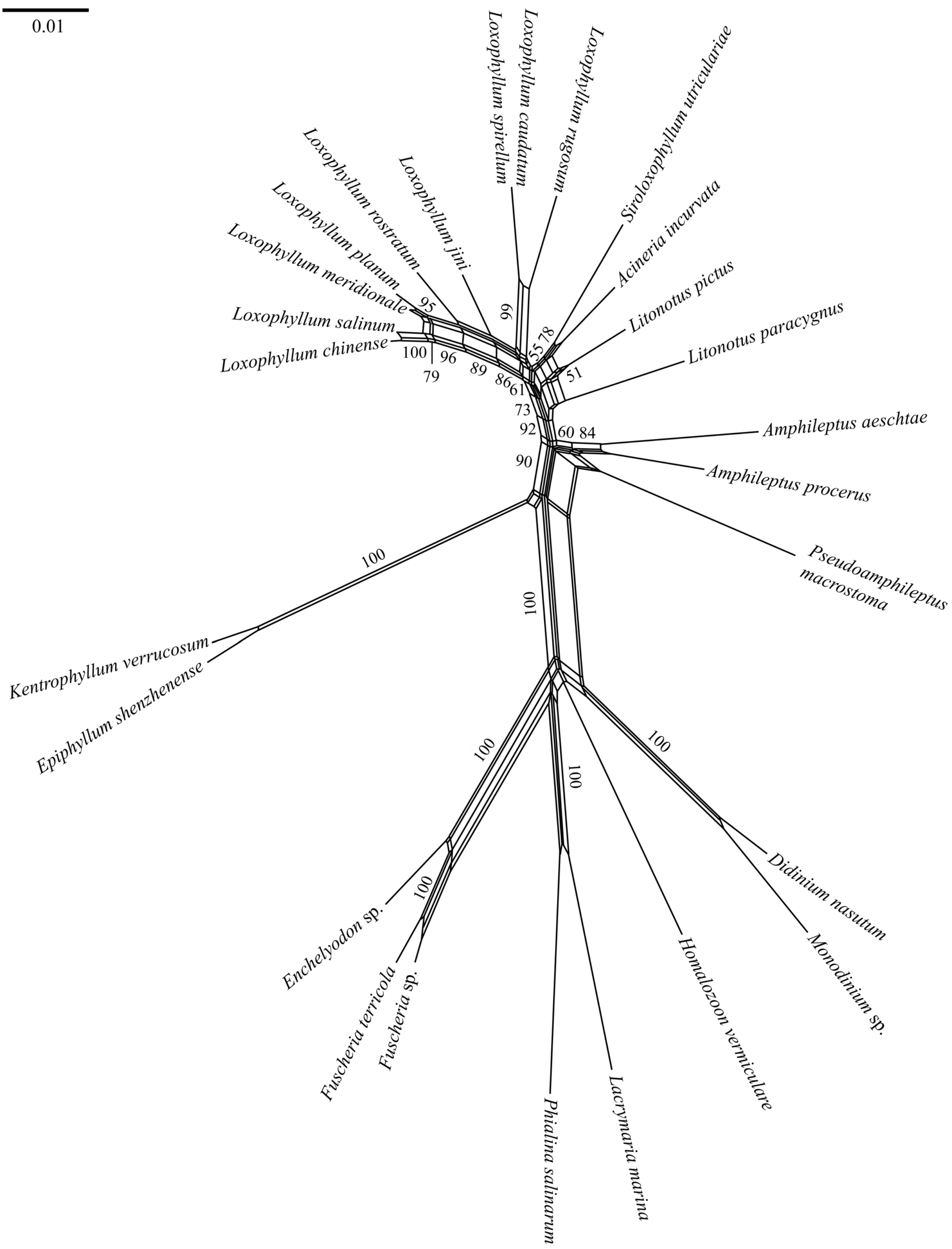


Fig. 4. Phylogenetic network based on 1462 nucleotide characters from 26 haptorian taxa. The split graph was constructed using the neighbornet algorithm and the uncorrected distances. Numbers along edges are bootstrap values coming from 1000 replicates. Values < 50% are not shown. The scale bar indicates one substitution per one hundred nucleotide positions.

