

Zoological Research

Running Title: Systematics and phylogeny of the class Litostomatea

**Evolutionary relationships within the ciliate class Litostomatea (Alveolata, Ciliophora):
Integrative morpho-molecular insights and the establishment of three new orders and two new
families**

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ABSTRACT

The class Litostomatea Small & Lynn, 1981 represents one of the most morphologically and ecologically diverse groups of ciliates. However, the evolutionary relationships within and among its subgroups remain poorly resolved, largely due to historical taxonomic confusions and sparse molecular data. In this study, we generated 88 new ribosomal DNA sequences spanning 10 orders and more than 17 families of litostomateans. Phylogenetic reconstructions were primarily conducted based on ribosomal gene datasets, supplemented by available genome/transcriptome-scale data, and subsequently interpreted using detailed morphological comparisons to elucidate order- and family-level relationships. Our main results are as follows: 1) thirteen free-living orders are identified, with the monophyly of most groups (excluding Spathidiida and Haptorida) being well-supported, and the order Helicoprordontida is recovered as the earliest-diverging lineage; 2) the “traditional haptorids” are separated from the species-rich and taxonomically complex order Spathidiida, and reassigned to the order Haptorida; 3) several anaerobic spathidiids and haptorids possessing unique morphological traits form an intermediate taxon that appears to bridge free-living and endosymbiotic lifestyles; 4) five new taxa are established, namely Chaeneida **ord. nov.**, Fuscheriida **ord. nov.**, Kamburophryida **ord. nov.**, Monilicaryonidae **fam. nov.** and Papillorhabdidae **fam. nov.**, and the previously described orders Homalozoonida and Pseudoholophryida are reinstated; 5) divergence times within the class Litostomatea are updated based on a robust phylogenetic framework and expanded taxon sampling; and 6) a new evolutionary hypothesis and an updated systematic classification for the class Litostomatea are proposed.

Keywords: Classification system; Evolutionary hypothesis; Multi-gene; New taxa; Phylogeny

INTRODUCTION

Ciliated protists (ciliates) are a highly specialized group of unicellular eukaryotes that serve as important model organisms in multiple disciplines such as cell biology, evolutionary biology, and ecology (Fokin et al., 2023; Košťířová et al., 2026; Lynn, 2008; Obert et al., 2025; Pan et al., 2024; Shi et al., 2025; Zhang et al., 2026). Members of the class Litostomatea Small & Lynn, 1981 constitute a morphologically diverse group comprising thousands of species, which are currently assigned to three subclasses: the free-living Haptoria Corliss, 1974 and Rhynchostomatia Jankowski, 1980, and the endosymbiotic Trichostomatia Bütschli, 1889 (Vďačný et al., 2011a; Vďačný et al., 2011b). The free-living forms act as predators in microbial food webs across a wide range of aquatic and terrestrial ecosystems, playing a pivotal role in the transfer of matter and energy (Zhou et al., 2024).

Historically, based on morphological characters, the free-living litostomateans were widely recognized as being represented by two orders, Haptorida Corliss, 1974 and Pleurostomatida Schewiakoff, 1896 (Corliss, 1979; Lynn, 2008). Nevertheless, despite their broad acceptance, a number of taxonomic uncertainties persisted. With the advent and increasing use of molecular techniques, some issues have been clarified to some extent, such as the validity of the order Helicoprordontida Grain, 1994, the systematic positions of the genera *Foissnerides* Song & Wilbert, 1989 and *Balantidium* Eberhard, 1862, and the justification for including the genus *Trachelotractus* Foissner, 1997 within Litostomatea (Chi et al., 2022; Huang et al., 2018; Zhang et al., 2012). In addition, a recent classification scheme based on SSU rDNA data has refined the systematics of free-living litostomateans, recognizing seven orders: Haptorida, Pleurostomatida, Lacrymariida Lipscomb & Riordan, 1990, Didiniida Jankowski, 1978, Spathidiida Foissner & Foissner, 1988, Tracheliida Vďačný et al., 2011, and Dileptida Jankowski, 1978 (Vďačný et al., 2011a).

Although molecular approaches have advanced our understanding, many key systematic issues within and among these taxa remain unresolved, such as which lineage is ancestral, which species should be included in the heterogeneous order Spathidiida, how free-living and endosymbiotic forms are evolutionarily connected, to which order *Homalozoon* Stokes, 1890 should belong, and cases in which even members of the same family or genus appear scattered across different branches of phylogenetic trees (Chi et al., 2024a; Huang et al., 2018; Jang et al., 2017; Vďačný et al., 2011a, 2012). These uncertainties likely stem from the extensive morphological diversity within the group, particularly in

body size and shape, somatic ciliary patterns, and oral structures. For example, some species with similar live morphology exhibit markedly different infraciliature patterns after silver staining, which may necessitate their classification into different families or even orders (Foissner et al., 2002; Foissner & Lei, 2004). This morphological variability makes identification challenging and may lead to misidentifications and misclassifications, further complicating phylogenetic interpretations. Additionally, many taxa occur at low abundances in natural environments, and their predatory lifestyle hampers laboratory cultivation, resulting in a significant lack of molecular data at the family and genus levels (Gao et al., 2025; Vďačný & Foissner, 2012). These limitations hinder the construction of a satisfactory phylogenetic framework.

To address these issues, 29 free-living litostomateans, representing 10 orders and more than 17 families, were isolated and identified, and 88 new ribosomal DNA sequences were obtained from these specimens. By combining molecular phylogenetic analyses with detailed morphological comparisons, the evolutionary relationships among all free-living litostomatean lineages were examined. This study aims to refine previous taxonomic ambiguities and to provide a referential phylogenetic and taxonomic framework based on currently available data, offering new insights for future studies on the biodiversity and evolution of this highly diverse ciliate group. In addition, the present study highlights the importance of accurate morphological identification in resolving systematic controversies within Litostomatea and provides a foundation for future efforts to refine the classification of this class at finer taxonomic levels, rather than amalgamating morphologically disparate taxa into broad heterogeneous assemblages, thereby promoting a more rational and phylogenetically meaningful taxonomy.

MATERIALS AND METHODS

Sample collection and identification

A total of 29 free-living litostomatean species (32 populations) were isolated from the Lake Weishan Wetland, China (Table 1). Species were identified based on observations of living cells under bright field and differential interference contrast (DIC) microscopy, as well as on protargol-stained preparations following Wilbert (1975). Morphological information for these species, including photomicrographs and brief descriptions, are provided in Supplementary Figure S1. Terminology and

systematic classification are mainly according to Lynn (2008) and Vďačný et al. (2011a).

DNA extraction, PCR amplification, and gene sequencing

For each species, either a single cell or a small number of conspecific cells were isolated and washed five times with 0.22 µm filtered *in situ* water. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Germany), following the manufacturer's protocol with a modification in which one-quarter of the recommended reagent volumes was used. Primers used for amplification of SSU rDNA, ITS1-5.8S-ITS2, and LSU rDNA are listed in Supplementary Table S1. PCR amplification was performed using Q5® Hot Start High-Fidelity 2× Master Mix DNA Polymerase (New England BioLabs, USA) to minimize experimental errors, under conditions described by Chi et al. (2024b). PCR products were verified via agarose gel electrophoresis and sequenced bidirectionally by Tsingke Biological Technology Company (Qingdao, China). Sequence fragments were assembled using Seqman (DNASar).

Datasets and alignments

Datasets 1–4, based on ribosomal DNA sequences (SSU rDNA, ITS1-5.8S-ITS2, LSU rDNA, and concatenated rDNA), included the newly generated ribosomal sequences along with representative litostomeans and CONthreeP taxa (outgroup) downloaded from GenBank database (Table 2; Supplementary Table S2). Initial alignments were performed using MAFFT with the E-INS-I strategy (Katoh & Standley, 2013). Ambiguously aligned regions were removed using BioEdit (Hall, 1999) prior to phylogenetic analyses. Concatenated datasets were compiled using SeaView v.4.0 (Gouy et al., 2009).

Dataset 5 was constructed from genomic and transcriptomic data based on the first 100 core genes identified for 17 litostomeans and five CONthreeP taxa (outgroup) by Wang et al. (2026) (<http://106.13.47.147:8216/siglCellData>) (Table 2; Supplementary Table S3). BLAST+ v.2.16.0 was employed to search orthologs in the genomic/transcriptomic data of nine newly sequenced litostomeans, with an e-value threshold of 1e-10. Of these 100 target core genes, orthologs were recovered for 89 genes, which were retained for subsequent phylogenomic analyses. The retained sequences were translated into amino acids using Augustus (Keller et al., 2011). Because not all taxa possessed all 89 retained genes, genes absent from particular taxa were represented by the missing

character (“?”) in the concatenated alignment. Multiple sequence alignments (MSAs) were generated for each gene using MAFFT v.7.525 (Kato et al., 2019) and concatenated with PhyKIT v.1.11.7, yielding a final alignment of 57 581 amino acid sites (Steenwyk et al., 2021). The concatenated alignment and the corresponding gene partition file defining the boundaries of the 89 concatenated genes are provided as Supplementary Data S1 and Data S2.

Phylogenetic analyses

For the four ribosomal datasets, maximum likelihood (ML) analyses were conducted using IQ-TREE v.2.0 with 10 000 ultrafast bootstrap replicates (Minh et al., 2020). The best-fit substitution models were automatically determined by IQ-TREE’s internal model-testing procedure (Table 2).

Bayesian inference (BI) analyses were performed using MrBayes v.3.2.7a (Ronquist et al., 2012) on ACCESS via the CIPRES Science Gateway (Miller et al., 2010). For BI analyses, the optimal nucleotide substitution model was evaluated using MrModeltest v.2.4 (Nylander, 2004) under the Akaike Information Criterion (AIC), and GTR+I+G was selected for all ribosomal datasets. Markov chain Monte Carlo (MCMC) simulations were run for 10 000 000 generations with the first 25% of samples discarded as burn-in. Convergence was confirmed when the average standard deviation of split frequencies was below 0.01. Phylogenetic trees were visualized using ChiPlot v.2.6.1 (Xie et al., 2023).

The phylogenomic dataset (Dataset 5) was analyzed separately. An ML tree was inferred using IQ-TREE v.2.0.3 under the optimal substitution model LG+F+I+G4, automatically selected via the -TEST option. Topological robustness was assessed with 1 000 ultrafast bootstrap replicates.

Calibration points selection and molecular clock analyses

Due to the complete absence of a primary fossil record for the class Litostomatea, our molecular dating analyses relied on a combination of secondary calibrations and a host-derived primary calibration. Vd’ačný (2015) first estimated the divergence time of Litostomatea based on the early molecular dating framework of Wright & Lynn (1997). Building on this foundation and incorporating updated molecular data, we selected three calibration points: 1) a maximum bound of 764 Ma (million years ago) for the class Litostomatea (secondary calibration); 2) a minimum bound of 522 Ma for the order Pleurostomatida (secondary calibration); and 3) a maximum bound of 145 Ma for the endosymbiotic

trichostomes, which serves as a host-derived calibration based on the primary fossil remnants of early therian mammals (Vďačný, 2018).