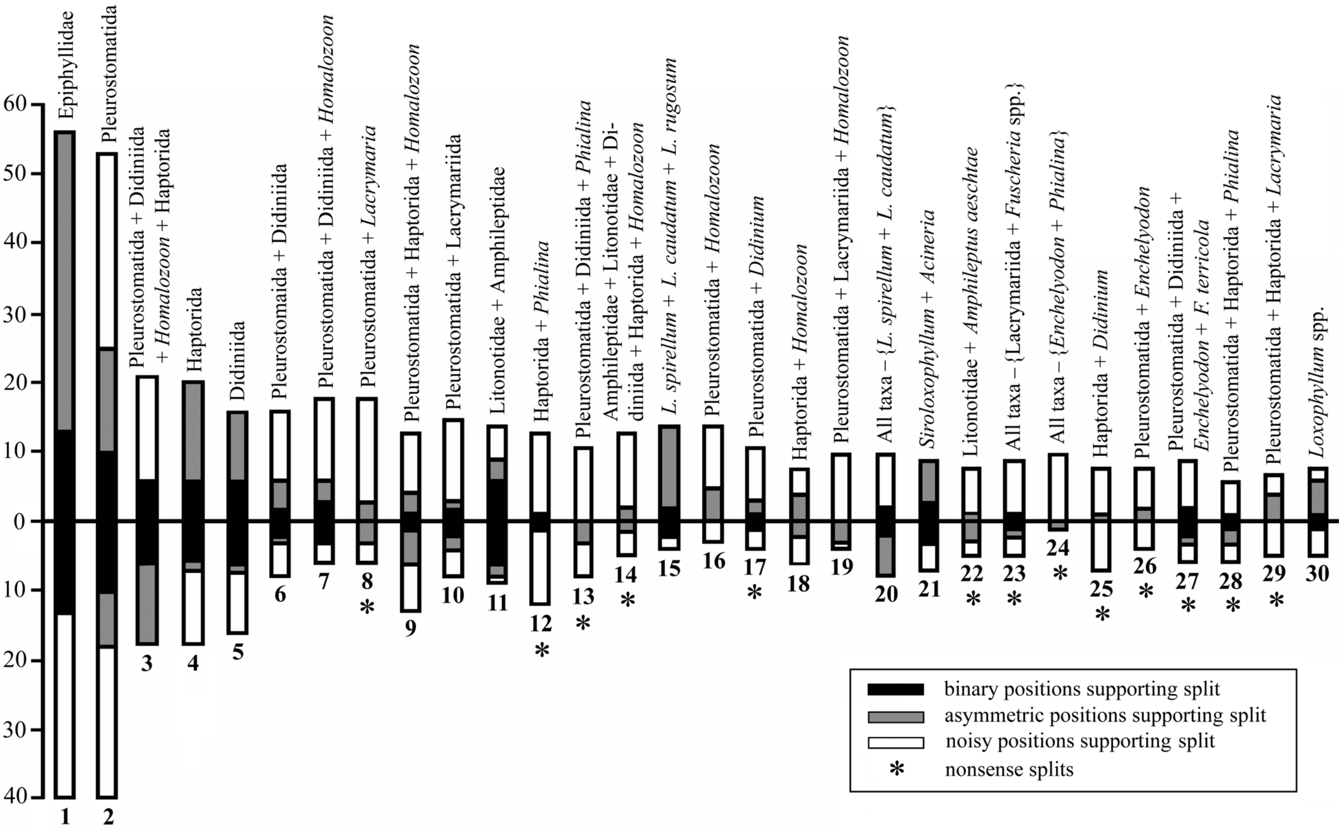


Fig. 5. Split support spectrum for the 18S rRNA gene alignment used to construct the phylogenetic network in Fig. 4. Column height represents the number of clade-supporting positions, i.e., putative primary homologies. Column parts above the y-axis represent the in-group partition, while those below the axis correspond to the out-group partition.

some fringe all around the body except for the oral region (proportional likelihood 0.9954). All other features present in the LCA of the Pleurostomatida remained virtually unchanged (proportional likelihoods ranging between 0.9985 and 1.0000) in the LCA of the Epiphyllidae. The genus *Epiphyllum* maintained the morphology of the LCA of the Epiphyllidae, while the genus *Kentrophyllum* evolved spines and its perioral kinety 1 was transformed into a peripheral kinety (Petz *et al.* 1995, Lin *et al.* 2005b).