Divergence times were estimated within a Bayesian framework using the MCMCTree in the PAML v.4.9j package (Yang, 2007). The ML tree inferred from Dataset 4, which exhibited strong support, was used as the reference topology. A likelihood ratio test in BaseML rejected the global molecular clock; therefore, a relaxed molecular clock was adopted in MCMCTree, using the independent rates model (clock = 2). Birth and death parameters were set as $\lambda = \mu = 2$ and $\rho = 0.1$, and the HKY85 + Γ_5 model was used. Priors were set as follows: a gamma prior $G(6, 2)$ for the transition/transversion rate ratio (κ), a gamma prior $G(1, 1)$ for the shape parameter (α), and a diffuse gamma prior $G(1, 75.47)$ for the nucleotide substitution rate (μ), corresponding to a mean of 0.01325 substitutions per site per 100 Myr, in line with previous studies (Vďačný, 2015; Wright & Lynn, 1997; Zhang & Vďačný, 2022). The variance of the log rate (σ^2) was given a diffuse gamma prior with a mean equal to the reciprocal of the prior root age, as recommended by Thorne et al. (1998). The subphylum Intramacronucleata was considered as the root, with its age defined based on estimates from Jiang et al. (2024) and Fernandes & Schrago (2019). MCMC chains were run for 150 000 iterations, with samples drawn every two iterations after a burn-in of 15 000 iterations.

RESULTS AND DISCUSSION

Summary of new sequences and phylogenetic trees

A total of 88 new sequences were generated in this study, including 26 SSU rDNA, 30 ITS1-5.8S-ITS2, and 32 LSU rDNA sequences from 32 populations representing 10 orders and more than 17 families. These sequences have been deposited in the GenBank database, and their lengths, GC contents, and accession numbers are summarized in Table 1. Notably, molecular data for several genera, namely *Enchelyotricha* Foissner, 1987, *Kamburophrys* Foissner & Oertel, 2009, *Myriokaryon* Jankowski, 1973, and *Neobryophyllum* Foissner in Foissner & Lei, 2004, are reported here for the first time.

Phylogenetic analyses were conducted using the newly obtained sequences together with carefully screened representative sequences from GenBank database. Multiple phylogenetic trees were constructed using both ribosomal genes and genome/transcriptome-scale datasets. As the topologies

inferred from ML analyses of ribosomal genes were largely congruent with those recovered from BI, only ML results are presented.

Phylogenetic overview of the class Litostomatea

The class Litostomatea is currently recognized to comprise three subclasses: the free-living Haptoria and Rhynchostomatia, and the endosymbiotic Trichostomatia. Although this study focuses primarily on the phylogenetic relationships among free-living lineages, representatives of the trichostomatian orders Entodiniomorphida Reichenow in Doflein & Reichenow, 1929 and Vestibuliferida de Puytorac et al., 1974 were included to clarify the position of the subclass.

Compared with the free-living forms, trichostomatians constitute a strongly supported monophyletic group that diverged relatively late in litostomatean evolution. However, in all phylogenetic reconstructions, Trichostomatia appears deeply nested within Haptoria, rendering the latter non-monophyletic. A similar pattern is observed for Rhynchostomatia, which also consistently nests within Haptoria (Figures 1–4).

In the present study, we recognize 13 orders of free-living litostomateans, including three that are newly established. With the exception of the orders Spathidiida (in all trees), Haptorida (in all trees), Pleurostomatida (in ITS1-5.8S-ITS2 and LSU rDNA trees), and Pseudoholophryida (in LSU rDNA tree), all remaining orders are recovered as monophyletic. In the following sections, we provide a detailed account of their phylogenetic positions and relationships, integrating both morphological and molecular evidence.

Ancestral lineage of the class Litostomatea

Due to the scarcity of genomic and transcriptomic data, the phylogenetic position of the order Helicoprordontida has remained undetermined in genome/transcriptome-scale analyses. However, all ribosomal-gene trees in this study consistently identify helicoprordontids as the earliest diverging litostomatean lineage with full support values (Figures 1–3). This result is congruent with recent phylogenetic studies incorporating helicoprordontid sequences (Bourland, 2023; Chi et al., 2022; Chi et al., 2024a; Chi et al., 2021; Huang et al., 2018; Jang et al., 2022; Omar et al., 2022; Pomahač et al., 2023; Rajter et al., 2019; Vďačný & Rataj, 2017). It contrasts, however, with early studies that

hypothesized the subclass Rhynchostomatia as the basal lineage (Vďačný et al., 2011a; Vďačný et al., 2011b), likely due to limited molecular sampling at that time.

The order Helicoprordontida comprises a single family, Helicoprordontidae Small & Lynn, 1985, and two genera, *Helicoprordon* Fauré-Fremiet, 1950 and *Trachelotractus* Foissner, 1997. Their most distinctive character is the presence of multiple somatic kineties that extend towards the apical region and spiral several times around the cytostome, clearly distinguishing them from other taxa (Foissner, 1997; Jiang et al., 2021). In addition, the key characters supporting their status as an ancestral lineage may include their typically vermiform shape and their inhabitation of marine sediments, reminiscent of the class Karyorelictea Corliss, 1974, which is widely regarded as preserving a number of ancestral morphological traits among ciliates.

Establishment of Chaeneida ord. nov. independent from Lacrymariida

The genus *Chaenea* Quennerstedt, 1867 appears as the second lineage to diverge after Helicoprordontida, based on both concatenated rDNA and genome/transcriptome-scale phylogenies, with full support values. It is followed by Lacrymariida, although the support values differ among trees (60/0.57 in concatenated rDNA tree vs. 99 in phylogenomic tree) (Figures 1, 4). By comparison, *Chaenea* clusters with Rhynchostomatia in the SSU rDNA tree and with Pleurostomatida in the ITS1-5.8S-ITS2 tree, both with low support (75/* and 84/0.62), and subsequently groups with Lacrymariida (76/* and 78/*). In addition, *Chaenea* forms a sister relationship with Lacrymariida in the LSU rDNA tree (90/1.00) (Figures 2, 3). Taken together, these results indicate that the genus *Chaenea* represents a distinct, early diverging lineage that frequently shows affinity to Lacrymariida in molecular phylogenetic analyses.

Historically, the systematic position of *Chaenea* has been contentious, with assignments to Enchelyidae Ehrenberg, 1838, Trachelophyllidae Kent, 1882, and Acropisthiidae Foissner & Foissner, 1988 (Corliss, 1979; Foissner, 1984; Lipscomb & Riordan, 1990). Kwon et al. (2014) later established the new family Chaeneidae to accommodate *Chaenea* and provisionally placed it within the order Lacrymariida due to the presence of cilium-like brush bristles resembling those of *Lacrymaria* species. However, our phylogenetic results do not support this ordinal placement and instead favor the recognition of chaeneids as an independent order. Morphologically, compared with lacrymariids,

chaeneids lack a differentiated head region bearing head kineties; instead, they possess narrowly spaced oralized somatic monokinetids. Although the head kineties of lacrymariids and oralized somatic monokinetids of chaeneids both arise immediately posterior to the circumoral kinety and may therefore be homologous, they differ markedly in their morphology and organization. Furthermore, chaeneids consistently possess four dorsal brush rows differentiated from the anterior portion of somatic kineties, whereas in lacrymariids, typically all somatic kineties exhibit anterior differentiation into a dorsal brush (Kwon et al., 2014; Tang et al., 2023; Vďačný & Rataj, 2017; Xu et al., 2025), although exceptions have recently been reported (Allievi et al., 2026). These differences strongly support the notion that chaeneids represent a morphologically distinct lineage. Therefore, based on both morphological distinctions and molecular phylogenetic support, we propose removing Chaeneidae from Lacrymariida and elevating it to a new order, Chaeneida **ord. nov.**

Reinstatement of Homalozoonida and its closely related taxa

The present phylogenetic analyses indicate a close relationship among the orders Homalozoonida Jankowski, 1980, Fuscheriida **ord. nov.** (established below), and Pleurostomatida. Specifically, Homalozoonida and Pleurostomatida are consistently recovered as sister groups within a moderately-to-strongly supported clade in the concatenated rDNA and SSU rDNA trees (85/0.98 and 90/0.98), while Fuscheriida **ord. nov.** branches peripherally to this clade with weak support (37/0.70 and 55/*) (Figures 1, 2). In contrast, the ITS1-5.8S-ITS2 tree places Homalozoonida and Fuscheriida **ord. nov.** together with *Protolitonotus magnus* (a pleurostomatid ciliate) on one branch (79/0.73), whereas the remaining pleurostomatids cluster with Chaeneida **ord. nov.** and Lacrymariida on a separate poorly supported branch (78/*), and these two branches have a weakly supported sister relationship (79/0.65). In the LSU rDNA tree, Fuscheriida **ord. nov.** is nested within Pleurostomatida, and Homalozoonida forms a separate branch (Figure 3). Due to the absence of genomic and transcriptomic data for both Fuscheriida **ord. nov.** and Homalozoonida, their positions remain unknown in the phylogenomic tree.

From a morphological perspective, the sister relationship of Homalozoonida and Pleurostomatida is plausible as these are the only two litostomatean taxa exhibiting left-right asymmetry in somatic ciliature, where the density of kineties differs between the two sides of the cell. Nevertheless, they differ from each other markedly in several key characters: 1) oral apparatus located along the ventral margin

in Pleurostomatida (vs. located apically in Homalozoonida); 2) presence of perioral kineties in Pleurostomatida (vs. absence in Homalozoonida); and 3) absence of circumoral kinety in Pleurostomatida (vs. presence in Homalozoonida) (Foissner, 1984; Zhang et al., 2024). In the most recent systematic revision of Litostomatea (Vďačný et al., 2011a), the genus *Homalozoon* Stokes, 1890 was treated as *incertae sedis*, although the authors, consistent with Foissner & Foissner (1988), speculated that it might represent a distinct order. Our results support this view, and because the order name was already proposed by Jankowski (1980), we reactivate it as Homalozoonida Jankowski, 1980.

Establishment of Fuscheriida ord. nov. as a morphologically haptorid-like lineage

Although the phylogenetic position of Fuscheriida **ord. nov.** remains uncertain, the present analyses consistently suggest potential relationships with Homalozoonida and Pleurostomatida. However, no specific morphological characters linking them have been identified. In contrast, members of Fuscheriida **ord. nov.** are morphologically more similar to haptorids. They exhibit a generally ellipsoidal body, meridionally arranged equidistant somatic kineties, an apical oral apparatus, and a circumoral kinety, traits which historically led to their classification within Haptorida (Foissner et al., 2002). Remarkably, in the most recent classification, fuscheriids were even recognized as “true haptorids”, while the “traditional haptorids” were assigned to the order Spathidiida (Vďačný et al., 2011a; Vďačný & Foissner, 2013).

This taxonomic confusion likely stemmed from the molecular clustering of *Fuscheria* sp. with *Enchelyodon* sp. (U80313), a haptorid sequence lacking morphological data, whereas the “traditional haptorids” were scattered within Spathidiida (Vďačný et al., 2011a). Morphologically, these two genera are similar *in vivo*, differing mainly in the dorsal brush (two rows in *Fuscheria* vs. three rows in *Enchelyodon*) (Foissner, 1984; Foissner et al., 2002). Such subtle differences often cause misidentification if based solely on live observation, emphasizing the necessity of silver-stained preparations. This is a persistent issue in the class Litostomatea, particularly within Haptorida and Spathidiida.

In addition, with the expanded molecular sampling of fuscheriids and representative haptorids (Chi et al., 2022; Chi et al., 2024a; Jang et al., 2022; Rajter & Vďačný, 2017), including data from this study, fuscheriids consistently form an independent lineage that is distant from haptorids. We therefore

establish a separate order for fuscheriids, namely Fuscheriida **ord. nov.**, and return the historically recognized “traditional haptorids” to the order Haptorida. Based on current knowledge, the two orders can be distinguished only by the dorsal brush (two rows in Fuscheriida **ord. nov.** vs. usually three rows, rarely one row, in Haptorida). Notably, the family Fuscheriidae Foissner et al., 2002 still includes taxa with three dorsal brush rows (e.g., *Coriplites* Foissner, 1988), which warrants additional molecular data to confirm their classification.

Reinstatement of Pseudoholophryida and its close relationship with Didiniida

The order Pseudoholophryida was established by Foissner & Foissner (1988) to encompass two new suborders, Pseudoholophryina and Helicoprordontina. Following the elevation of Helicoprordontina to ordinal rank (Vďačný & Rataj, 2017), Pseudoholophryida was reduced to a monotypic order containing only the family Pseudoholophryidae Berger et al., 1984. Due to a lack of molecular data, this order was provisionally treated as *incertae sedis* in the classification of the class Litostomatea proposed by Vďačný et al. (2011a). Subsequent phylogenetic analyses based on SSU rDNA sequences indicated that pseudoholophryids are nested within the Spathidiida lineage; consequently, Pseudoholophryida was suppressed and its sole family transferred to Spathidiida (Rajter & Vďačný, 2016, 2017). A consistent result was recovered in the present SSU rDNA phylogeny, with all pseudoholophryids clustering with Clade 8 of the Spathidiida + Haptorida assemblage. However, this grouping lacked strong statistical support in both previous and current analyses (Figure 2).

In contrast, most analyses in this study recovered a close relationship between Pseudoholophryida and Didiniida, followed by members of the Spathidiida + Haptorida assemblage. Although the concatenated rDNA tree provides only weak support for a pseudoholophryids + didiniids clade (46/0.84), support is better in the LSU rDNA tree (93/0.97) and phylogenomic tree (99). In the ITS1-5.8S-ITS2 tree, however, the positions of both taxa remain uncertain due to the weak resolution with other litostomateans (Figures 1, 3, 4).

From a morphological perspective, these two taxa share characters consistent with a close relationship, such as small to medium size, fast-swimming, and the presence of multiple dorsal brush rows (more than three rows). Nevertheless, each taxon exhibits distinct morphological characters. For example, pseudoholophryids are characterized by spiral somatic kinetics and a highly aberrant dorsal

brush composed of numerous minute kinetofragments interspersed between ordinary somatic kinetosomes (except in *Acaryophrya* André, 1915) (Foissner et al., 2002). These characters differ markedly from those of typical spathidiids (e.g., *Spathidium* and related taxa) and do not conform to the diagnosis of Spathidiida. Based on combined morphological and molecular evidence, we recommend transferring the family Pseudoholophryidae out of Spathidiida and reinstating the order Pseudoholophryida. Similarly, the order Didiniida is morphologically distinctive, being characterized by its prominent oral dome and ciliary girdles encircling the entire cell (Foissner et al., 1999). These unique characters clearly support its recognition as a separate clade, even though its phylogenetic position has long remained ambiguous (e.g., Bourland, 2023; Chi et al., 2024a; Jang et al., 2022; Kwon et al., 2014; Omar et al., 2022; Pomahač et al., 2023; Vďačný et al., 2011; Vďačný & Rataj, 2017; Zhang et al., 2012).

Although our results tentatively indicate that Pseudoholophryida and Didiniida are closely related, this relationship requires further investigation. First, the present phylogenetic results have not yet achieved maximal support. Second, in the LSU rDNA tree, *Paraenchelys terricola* clusters with Didiniida as a sister branch, a relationship also recovered in the Bayesian analysis of the concatenated rDNA tree (not shown). This highlights the need for additional data to better define their boundaries and clarify their evolutionary relationships.

Phylogenetic instability of the subclass Rhynchostomatia

The subclass Rhynchostomatia, established by Jankowski (1980), was later redefined by Vďačný et al. (2011b) and subdivided into two orders: Dileptida and Tracheliida. Members of both orders are characterized by a conspicuous proboscis bearing oral ciliature (including the circumoral kinety, perioral kinety, and preoral kineties), well-developed extrusomes, and a dorsal brush. Their oral opening is situated at the base of the proboscis, opening ventrally and obliquely (Chi et al., 2021; Vďačný & Foissner, 2012).

Our phylogenetic analyses, however, revealed highly inconsistent placements of rhynchostomatians. In the SSU rDNA, ITS1-5.8S-ITS2, and phylogenomic trees, Dileptida and Tracheliida consistently formed a fully supported sister branch, but the placement of this branch remained unstable due to weak support for its links with neighboring lineages (75/*, 52/* and 54,

respectively; Figures 2–4). In contrast, the concatenated rDNA tree robustly recovered Dileptida and Tracheliida as the sister group to Kamburophryida **ord. nov.** (established below) with full statistical support (100/1.00; Figure 1), which collectively clustered with spathidiids and haptorids. This close affinity is independently reaffirmed in the LSU rDNA tree, where Tracheliida and Kamburophryida **ord. nov.** form a fully supported cluster (100/1.00) within the Spathidiida + Haptorida assemblage, whereas Dileptida emerged as an early diverging lineage after Helicoprordontida (Figure 3).

Concatenated multi-gene datasets typically enhance phylogenetic signals and yield more robust tree topologies than single markers (Chi et al., 2021). Our concatenated rDNA analysis strongly supports a close relationship among rhynchostomatians, spathidiids, and haptorids. Rhynchostomatians possess a relatively complex oral ciliature, typically comprising a circumoral kinety (~paroral membrane), a perioral kinety and numerous preoral kineties (~adoral zone of membranelles), the latter two of which are absent in other litostomateans. These morpho-molecular data suggest that Rhynchostomatia might represent a highly specialized lineage that diverged relatively recently within the evolutionary history of litostomateans. Nonetheless, additional taxon sampling with both morphological and multi-gene data are required to test this hypothesis.

Establishment of Kamburophryida ord. nov. as a basal lineage of Rhynchostomatia

As noted above, our analyses indicate a close relationship between the genus *Kamburophrys* and the previously classified rhynchostomatians (i.e., Dileptida and Tracheliida) (Figures 1, 3). *Kamburophrys* was established by Foissner & Oertel (2009) based on three peculiar characters: a subapical hump carrying the dorsal brush and brush membranoid, a bulge cone, and an oblique circumoral kinety. However, its ordinal affiliation has remained uncertain. The original authors noted that the oblique circumoral kinety of *Kamburophrys* resembled that of spathidiids, but could find no other evidence of a relationship between them. They also proposed that the unique brush membranoid, which is separated from the somatic kineties, might share an ontogenetic origin with the preoral kineties of *Dileptus* (Foissner & Oertel, 2009; Vďačný & Foissner, 2009).

We argue that *Kamburophrys* does share significant morphological and behavioral affinities with rhynchostomatians. First, although *Kamburophrys* lacks a conspicuous proboscis, it possesses a subapical hump carrying the dorsal brush and brush membranoid (~preoral kineties). To some extent,

the hump serves a functionally analogous role to a proboscis. Second, the orientation of the circumoral kinety of *Kamburophrys* indicates a ventrally and obliquely positioned oral opening, similar to that in dileptids and tracheliids. Furthermore, the prominent proboscis of dileptids and tracheliids leads to a forward-swinging or whipping motion during locomotion, rather than the axial rotation of symmetrical ciliates. This distinct movement is also observed in *Kamburophrys*, likely attributable to the presence of the subapical hump (~proboscis).

Based on these morphological characters and molecular evidence, we transfer *Kamburophrys* to the subclass Rhynchostomatia and establish a new order, Kamburophryida **ord. nov.** for it due to its significant morphological divergence from existing orders. Phylogenetic reconstructions further suggest that Kamburophryida **ord. nov.** represents the basal branch of Rhynchostomatia and is more closely related to Tracheliida than to Dileptida. This implies an evolutionary trajectory wherein ancestral rhynchostomatians underwent a gradual reduction of the oral bulge and cone, while the dorsal region specialized into a proboscis. This emergent organelle likely not only inherited the functions of the oral bulge but also became increasingly complex and specialized.

Establishment of Monilicaryonidae fam. nov.

With the inclusion of Kamburophryida **ord. nov.**, Rhynchostomatia is redefined to comprise three orders, which can be distinguished by general appearance following the criteria of Vd'ačný & Foissner (2012): 1) Dileptida, oblong body with agile proboscis; 2) Tracheliida, ovoidal body with immobile or slightly mobile proboscis; 3) Kamburophryida **ord. nov.**, slender obpyriform body with a dorsal hump (~proboscis) and a bulge cone. Tracheliida and Kamburophryida **ord. nov.** are monotypic, whereas Dileptida contains two families, primarily differentiated by macronuclear morphology: 1) Dileptidae Jankowski, 1980 having at least four moniliform or scattered nodules; 2) Dimacrocarionidae Vd'ačný et al., 2011 having one or two nodules (Vd'ačný et al., 2011b).

Interestingly, the Chinese population of *Monilicaryon monilatum* (the type species of *Monilicaryon*) that we isolated fits Dileptidae in having a moniliform macronucleus, yet it consistently clusters at the periphery of the Dileptidae + Dimacrocarionidae clade in all phylogenetic trees (Figures 1–3). Morphologically, it is exceptional among rhynchostomatians, featuring a perioral-like kinety made by linearly arranged preoral kineties and a single dorsal brush row (Vd'ačný & Foissner, 2012) (Figure 2C).

Given the taxonomic importance of these characters within Rhynchostomatia and even Litostomatea, together with the molecular phylogenetic results, we propose removing *Monilicaryon* from Dileptidae and transferring it to a new family, Monilicaryonidae **fam. nov.**

Intricate and interwoven phylogeny between Spathidiida and Haptorida

With the increasing availability of molecular data, the phylogeny of Spathidiida has emerged as a melting pot that encompasses morphologically diverse taxa (Barmshuri et al., 2023; Chi et al., 2022; Chi et al., 2024a; Pomahač et al., 2023). This complexity has led to persistent confusion in both morphological taxonomy and molecular systematics. Based on the analyses presented above, we proposed two taxonomic revisions: 1) reinstatement of pseudoholophryids, formerly included in Spathidiida, to ordinal rank; and 2) reassignment of the “traditional haptorids”, previously placed in Spathidiida, to the order Haptorida. Across all phylogenetic trees, Pseudoholophryida is generally monophyletic, whereas Spathidiida and Haptorida form an intermingled assemblage.