The last common ancestor of the family Amphileptidae remained almost morphologically unchanged with respect to the reconstructed morphology of the LCA of the Pleurostomatida (proportional likelihoods ranging between 0.8726 and 1.0000). Given the 26 terminal taxa, the present reconstruction analyses strongly suggested that the moniliform macronucleus of the LCA of the Pleurostomatida was transformed into a binucleate pattern (proportional likelihood 0.9812) in the LCA of the Amphileptidae. Our detailed comparison revealed no other further changes in the ground pattern of the LCA of the Amphileptidae and its name-bearing genus *Amphileptus*. On the other hand, the dorsal brush became dislocated near the ventral side in the genus *Pseudoamphileptus* (Foissner 1983).

In the last common ancestor of the family Litonotidae, there were several conspicuous morphological changes: the oral extrusomes became attached to the whole length of the oral bulge (proportional likelihood 0.9869), a third perioral kinety evolved left of the second perioral kinety (proportional likelihood 0.9807), and the right side somatic kineties became gradually shortened along the pleurostome, a process that erased the ancestral suture-like pattern (proportional likelihood 0.9878). The binodulated macronucleus (proportional likelihood 0.9618) and the single subterminal

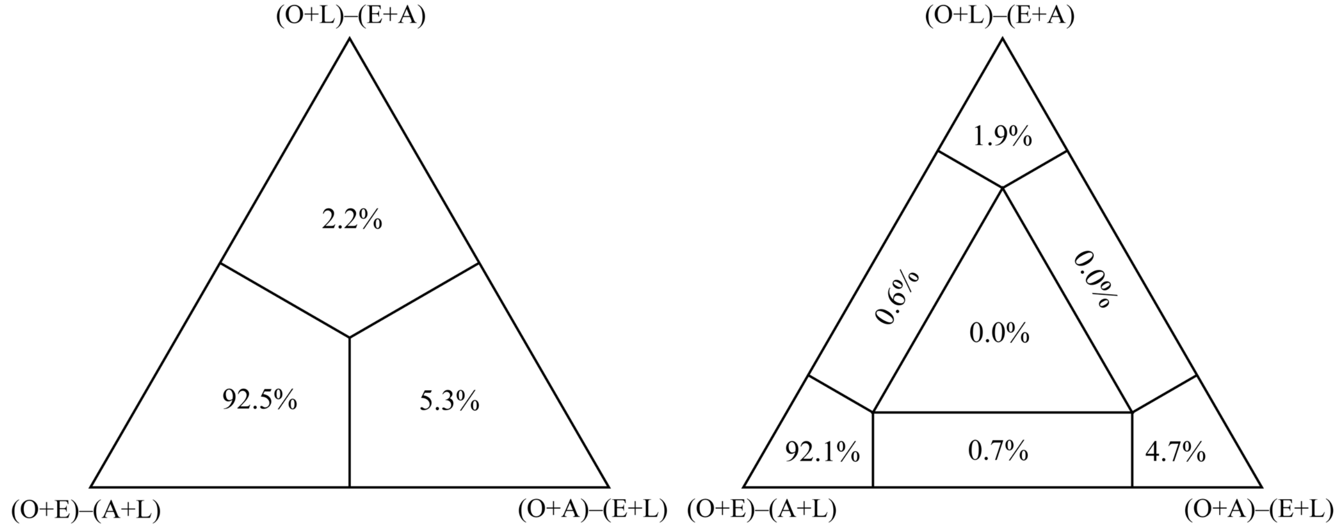


Fig. 6. Quartet likelihood mapping showing distribution of phylogenetic signal in the 18S rRNA gene alignment for three possible relationships among pleurostomatid families. Coding of groups: A – Amphileptidae, E – Epiphyllidae, L – Litonotidae, O – outgroup.

contractile vacuole (proportional likelihood 0.4856) were also very likely properties of the LCA of the Litonotidae. No further morphological apomorphies were found in the genus *Litonotus*. This explains very well its paraphyly observed in the molecular and morphological trees. On the other hand, the pleurostome became anteriorly hook-like curved in *Acineria* (Augustin *et al.* 1987, Foissner *et al.* 1995), while the genus *Loxophyllum* evolved dorsal warts armored with extrusomes (Foissner and Leipe 1995, Lin *et al.* 2009).