In the concatenated rDNA tree, the Spathidiida + Haptorida assemblage is resolved into eight clades (excluding Clade 1, which is treated as a special case in section 3.2.10), and these are largely congruent with those recovered in single-gene trees. Some clades contain species exclusively from one order, while others comprise taxa from both orders; nonetheless, all clades are well supported. A few taxa, however, such as *Myriokaryon lieberkuehnii*, for which molecular data are reported here for the first time, still occupy uncertain positions due to weak statistical support.

Building on this phylogenetic framework, we examine several clades in detail below, highlighting new findings and perspectives. At the same time, we note that many issues remain unresolved within this complex assemblage, e.g., congeners scattered across multiple clades, and discrepancies between the phylogenetic position and morphological classification of certain species. Addressing these challenges will require broader taxon sampling combined with molecular data derived from accurately identified species.

Intermediate taxa between free-living and endosymbiotic lifestyles

The endosymbiotic subclass Trichostomatia represents a well-supported monophyletic taxon. Previous and present phylogenetic analyses consistently indicate that this subclass is nested within the Spathidiida

+ Haptorida assemblage (Barmshuri et al., 2023; Bourland, 2023; Huang et al., 2018; Jang et al., 2015; Pomahač et al., 2023; Rajter et al., 2019; Rajter & Vďačný, 2016; Vďačný, 2015; Vďačný et al., 2011a, 2012; Vďačný et al., 2014; Weiss & Esteban, 2024). In our concatenated rDNA, SSU rDNA, and LSU rDNA trees, trichostomatians cluster with species of Clade 1. In contrast, the phylogenetic analysis based on ITS1-5.8S-ITS2 sequences places trichostomatians with Clade 8 species, although this relationship is weakly supported (58/*). Furthermore, in the phylogenomic tree, trichostomatians form a fully supported clade with *Epispathidium* sp., and coincidentally, a congener of *Epispathidium* sp. is likewise recovered in Clade 1 of the trees inferred from ribosomal datasets (Figures 1–4).

Interestingly, species belonging to Clade 1 exhibit morphologies that deviate markedly from conventional haptorians. They possess distinctive characters that separate them from other free-living litostomateans, including *Spathidium papilliferum* with prominent papillae, *Neobryophyllum* sp. bearing a long “tail”, *Legendrea* spp. with flexible tentacles, *Dactylochlamys pisciformis* with rigid tentacles and spiral somatic kinetics, and *Apertospathula* spp. with an incomplete and shortened circumoral kinety (Bourland, 2023; Jang et al., 2017; Pomahač et al., 2023; Weiss et al., 2022; Weiss & Esteban, 2024; Yıldız, 2018) (Figure 1A). Although these species do not form an independent clade, we collectively refer to them as Clade 1, given their close phylogenetic relationship to Trichostomatia and their morphological distinctiveness.

Furthermore, and importantly, all Clade 1 species have been isolated from anaerobic or microaerophilic environments. Although it remains unclear whether their unique morphologies represent specific adaptations to an anaerobic or microaerophilic lifestyle, their intermediate phylogenetic position and suite of unusual traits suggest that Clade 1 may represent a transitional lineage bridging free-living and endosymbiotic forms within Litostomatea.

Taxonomic revision of Bryophyllidae

The family Bryophyllidae Jankowski, 1980 represents a spathidiid lineage that is morphologically similar to Pleurostomatida, as its members possess an oral apparatus extending meridionally or spirally along the left side of the cell toward the posterior end. Foissner & Lei (2004) assigned three genera to this family: *Bryophyllum* Kahl, 1931, *Apobryophyllum* Foissner, 1998, and *Neobryophyllum* Foissner in Foissner & Lei, 2004.

The present study provides the first molecular data for *Neobryophyllum*, revealing that it does not cluster with *Bryophyllum* and *Apobryophyllum*. Instead, it falls within Clade 1, contradicting its current taxonomic classification (Figures 1–3). Morphologically, these three genera were originally distinguished by the pattern (isomorphic in *Bryophyllum* and *Neobryophyllum* vs. heteromorphic in *Apobryophyllum*) and number of rows (three in *Bryophyllum* vs. more than three in *Apobryophyllum* and *Neobryophyllum*) of the dorsal brush (Foissner & Lei, 2004). Our morphological comparison further demonstrates that the dorsal brush of *Bryophyllum* and *Apobryophyllum* originates from the anterior somatic kineties adjacent to the left side of the circumoral kinety. In contrast, the dorsal brush of *Neobryophyllum* is derived from somatic kineties situated in the mid-left region of the cell, indicating a distinct positional difference (left-lateral vs. dorso-lateral) (Foissner et al., 2002; Foissner & Berger, 2021; Foissner & Lei, 2004) (Figure 2B). Given that the location of the dorsal brush is a key character used to distinguish high-rank taxa, we propose transferring *Neobryophyllum* out of Bryophyllidae. However, due to the lack of robust support for its phylogenetic position, we tentatively treat it as *incertae sedis*, potentially representing a novel family within the order Spathidiida.

Berger et al. (2025) recently noted that *Cultellothrix* Foissner, 2003 (a member of the family Arcuospathidiidae Foissner & Xu, 2007) was an unavailable name due to the lack of a fixed holotype. Consequently, *Neocultellothrix* Foissner in Berger et al., 2025 was established to accommodate the members of this genus. Our phylogenies show that *Neocultellothrix lionotiformis* clusters with *Apobryophyllum* with full support (Figures 1–3). Although the oral apparatus of *N. lionotiformis* extends only to the middle region of the cell (vs. to the posterior end in typical bryophyllids), its three dorsal brush rows are isomorphic and positioned left-laterally, as in *Bryophyllum*, being derived from somatic kineties adjacent to the left side of the circumoral kinety (Foissner & Xu, 2007). The type species, *Neocultellothrix velhoi*, exhibits a similar morphology and infraciliature (Foissner, 2003). Accordingly, we propose transferring *Neocultellothrix* to Bryophyllidae. Notably, several other species currently assigned to this genus possess a shorter, spatula-shaped oral apparatus confined to the anterior region (resembling typical *Spathidium*), which obscures the left-side distribution of the dorsal brush. Additional molecular data are required to clarify the systematic positions of these species.

Phylogenetic relationships within Trachelophyllina

Currently, the defining diagnostic character of the suborder Trachelophyllina Grain, 1994 is the presence of a layer of epicortical scales (lepidosomes) covering the cell surface. This suborder presently comprises two families: Trachelophyllidae Kent, 1881 and Lingulotrichidae Foissner, 2016 (Foissner, 2016; Foissner et al., 2002). In this study, molecular data were obtained for four trachelophyllinan species. Two of these taxa (labeled *Trachelophyllum brachypharynx* pop. 1 and *Trachelophyllum* (?) sp. 1 in the phylogenetic trees) conform well to the diagnosis of Trachelophyllidae, particularly in possessing a dorsal brush composed of two dikinetidal rows and one monokinetidal row. Moreover, lepidosomes were readily observable in both living and protargol-stained specimens of these populations. The remaining two taxa (labeled *Enchelyotricha* sp. 1 and *Enchelyotricha* sp. 2) exhibit the characteristic morphology attributed to the genus *Enchelyotricha*, including a highly contractile, symmetrical obclavate body, and a dorsal brush comprising more than three rows. However, no lepidosomes were detected in either living or protargol-stained specimens.

Although lepidosomes are frequently observable under light microscopy in trachelophyllinans (Bourland et al., 2026; Foissner, 2016; Foissner et al., 2002; Jang et al., 2015; Omar et al., 2022), we cannot exclude the possibility that they are present in these taxa but too small or inconspicuous to be detected using this approach. Consequently, the apparent absence of lepidosomes cannot be confirmed without scanning electron microscopy (SEM), which remains the most reliable method for characterizing their presence and morphology. Therefore, the taxonomic identifications of all four populations examined here should be regarded as provisional pending SEM observations.

Despite this uncertainty, across all phylogenetic analyses based on the four rDNA datasets (Figures 1–3), the newly sequenced trachelophyllid populations clustered with previously reported trachelophyllid sequences to form a fully supported clade, confirming the family Trachelophyllidae as a robust monophyletic group. Furthermore, our phylogenetic analyses consistently place *Enchelyotricha* in close association with trachelophyllids. In the concatenated rDNA and SSU rDNA trees, *Enchelyotricha* forms a well-supported sister lineage to Trachelophyllidae (99/1.00 and 92/0.94, respectively), whereas in the ITS1-5.8S-ITS2 and LSU rDNA trees it remains embedded within the same broader clade, although without a direct sister relationship.

From a morphological perspective, *Enchelyotricha* also exhibits substantial similarity to members of Lingulotrichidae, the other currently recognized family within Trachelophyllina. Apart from the

uncertain occurrence of lepidosomes, its overall body form, nuclear apparatus, and especially its uniquely complex dorsal brush correspond closely with the diagnosis of that family. This interpretation is likewise consistent with previous taxonomic hypotheses. The genus *Enchelyotricha* was originally assigned to Trachelophyllidae (Foissner, 1987), but following the redefinition of that family by Foissner et al. (2002), it was excluded because of its highly differentiated dorsal brush, and those authors suggested that it might represent a distinct family within Trachelophyllina. Subsequently, when establishing Lingulotrichidae, Foissner (2016) again proposed that *Enchelyotricha* might belong to that family, although molecular evidence was unavailable at the time. Considering the currently uncertain familial placement of *Enchelyotricha*, together with the morphological observations and molecular phylogenetic results presented here, we tentatively assign the genus *Enchelyotricha* to Lingulotrichidae. We emphasize, however, that this taxonomic placement remains provisional, pending future corroboration from definitive SEM observations and broader molecular sampling.

Establishment of Papillorhabdidae fam. nov.

Our previous study reviewed the genus *Papillorhabdos* Foissner, 1984 and elucidated its phylogenetic position for the first time based on the SSU rDNA sequence of *Papillorhabdos foissneri* (Chi et al., 2024a). Although molecular phylogenetic analyses suggested a close relationship between *Papillorhabdos* and *Actinobolina* Strand, 1928, the pronounced morphological differences between these two genera led us to conservatively retain *Papillorhabdos* within the family Enchelyidae.