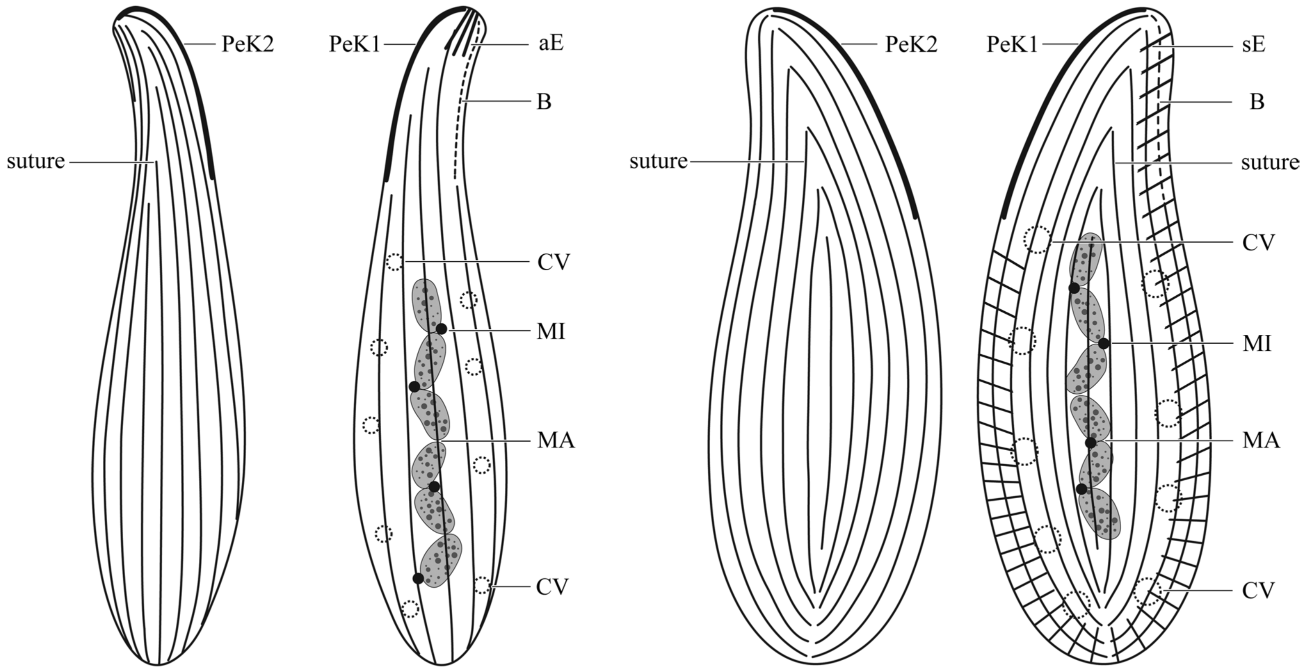
DISCUSSION

Comparison of morphological and molecular phylogenies

Morphological trees are consistent with molecular ones in that the orders Didiniida, Lacrymariida, Haptorida, and Pleurostomatida are monophyletic each. Further, monophyly of the pleurostomatid family Epiphyllidae is strongly supported by all morphological and molecular analyses. However, the family Amphileptidae is not recovered as a monophyletic group in the morphological trees, while its monophyly is comparatively strongly sustained in the molecular and combined trees (compare Fig. 1 with Figs 2 and 3). The same applies to the family Litonotidae whose monophyletic origin received very poor or no support in morphological analyses, while a very strong support in molecular and combined phylogenies. This indicates that phylogenetic relationships within and between the families Amphileptidae and Litonotidae could be biased by the homoplastic nature of several characters and/or morphological apomorphies were outnumbered by homoplasies. Another possible explanation is provided by the likelihood method of reconstruction of character history. This approach shows that features traditionally used to define the family Amphileptidae are apomorphies of the order Pleurostomatida (Fig. 7), i.e., features that are not appropriate to support monophyly of amphileptids in morphological trees because they are plesiomorphies at this level. Analogous problems have been revealed also within the family Litonotidae whose name-bearing genus, *Litonotus*, is based only on the same apomorphies as its family.