In the present study, we provide additional ITS1-5.8S-ITS2 and LSU rDNA sequences for *Papillorhabdos foissneri*. Phylogenetic analyses based on these new data corroborate our previous findings: *Papillorhabdos* shows no affinity with Enchelyidae but instead consistently forms a fully supported clade with *Actinobolina* (Figures 1, 3). Morphologically, *Papillorhabdos* differs markedly from well-characterized enchelyids in several key morphological characters, including the number of dorsal brush rows (four in *Papillorhabdos* vs. two or three in enchelyids) and the distribution of extrusomes (arranged perpendicularly along the somatic cortex in *Papillorhabdos* vs. restricted to the oral apparatus in enchelyids) (Chi et al., 2024a; Foissner, 1984; Foissner et al., 2002). In light of these distinctive morphological and molecular characteristics, we herein establish a new family, Papillorhabdidae **fam. nov.**, to accommodate *Papillorhabdos*.

Evolutionary hypothesis of the class Litostomatea

Based on the phylogenetic analyses integrating both molecular data and morphological characteristics, we propose an evolutionary hypothesis for the class Litostomatea (Figure 5). The key insights are as follows:

1) The order Helicoprordontida represents the earliest diverging lineage within Litostomatea. Its morphological and ecological similarities (e.g., cell shape and habitat preference) to Karyorelictea, a ciliate class retaining many plesiomorphic traits, support this interpretation.

2) The newly established order Chaeneida **ord. nov.** constitutes the second diverging lineage, and it frequently shows morphological and molecular affinity to the subsequently diverging order Lacrymariida.

3) The orders Homalozoonida and Pleurostomatida, both characterized by left-right asymmetry in their somatic ciliature, are generally closely related. The haptorid-like order Fuscheriida **ord. nov.** also exhibits affinity with these taxa; however, current phylogenetic evidence indicates that the precise placement of Fuscheriida **ord. nov.** remains uncertain.

4) Members of the orders Spathidiida and Haptorida appear to have undergone rapid evolutionary radiation, forming a morphologically diverse and phylogenetically intricate assemblage that diverged relatively late in the evolutionary history of the class Litostomatea.

5) The order Pseudoholophryida is typically associated with the Spathidiida + Haptorida assemblage, although the order Didiniida, which resembles pseudoholophryids in appearance and locomotion, may share an even closer evolutionary relationship to this lineage.

6) The subclass Rhynchostomatia, distinguished by its distinctive proboscis, likely represents another late-diverging lineage. The evolution of the proboscis may serve as a key driver for the diversification of rhynchostomatians.

7) Several taxa within the Spathidiida + Haptorida assemblage that are closely related to the subclass Trichostomatia show adaptations to anaerobic or microaerophilic habitats. These forms likely represent transitional stages on the path to endosymbiosis, suggesting that Trichostomatia constitutes the most recently diverging lineage within Litostomatea.

Compared with previous evolutionary hypotheses (e.g., Kwon et al., 2014, which was based on the

evolution of the dorsal brush), our scenario relies on expanded taxon sampling and a more robust phylogenetic framework, resulting in several revised relationships among major litostomatean lineages. Although the dorsal brush is an important morphological character for delineating free-living litostomateans and may have played a significant role in their evolutionary history, the remarkable diversity of litostomateans indicates that many key morphological characters have undergone extensive adaptive radiation and convergent evolution across different lineages. Consequently, we consider the currently available evidence insufficient to explain the evolutionary history of the entire class using a framework primarily driven by a single morphological character. Future studies incorporating additional molecular data from accurately identified taxa, particularly from phylogenetically critical and currently undersampled lineages, will be essential for further refining the evolutionary framework of this class.

Estimation of divergence times

Vd'áčný (2015) first estimated the divergence times of the major litostomatean lineages and later focused specifically on the endosymbiotic trichostomatians (Vd'áčný, 2018). Building on these studies and incorporating newly available molecular data (including sequences generated here), we established an updated molecular dating framework for Litostomatea (Figure 6A). The posterior means and 95% credibility intervals (CI) for the divergence times of major lineages are summarized in Figure 6B.

The common ancestor of Litostomatea is estimated to have originated around 964 Ma with a 95% CI of 818–1073 Ma, consistent with previous estimates based on phylogenomic data (Jiang et al., 2024). Radiation of the class is inferred to have occurred during the Neoproterozoic, approximately 757 Ma (95% CI: 687–828 Ma), marking the emergence of the earliest haptorian lineage, the order *Helicoprordontida*, which diverged around 452 Ma (95% CI: 229–668 Ma). The second-oldest haptorian lineage, the newly proposed order *Chaeneida* **ord. nov.**, appeared around 681 Ma (95% CI: 617–750 Ma) and diverged approximately 488 Ma (95% CI: 307–639 Ma), whereas the subsequently diverging order *Lacrymariida*, radiated around 384 Ma (95% CI: 216–559 Ma).

Consistent with Vd'áčný (2015), the earliest radiating haptorian lineages are the order *Pleurostomatida* and the *Spathidiida* + *Haptorida* assemblage, which date back to the early Paleozoic, approximately 530 Ma (95% CI: 496–574 Ma) and 533 Ma (95% CI: 413–629 Ma), respectively. The split between *Pleurostomatida* and *Homalozoonida* occurred around 555 Ma (95% CI: 516–608 Ma),

whereas the divergence between the newly established order Fuscheriida **ord. nov.** and the Homalozoonida + Pleurostomatida assemblage occurred earlier, around 583 Ma (95% CI: 537–642 Ma). Within the Spathidiida + Haptorida assemblage, the common ancestor of Pseudoholophryida and Didiniida diverged around 284 Ma (95% CI: 173–405 Ma), with subsequent radiations at 214 Ma (95% CI: 109–343 Ma) and 93 Ma (95% CI: 44–159 Ma), respectively.

The subclasses Trichostomatia and Rhynchostomatia are also nested within the Spathidiida + Haptorida assemblage. Trichostomatia radiated approximately 135 Ma (95% CI: 108–153 Ma), while Rhynchostomatia diversified around 246 Ma (95% CI: 181–321 Ma), marking the divergence of Kamburophryida **ord. nov.** from other rhynchostomatian lineages. Subsequently, the divergence between the remaining two rhynchostomatian orders, Dileptida and Tracheliida, occurred approximately 205 Ma (95% CI: 147–275 Ma).

The divergence estimates inferred here differ from those reported by Vďačný (2015) for certain lineages. These discrepancies likely reflect differences in taxon sampling and the availability of molecular data at that time, as well as the fact that Vďačný (2015) relied solely on SSU rDNA, whereas the present analyses are based on a concatenated three-gene rDNA dataset and incorporate additional ciliate outgroups. It should be noted, however, that the current dating framework is subject to inherent uncertainties derived from the calibration methods. Because no confirmed primary fossil record exists for Litostomatea, our timeline relies heavily on two secondary calibrations adopted from Vďačný (2015), which were themselves derived from the early estimates of Wright & Lynn (1997), together with one host-derived fossil calibration following Vďačný (2018). While recent studies have employed primary ciliate fossils for molecular dating (Costa et al., 2021, 2025), these taxa belong to entirely different, deeply divergent classes. Incorporating such distant outgroups to utilize their fossil records would require expanding the taxonomic scope of the dataset to the subphylum Intramacronucleata, potentially introducing additional rate heterogeneity and increasing uncertainty in the inferred relationships within Litostomatea. Therefore, while our combined use of secondary and host-derived calibrations provides the most plausible evolutionary timeline currently available for litostomateans, these estimates remain provisional hypotheses that may be further refined as new methodologies or direct fossil evidence become available.

661 **Taxonomic summary**

662 Order Chaeneida **ord. nov.**

663 Diagnosis: Haptoria with narrowly spaced oralized somatic monokinetids adjacent to the circumoral
664 kinety, and four dorsal brush rows separated from the anterior body end by oralized somatic
665 monokinetids.

666 Family assigned: Chaeneidae Kwon et al., 2014 [Type].

667 Etymology: Composite of the stem of the name of type family and the order suffix “-ida”.

668

669 Order Fuscheriida **ord. nov.**

670 Diagnosis: Haptoria with general haptorid-like appearance; circumoral kinety composed of dikinetids;
671 somatic kineties meridionally arranged; usually two dorsal brush rows.

672 Family assigned: Fuscheriidae Foissner et al., 2002 [Type].

673 Etymology: Composite of the stem of the name of type family and the order suffix “-ida”.

674

675 Order Kamburophryida **ord. nov.**

676 Diagnosis: Rhynchostomatia with a small oral cone; a subapical dorsal hump (~proboscis) carrying a
677 dorsal brush and a brush membranoid (~preoral kinety); circumoral kinety distinctly oblique, composed
678 of dikinetids.

679 Family assigned: Kamburophryidae Foissner & Oertel, 2009 [Type].

680 Etymology: Composite of the stem of the name of type family and the order suffix “-ida”.

681

682 Family Monilicaryonidae **fam. nov.**

683 Diagnosis: Dileptida with a single dorsal brush row; preoral kineties linearly arranged to form a perioral-
684 like kinety.

685 Genus assigned: *Monilicaryon* Jankowski, 1967 [Type].

686 Etymology: Composite of the stem of the name of type genus and the family suffix “-idae”.

687 ZooBank registration: urn:lsid:zoobank.org:act:FCD58458-10F6-4E2A-9994-2EE06E6BE952.

688

689 Family Papillorhabdidae **fam. nov.**

690 Diagnosis: Haptorida with four dorsal brush rows; circumoral kinety absent; extrusomes vertically
691 attached to somatic cortex.

692 Genus assigned: *Papillorhabdos* Foissner, 1984 [Type].

693 Etymology: Composite of the stem of the name of type genus and the family suffix “-idae”.

694 ZooBank registration: urn:lsid:zoobank.org:act:066E5420-6334-4B03-9475-A48D40F34E27.

695

696 Order Homalozoonida Jankowski, 1980

697 Improved diagnosis: Haptoria with left-right asymmetry in somatic ciliature, i.e., right somatic kineties
698 densely arranged, left somatic kineties loosely arranged and anteriorly differentiated into a dorsal brush;
699 oral apparatus apical; circumoral kinety composed of dikinetids; three dorsal brush rows.

700 Family assigned: Homalozoonidae Jankowski, 1980 [Type].

701

702 Order Pseudoholophryida Foissner & Foissner, 1988

703 Improved diagnosis: Haptoria with spiral somatic kineties; multiple (> 3) dorsal brush rows, composed
704 of numerous minute kinetofragments interspersed between ordinary somatic kinetosomes.

705 Family assigned: Pseudoholophryidae Berger et al., 1984 [Type].

706

707 Family Bryophyllidae Jankowski, 1980

708 Improved diagnosis: Spathidiida with general pleurostomatid-like appearance; oral apparatus extending
709 to middle region or posterior end of cell; three or more dorsal brush rows, derived from anterior somatic
710 kineties adjacent to left side of circumoral kinety.

711 Genera assigned: *Apobryophyllum* Foissner, 1998; *Bryophyllum* Kahl, 1931 [Type]; *Neocultellothrix*
712 Foissner in Berger et al., 2025.

713

714 **An updated classification of free-living litostomeans**

715 The following list outlines the subclasses and their constituent orders, along with the families included
716 within each order.