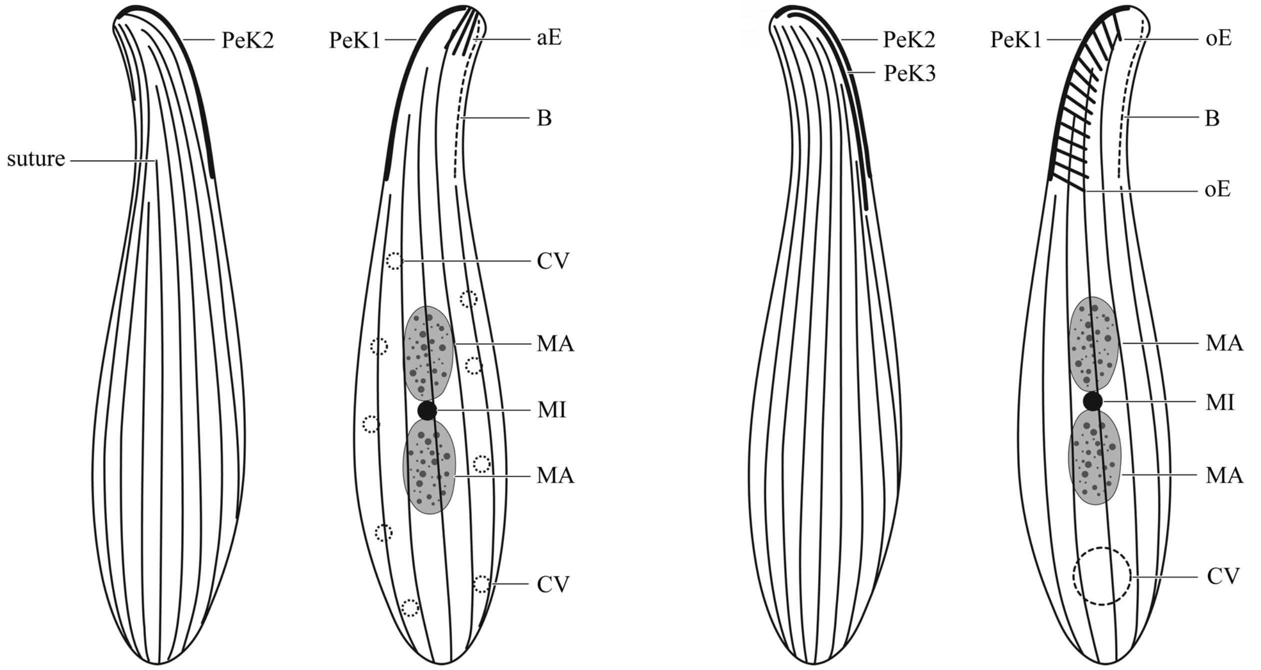
Establishing a new pleurostomatid family: Epiphyllidae

Based on the present morphological and molecular analyses, we have recognized that the genera *Epiphyllum* and *Kentrophyllum* represent a very distinct lineage within the order Pleurostomatida. Since their discovery, they were classified within the family Amphileptidae due to the presence of only two perioral kineties and a suture on the right side of the body (Petz *et al.* 1995,



Reconstruction of the last common ancestor (LCA) of the Pleurostomatida

Reconstruction of the LCA of the Epiphyllidae



Reconstruction of the LCA of the Amphileptidae

Reconstruction of the LCA of the Litonotidae

Fig. 7. Reconstruction of ancestral morphologies in pleurostomatids. Schematic drawings are based on the results of the likelihood method in combination with the Markov evolutionary model implemented in the computer program Mesquite. aE – apical group of oral extrusomes, B – dorsal brush, CV – contractile vacuoles, MA – macronucleus, MI – micronucleus, oE – extrusomes attached along the whole length of the oral bulge, PeK1–3 – preoral kineties 1–3, sE – extrusomes attached to the somatic cortex.

Lin *et al.* 2005b, Pan *et al.* 2010). All subsequent authorities followed this framework (Jankowski 2007, Lynn 2008), though epiphyllids display some peculiar morphological features not present in typical amphileptids, such as a bipolar suture on both the right and the left side of the body or loss of the oral bulge extrusomes (Petz *et al.* 1995, Lin *et al.* 2005b, Pan *et al.* 2010).