717

718 Subclass Haptoria Corliss, 1974

- 1) Order Chaeneida **ord. nov.** (Chaeneidae Kwon et al., 2014)
- 2) Order Didiniida Jankowski, 1978 (Didiniidae Poche, 1913)
- 3) Order Fuscheriida **ord. nov.** (Fuscheriidae Foissner et al., 2002)
- 4) Order Lacrymariida Lipscomb & Riordan, 1990 (Lacrymariidae de Fromentel, 1876)
- 5) Order Haptorida Corliss, 1974 (Acropisthiidae Foissner & Foissner, 1988; Actinobolinidae Kahl, 1930; Enchelyidae Ehrenberg, 1838; Enchelyodontidae Foissner et al., 2002; Lingulotrichidae Foissner, 2016; Papillorhabdidae **fam. nov.**; Pleuroplitidae Foissner, 1996; Trachelophyllidae Kent, 1882)
- 6) Order Helicoprordontida Grain, 1994 (Helicoprordontidae Small & Lynn, 1985)
- 7) Order Homalozoonida Jankowski, 1980 (Homalozoonidae Jankowski, 1980)
- 8) Order Pleurostomatida Schewiakoff, 1896 (Amphileptidae Bütschli, 1889; Epiphyllidae Vd'ačný et al., 2015; Litonotidae Kent, 1882; Paralitonotidae Zhang et al., 2022; Protolitonotidae Wu et al., 2017)
- 9) Order Pseudoholophryida Foissner & Foissner, 1988 (Pseudoholophryidae Berger et al., 1984)
- 10) Order Spathidiida Foissner & Foissner, 1988 (Apertospathulidae Foissner et al., 2005; Arcuospathidiidae Foissner & Xu, 2007; Bryophyllidae Jankowski, 1980; Myriokaryonidae Jankowski, 1980; Pharyngospathidiidae Foissner et al., 2025; Protospathidiidae Foissner & Xu, 2007; Spathidiidae Kahl in Doflein & Reichenow, 1929; Teuthophryidae Chatton & de Beauchamp, 1924)
- 11) *Incertae sedis* genera: *Dactylochlamys* Lauterborn, 1901; *Enchelyothrix* Barmshuri et al., 2023; *Legendrea* Fauré-Fremiet, 1908; *Neobryophyllum* Foissner in Foissner & Lei, 2004.

737

738 Subclass Rhynchostomatia Jankowski, 1980

739 1) Order Dileptida Jankowski, 1978 (Dileptidae Jankowski, 1980; Dimacrocaryonidae Vd'ačný et al.,
740 2011; Monilicaryonidae **fam. nov.**)

741 2) Order Kamburophryida **ord. nov.** (Kamburophryidae Foissner & Oertel, 2009)

742 3) Order Tracheliida Vd'ačný et al., 2011 (Tracheliidae Ehrenberg, 1830)

743

744

745 CONCLUSION

746 The present study, which reconstructs the phylogeny of the class Litostomatea based on a large number
747 of newly obtained sequences combined with carefully screened data from the GenBank database, once

again highlights the remarkable diversity within this class. Although statistical support for some nodes remains limited, the phylogenetic relationships inferred from multi-gene datasets (i.e., concatenated rDNA and 89 core-gene) provide a more robust and reliable framework for Litostomatea than those derived from single-gene analyses. By interpreting the molecular phylogeny in the context of detailed morphological evidence, we further clarify the evolutionary relationships among free-living litostomatean taxa, refining their systematics, classification, and divergence time estimations. Given the pronounced morphological diversity and apparent evolutionary radiation, particularly within the Spathidiida + Haptorida assemblage, we emphasize the importance of accurate morphological identification to minimize errors introduced by misidentified molecular data. Looking ahead, taxonomic efforts should aim to refine the classification of Litostomatea at finer levels (e.g., suborders and families) rather than amalgamating morphologically disparate taxa into broad, heterogeneous groups, thereby establishing a more rational and phylogenetically meaningful taxonomy for this diverse and complex class.

NOMENCLATURAL ACTS REGISTRATION

The electronic version of this article in portable document format will represent a published work according to the International Commission on Zoological Nomenclature (ICZN), and hence the new names contained in the electronic version are effectively published under that Code from the electronic edition alone (see Article 8.5–8.6 of the Code). The published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information can be viewed through any standard web browser by appending the LSID to prefix <http://zoobank.org/>.

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DATA AVAILABILITY

All datasets generated in this study were deposited in GenBank (accession numbers: PX629974–PX630061), Science Data Bank (doi: 10.57760/sciencedb.j00139.000ag), and Genome Sequence Archive (<https://ngdc.cncb.ac.cn/gsa/>) (accession numbers: C_AA494427.1–C_AA494514.1).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

W.B.S. and B.R.L. designed and supervised the research. Y.C. performed the experiments, prepared the figures, and wrote the original manuscript. Y.R.W., G.A.T.Z., and S.A.A. checked all the data and helped to write the manuscript. Y.C., A.W., B.R.L., and W.B.S. contributed to the manuscript development and revisions. All authors read and approved the final version of the manuscript.

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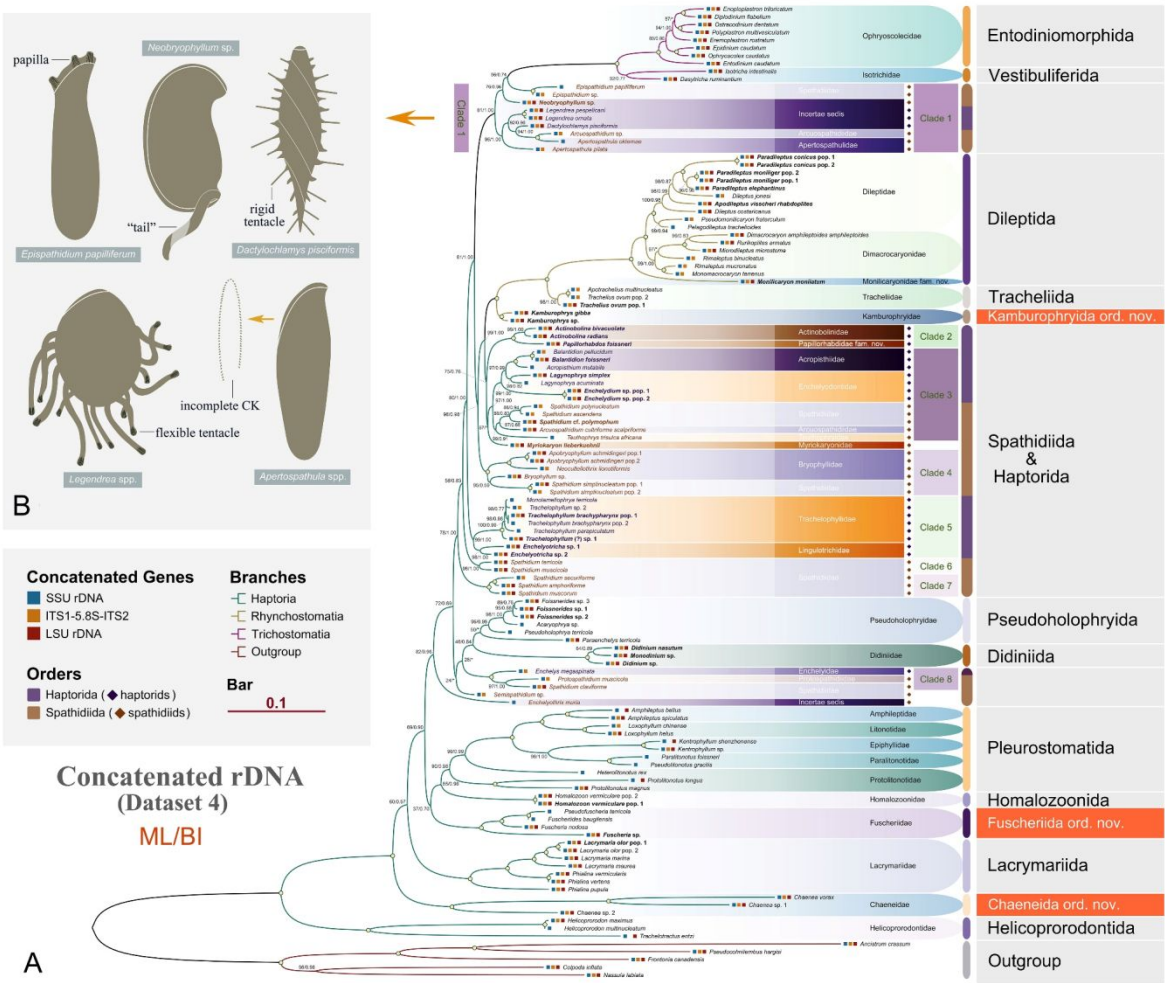
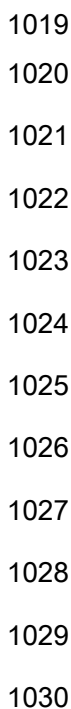


Figure 1 Concatenated rDNA phylogenetic tree and schematic illustrations of representative species

A: Maximum likelihood (ML) tree inferred from concatenated sequences of SSU rDNA, ITS1-5.8S-ITS2, and LSU rDNA. Newly sequenced species are shown in bold. Numbers at nodes indicate bootstrap values from ML and posterior probabilities from Bayesian inference (BI). Asterisks indicate topological disagreements between ML and BI. Solid circles represent fully supported branches (100/1.00). The scale bar represents 10 substitutions per 100 nucleotide positions. B: Schematic diagrams of species within Clade 1, highlighting their unique morphological characters.



A: Maximum likelihood (ML) tree inferred from SSU rDNA sequences. Newly sequenced species are shown in bold. Numbers at nodes indicate bootstrap values from ML and posterior probabilities from Bayesian inference (BI). Asterisks indicate topological disagreements between ML and BI. Solid circles represent fully supported branches (100/1.00). The scale bar represents 10 substitutions per 100 nucleotide positions. Clade designations follow the groupings defined in Figure 1. B: Comparative morphology of the dorsal brush (DB) and circumoral kinety (CK) between *Bryophyllum* and *Neobryophyllum*, illustrating the distinct localization of the DB. C: Oral infraciliature of *Monilicaryon* compared with other dileptids, noting that the preoral kinety (PR) on the left side of the circumoral kinety (CK) in *Monilicaryon* consists of a single kinety, similar to the perioral kinety (PE).

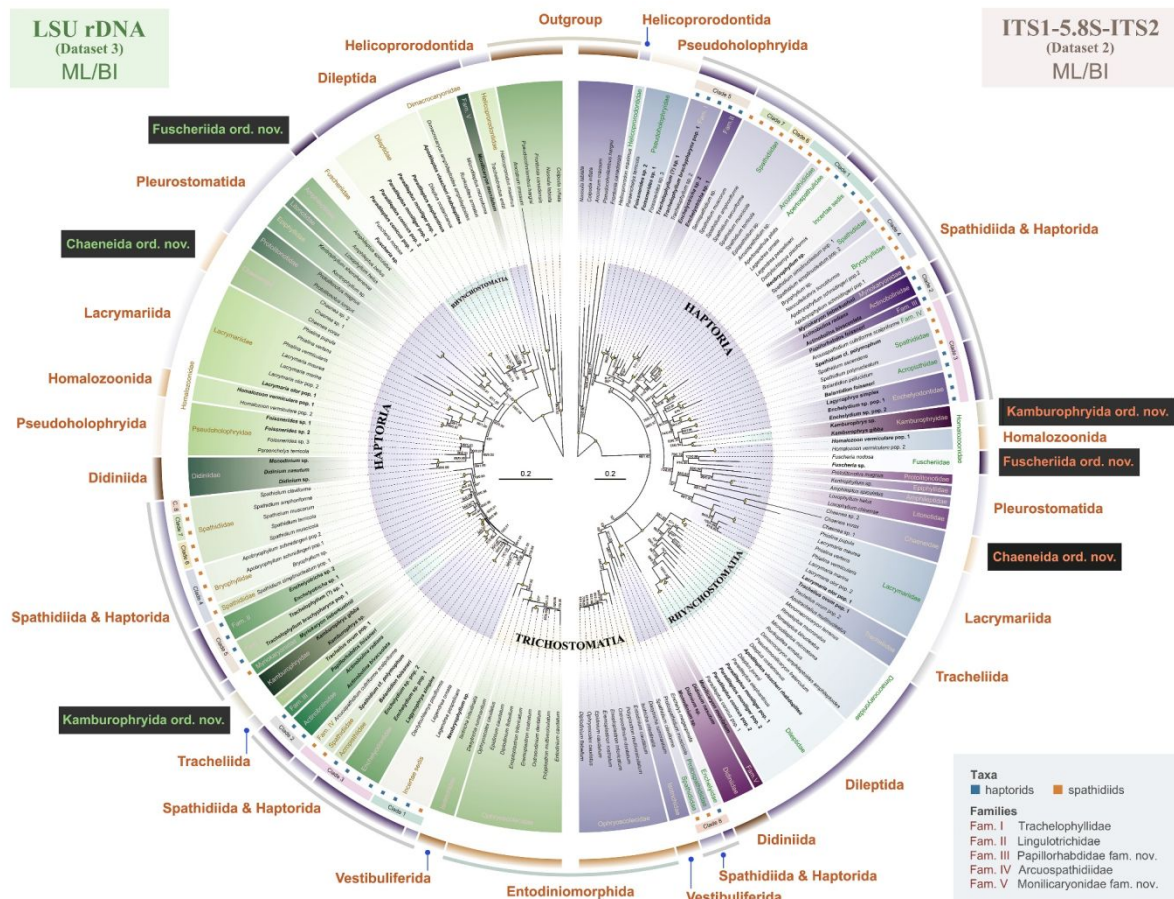


Figure 3 Maximum likelihood (ML) trees inferred from ITS1-5.8S-ITS2 and LSU rDNA sequences

Newly sequenced species are shown in bold. Numbers at nodes indicate bootstrap values from ML and posterior probabilities from Bayesian inference (BI). Asterisks indicate topological disagreements between ML and BI. Solid circles represent fully supported branches (100/1.00). The scale bars represent 20 substitutions per 100 nucleotide positions. Clade designations follow the groupings defined in Figure 1.

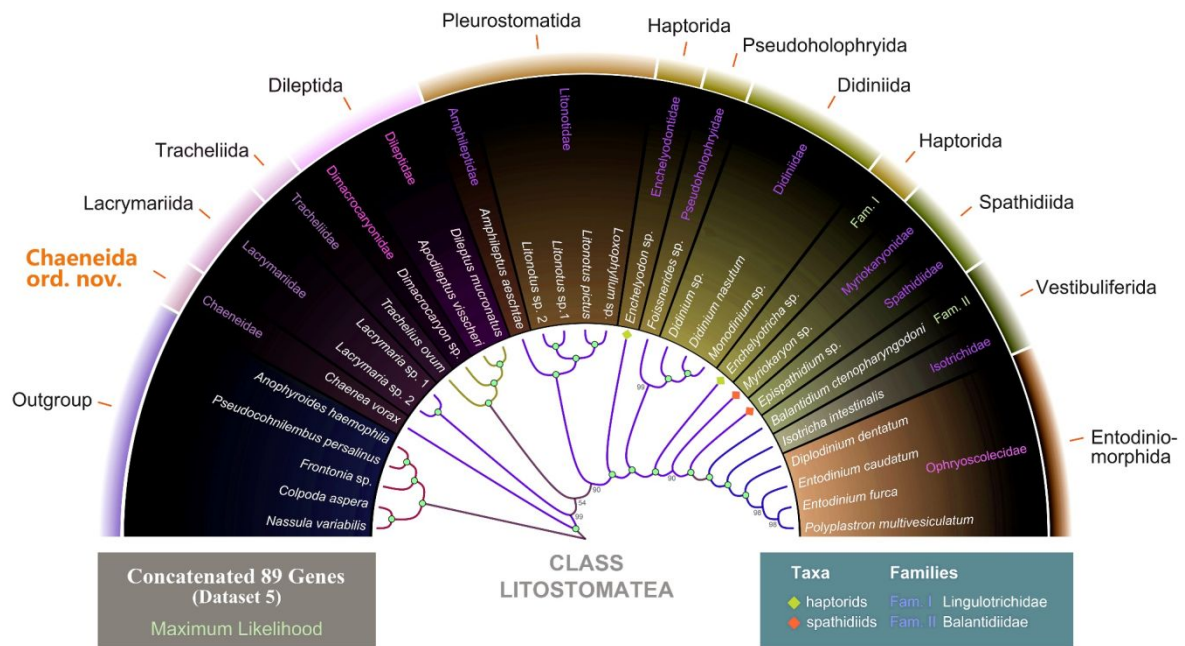


Figure 4 Phylogenomic maximum likelihood (ML) tree inferred from concatenated sequences of 89 core genes

Numbers at nodes indicate bootstrap values from ML. Solid circles represent fully supported branches.

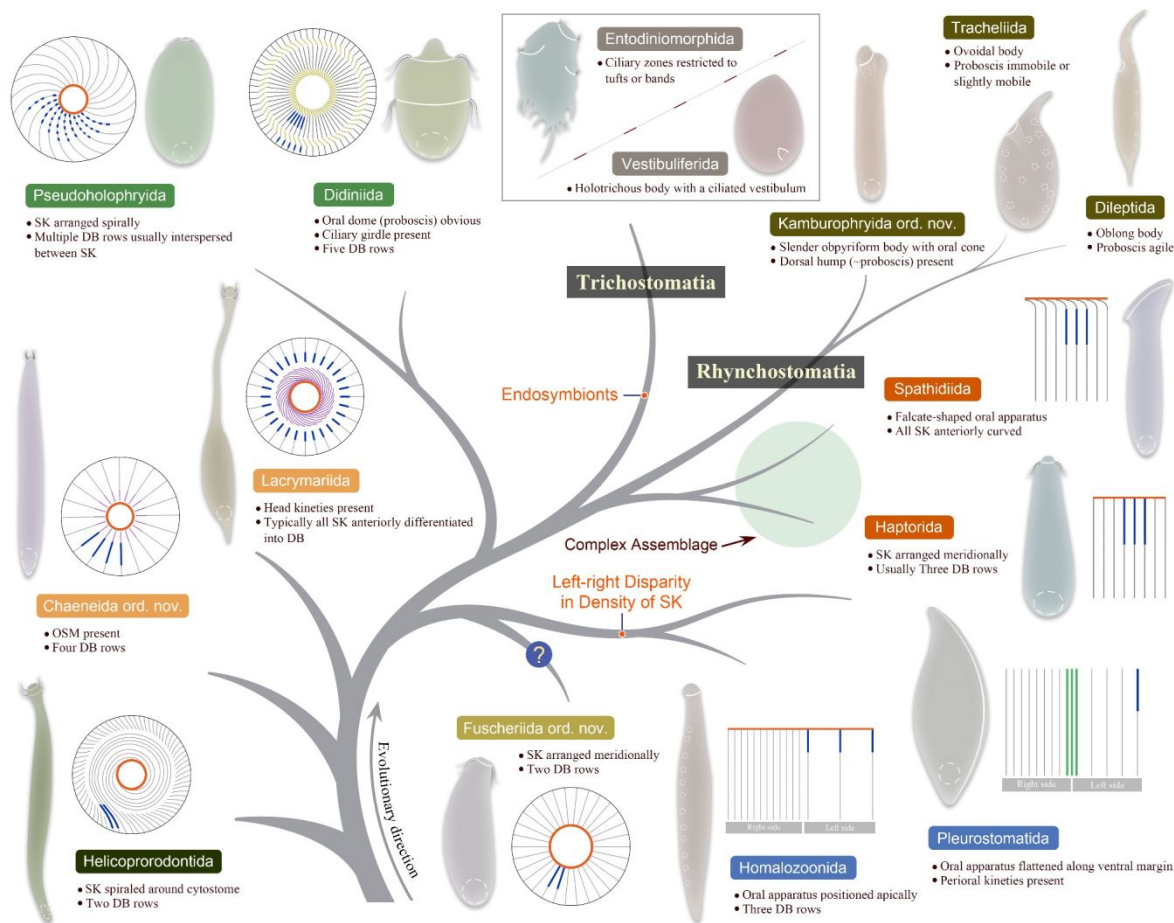


Figure 5 Hypothetical evolutionary framework of the class Litostomatea based on morpho-molecular data

Representative species and key diagnostic characters for each order are illustrated, with their corresponding infraciliature patterns. Color annotations in infraciliature diagrams: orange, circumoral kinety; blue, dorsal brush; grey, somatic kineties; green, perioral kineties (in Pleurostomatida); purple, head kineties (in Lacrymariida) and oralized somatic monokinetids (in Chaeneida **ord. nov.**); yellow, ciliary girdle (in Didiniida). DB, dorsal brush; OSM, oralized somatic monokinetids; SK, somatic kineties.