18S rRNA gene phylogenies have shown that the genus *Epiphyllum* does not cluster together with other amphileptids, but is depicted as a sister taxon of all other pleurostomatids causing a paraphyly of the traditional family Amphileptidae (Pan *et al.* 2010, 2013; Wu *et al.* 2013). This observation was questioned by Pan *et al.* (2010) in that the statistical tree topology tests do not reject monophyletic grouping of *Epiphyllum* with amphileptids. We have obtained the same result from the present topology tests, though we included a further related genus, *Kentrophyllum*, into the analyses (Table 3). However, a close relationship of epiphyllids and amphileptids has not been depicted in either molecular or combined trees (Figs 2 and 3). Phylogenetic networks have also clearly confirmed the basal position and a long independent evolution of *Epiphyllum* and *Kentrophyllum* within the order Pleurostomatida (Fig. 4). In split spectrum analyses, epiphyllids represent the best split with a total support of 56 conserved nucleotide positions. On the other hand, there is no distinct nucleotide pattern supporting a sister relationship of the families Epiphyllidae and Amphileptidae within the 200 best splits, but the sister relationship of Amphileptidae and Litonotidae is supported by 14 conserved nucleotide positions (Fig. 5). Quartet mapping also strongly supports the basal position of epiphyllids within the order Pleurostomatida with 92.1% of data points, while monophyletic grouping of epiphyllids and amphileptids received a negligible support of 1.9% of data points (Fig. 6). Based on this body of evidence along with three unique morphological apomorphies, we establish a new family, Epiphyllidae, which unites *Epiphyllum* and *Kentrophyllum* (for further details, see Taxonomic summary).

Paraphyly of *Litonotus*

Litonotus represents a taxonomically difficult and species-rich genus that is typically depicted as paraphyletic in phylogenetic trees. Although no primary homologies corroborating its monophyly have been found within the 200 best splits, its monophyly is not rejected by the statistical tree topology tests given the selected GTR + I + Γ evolutionary model (Table 3). This problem can be explained either by incomplete lineage sorting at the rRNA locus or by a rapid radiation event that did not allow primary nucleotide homologies to be fixed for *Litonotus* species. A similar problem was already encountered in spathidiids by Vďačný *et al.* (2014) who used these two evolutionary phenomenons to interpret the confusing haptorian phylogeny. On the other hand, the present likelihood reconstruction of ancestral morphologies provides also a different view on the paraphyly problem of *Litonotus*. Specifically, the ground pattern of the genus *Litonotus* remained virtually unchanged with respect to that of the last common ancestors of the family Litonotidae (Fig. 7). In other words, *Litonotus* is defined solely by apomorphies of its own family, which makes it a 'collective group' for more or less closely related taxa that maintained the ancestral litonotid ground pattern, but underwent a comparatively long independent evolution.

TAXONOMIC SUMMARY

Epiphyllidae fam. nov.

Diagnosis: Leaf-shaped Pleurostomatida without conspicuous neck-like region. Somatic ciliature of both right and left side forming sutures; a single dorsal brush row. One right and one left perioral kinety which can form a peripheral kinety. Oral bulge extrusomes lacking. Somatic extrusomes arranged around whole body except for oral region.

Type genus: *Epiphyllum* Lin, Song and Warren, 2005.

Etymology: Composite of the stem of the name of the type genus and the family suffix -idae [Articles 29.1 and 29.2 of the ICZN (1999)].

Genera assignable: *Epiphyllum* Lin, Song and Warren, 2005; *Kentrophyllum* Petz, Song and Wilbert, 1995.

Remarks: We have chosen *Epiphyllum* as the type genus of the family Epiphyllidae because its actual morphology is closer, according to the present likelihood reconstruction of ancestral ground patterns, to that of the last common progenitor of the Epiphyllidae than the actual morphology of *Kentrophyllum*.

The family Epiphyllidae differs from the family Amphileptidae by the presence of a suture also on the left side of the body, by the lack of oral extrusomes, and by somatic extrusomes forming a fringe all around the whole body except for the oral region. The two epiphyllid genera are easily separated by the absence/presence (*Epiphyllum*/*Kentrophyllum*) of spines along the ventral and dorsal body margin.

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