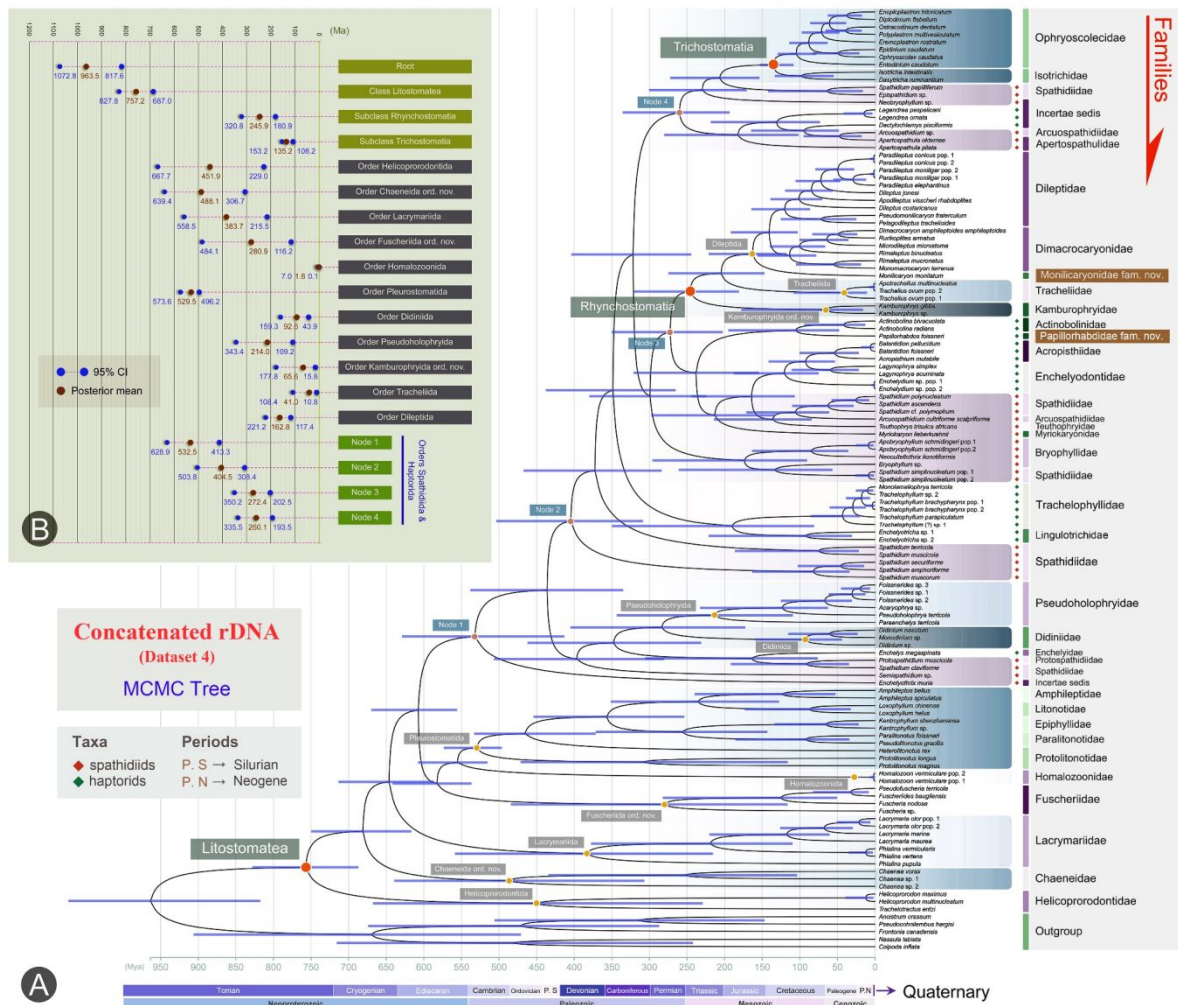


Figure 6 Divergence time estimation of the class Litostomatea

A: Chronogram was inferred from the concatenated rDNA tree. Solid circles indicate the mean divergence times for major nodes (e.g., class, subclass, order). The blue bars at nodes represent the 95% credibility intervals. The lower axis indicates the geological time scale (eras and periods) in millions of years. B: Posterior means and 95% credibility intervals for the divergence times of major lineages.

1063 TABLES

1064 Table 1 List of taxa for which SSU rDNA, ITS1-5.8S-ITS2, and LSU rDNA were newly sequenced
 1065 in this study (in bold)

Taxon	SSU rDNA			ITS1-5.8S-ITS2			LSU rDNA		
	Length	GC (%)	Accession No.	Length	GC (%)	Accession No.	Length	GC (%)	Accession No.
<i>Actinobolina bivacuolata</i>	1 518	43.08	OQ859614	390	37.18	PX630000	1 682	45.84	PX630030
<i>Actinobolina radians</i>	1 598	42.99	OQ859613	391	35.55	PX630001	1 701	46.15	PX630031
<i>Apodileptus visscheri rhabdoplites</i>	1 518	42.29	PX629974	383	37.08	PX630002	1 721	46.66	PX630032
<i>Balantidium foissneri</i>	1 591	42.99	ON158511	389	36.76	PX630003	1 683	48.19	PX630033
<i>Didinium nasutum</i>	1 597	41.77	PX629975	387	34.88	PX630004	1 716	44.29	PX630034
<i>Didinium</i> sp.	1 516	42.41	PX629976	379	33.25	PX630005	1 682	44.95	PX630035
<i>Enchelydium</i> sp. pop. 1	1 517	43.57	PX629977	379	36.94	PX630006	1 715	47.41	PX630036
<i>Enchelydium</i> sp. pop. 2	1 517	43.57	PX629978	379	36.94	PX630007	1 679	47.71	PX630037
<i>Enchelyotricha</i> sp. 1	1 516	43.14	PX629979	384	34.38	PX630008	1 683	47.65	PX630038
<i>Enchelyotricha</i> sp. 2	1 594	42.85	PX629980	382	35.34	PX630009	1 674	47.73	PX630039
<i>Foissnerides</i> sp. 1	1 596	42.48	PX629981	400	30.75	PX630010	1 691	45.65	PX630040
<i>Foissnerides</i> sp. 2	1 596	42.36	PX629982	397	31.49	PX630011	1 683	45.75	PX630041
<i>Fuscheria</i> sp.	1 516	41.03	PX629983	363	34.44	PX630012	1 703	44.57	PX630042
<i>Homalozoon vermiculare</i>	1 590	42.33	PX629984	373	40.75	PX630013	1 679	46.52	PX630043
<i>Kamburophrys gibba</i>	1 595	43.07	PX629985	388	36.08	PX630014	1 689	47.42	PX630044
<i>Kamburophrys</i> sp.	1 591	43.43	PX629986	389	39.33	PX630015	1 691	47.90	PX630045
<i>Lacrymaria olor</i>	1 518	42.49	PX629987	389	34.96	PX630016	1 685	45.88	PX630046
<i>Lagynophrya simplex</i>	1 516	43.54	PX629988	403	39.95	PX630017	1 696	48.35	PX630047
<i>Monilicaryon monilatum</i>	1 582	42.29	PX629989	379	33.77	PX630018	1 719	45.08	PX630048
<i>Monodinium</i> sp.	1 519	42.20	PX629990	389	32.13	PX630019	1 683	44.33	PX630049
<i>Myriokaryon lieberkuehnii</i>	1 513	43.56	PX629991	387	38.24	PX630020	1 718	48.20	PX630050
<i>Neobryophyllum</i> sp.	1 593	43.25	PX629992	387	37.47	PX630021	1 718	47.61	PX630051
<i>Papillorhabdos foissneri</i>	1 516	43.14	OQ859612	386	36.79	PX630022	1 689	47.48	PX630052
<i>Paradileptus conicus</i> pop. 1	1 518	41.83	MZ147013	391	31.71	MZ574467	1 713	44.37	PX630053
<i>Paradileptus conicus</i> pop. 2	1 548	41.60	PX629993	391	31.97	PX630023	1 713	44.37	PX630054
<i>Paradileptus elephantinus</i>	1 518	42.23	MZ147012	392	33.42	MZ574468	1 722	45.99	PX630055
<i>Paradileptus moniliger</i> pop. 1	1 459	42.63	PX629994	392	34.44	PX630024	1 685	46.53	PX630056
<i>Paradileptus moniliger</i> pop. 2	1 505	42.39	PX629995	392	34.44	PX630025	1 682	46.55	PX630057
<i>Spathidium</i> cf. <i>polymorphum</i>	1 595	43.26	PX629996	381	40.16	PX630026	1 682	48.45	PX630058
<i>Trachelius ovum</i>	1 522	42.05	PX629997	382	32.20	PX630027	1 674	48.03	PX630059
<i>Trachelophyllum brachypharynx</i>	1 596	43.05	PX629998	383	34.99	PX630028	1 699	46.91	PX630060
<i>Trachelophyllum</i> (?) sp.	1 594	43.04	PX629999	382	34.55	PX630029	1 686	47.03	PX630061

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1067 **Table 2 Information of datasets analyzed in this study**

Dataset	Data sources	Number of taxa	Number of characters	Best-fit models for ML analyses	Descriptions
Dataset 1	SSU rDNA	125	1 749	GTR+F+I+R5	120 litostomateans and five outgroup taxa
Dataset 2	ITS1-5.8S-ITS2	103	424	GTR+F+I+G4	98 litostomateans and five outgroup taxa
Dataset 3	LSU rDNA	86	1 947	GTR+F+I+R4	81 litostomateans and five outgroup taxa
Dataset 4	Concatenated rDNA	125	4 120	GTR+F+I+R5	120 litostomateans and five outgroup taxa from the concatenated Datasets 1–3
Dataset 5	Concatenated core-gene set	31	57 581	LG+F+I+G4	26 litostomateans and five outgroup taxa with the concatenation of 89 genes

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裂口纲纤毛虫的系统演化关系：基于形态与分子整合证据的新认知，兼论

三新目两新科的建立

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摘要

裂口纲 (Litostomatea Small & Lynn, 1981) 是纤毛门中形态和物种多样性最为丰富的类群之一。然而, 受长期积累的分类混乱与分子数据匮乏的限制, 该纲内部各类群之间, 尤其是自由生支系的系统演化关系长期模糊不清。本研究聚焦该类群, 新获得了涵盖 10 个目、17 个科的 88 条核糖体 DNA 序列。在此基础上, 以核糖体基因数据集为核心, 结合已有的基因组/转录组数据开展了系统发育的重建研究, 并通过形态学等信息比较, 探讨了目级和科级水平的亲缘关系。主要结果如下: (1) 厘定和廓清了 13 个自由生目级阶元, 除刀口目 (Spathidiida) 和钩刺目 (Haptorida) 外, 其余类群的单系性均得到高度支持, 且确认旋前管目 (Helicoprordontida) 为该纲最早分化的类群; (2) 将部分“传统钩刺类”从物种丰富且分类关系复杂的刀口目中分离出来, 并重新划归入钩刺目; (3) 我们的工作显示, 部分具独特形态特征的厌氧型刀口类和钩刺类, 代表了自由生活与内共生生活方式之间的过渡类群; (4) 建立了 5 个新分类单元, 即纤口目 (Chaeneida ord. nov.)、富舍尔目 (Fuscheriida ord. nov.)、驼眉目 (Kamburophryida ord. nov.)、链核科 (Monilicaryonidae fam. nov.) 和乳突短柱科 (Papillorhabdidae fam. nov.), 恢复了平带目 (Homalozoonida) 和伪裸口目 (Pseudoholophryida) 的目级分类地位; (5) 基于系统发育框架与更广泛的类群取样, 更新了裂口纲内部各支系的分化时间; (6) 综合上述信息, 提出了裂口纲的演化新假说及修订的分类系统。

关键词: 分类系统; 演化假说; 多基因; 新分类单元; 系统发育

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