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Increased microgastropoda sampling give new insights into the phylogenetic relationships of Littorinoidea (Littorinimorpha)

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ABSTRACT

Littorinoidea is one of the most diverse radiations and the most successful group that evolutionary transitions from marine to terrestrial within Littorinimorpha. With such an unmatched diversity, few phylogenetic investigations have attempted to understand their evolutionary relationships, and existing research has primarily focused on typical intertidal species. To address this gap, we conducted the first phylogenomic analysis of the Littorinoidea, leveraging 35 transcriptomes to investigate their internal relationships. Our analyses revealed significant revisions necessary within the Littorinoidea: 1) *Pomatias* appears distantly related to Littorinidae, suggesting a potential ancestral origin outside of Littorinoidea, challenging traditional classification. The homology of penial innervation within Littorinoidea warrants reevaluation. 2) *Lacuna*'s placement indicates a close relationship with Naticidae, prompting consideration for its removal from Littorinidae. 3) Based on the current phylogenetic research, *Peasiella* may belong to a distinct family separate from Littorinidae. 4) Our findings support revising the placement of Pteropods within the Littorinimorpha, which is situated phylogenetically between the families Littorinoidea and Naticoidea. Additionally, we highlight the impact of site heterogeneity and evolutionary rate variation on phylogenetic inference. Our study provides a robust phylogenomic framework for the Littorinoidea, emphasizing the importance of including microgastropoda taxa in molecular phylogenetic reconstructions of gastropod subgroups.

1. Introduction

The Littorinoidea, a part of the broader order Littorinimorpha and with more than 400 described living species, comprises the mainly marine families: Littorinidae, Zerotulidae, and Skeneopsidae, as well as terrestrial families: Pomatiidae and Annulariidae (WoRMS). They are up to 50 mm in height and are turbinate, trochoidal, or conical in shape. Littorinidae and Pomatiidae are probably the best-studied prosobranch family within this superfamily. The terrestrial Pomatiidae, particularly *Pomatias elegans* (Müller, 1774), is often used as research material for population genetics (Jordaens et al., 2001), which was considered a probable sister group of the Littorinidae (Reid, 1989) because the *Pomatias* (Pomatiidae) and most littorinid genera share the character of the penial nerve originates from the right pedal gangliond (Garnault, 1887; Creek, 1951; Ponder, 1988). Reid (1989) confirmed the origin of the penial nerve in the pedal ganglion for most littorinid genera. However, as the penial nerve of *Cremnoconchus* (Littorinidae) and *Pomatias* (Pomatiidae) arises at the base of the pleuropedal connective (Linke, 1935; Reid, 1989), the penial innervation as homologous information was questioned (Barker, 2001). Despite inconclusive evidence, Pomatiidae has been classified as an outgroup of Littorinidae in multiple studies (Reid, 1989; Fehér et al., 2009; Saha et al., 2022).

Within the Littorinoidea, the systematics and evolution of the Littorinidae have been widely studied than that of Pomatiidae (Reid et al., 2012; Williams et al., 2009; Winnepenninckx et al., 1998), as it is abundant and familiar members of the coastal community on worldwide seashores. Some members of this group also comprise attractive models for studying environmental adaptation and evolutionary ecology due to

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their wide geographic distribution, large populations, and diversity of habitats. (Rolán-Alvarez et al., 2015; Santos et al., 2022).

The traditional classification of the Littorinidae primarily relied heavily on characters of the shell, operculum, and radula (Wenz, 1938; Rosewater, 1970,1972,1981). Taxonomists turned to anatomical characters because the shells are generalized in form and subject to genetic and ecophenotypic variability. Subsequently, the family Littorinidae was divided into three subfamilies: Lacuninae, Laevilitorininae, and Littorininae, which produced by cladistic analysis of a range of anatomical characters morphological data (Reid, 1989). However, some taxonomists have raised concerns regarding interpreting polarity for numerous character states in Reid's (1989) phylogenetic analysis (Barker, 2001). With the advent of molecular phylogenetics, a series of phylogenetic studies within Littorinidae have been conducted (Reid et al., 2012, 2010; Williams et al., 2009; Winnepenninckx et al., 1998). The subfamily Littorininae is probably the best-studied group, and the intertidal genus (*Echinolittorina*, *Littoraria*, and *Littorina*) has received particular attention (Reid et al., 2012). Phylogenetic analyses based on single sequences have advanced our understanding of interfamilial relationships within the Littorininae. However, there has yet to be a molecular phylogenetic framework for the superfamily Littorinoidea. It's worth noting that microgastropods (with an adult size of less than 5 mm, e.g. *Lacuna*, *Peasiella*, *Mainwaringia*) from subfamily Lacuninae and Littorininae have received limited attention, possibly due to their diminutive size and seagrass habitat (González-Wevar et al., 2022; Saha et al., 2022), which prevents recovery of systematic framework for this large and highly diverse gastropod group. Therefore, integrating these microgastropods, into the Littorinoidea phylogeny holds significant importance for understanding the phylogenetic relationships within this group.

In this study, our goal was to understand the phylogenetic relationships within Littorinoidea by increasing taxon sampling, particularly the often overlooked microgastropods, in the systematic framework of Littorinimorpha. We present an extended sample of Littorinidae by producing new transcriptomes and complement the dataset with the latest published Littorinimorpha transcriptomes. We employ various methods and models with strategic gene subsampling to mitigate the impact of systematic error on phylogenetic inference.

2. Methods

2.1. Taxon sampling and morphological work

The transcriptomes of 13 Littorinoidea and 1 Barleeiidae were generated from specimens collected from shallow intertidal of China in 2022 (Table 1). All samples were initially stored in liquid nitrogen and later transferred to institutional laboratories, where they were preserved at − 80℃. The identification of micromolluscs involved the examination of the shell, radular, and operculum, which were directly mounted on aluminum stubs using a conducting carbon adhesive tab, sputter-coated with gold, and observed using a scanning electron microscope (Flexsem 1000II). The voucher specimens of species sampled herein were deposited in the Laboratory of Shellfish Genetics and Breeding (LSGB) at Ocean University of China in Qingdao, China.

2.2. Molecular techniques and data collection

The total RNA for each species was extracted from the whole body of adult specimens by using the RNeasy Plus Universal Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Residual genomic DNA was removed by the RNase-free DNase (Qiagen, Germany). RNA concentration was measured by a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). RNA integrity was assessed by 1.5 % agarose gel electrophoresis and was analyzed by an Agilent Bioanalyzer 2100 system (Agilent Technologies). Illumina paired-end library (2 × 100 bp) for each of 14 species was prepared and sequenced on the Illumina Hiseq 2500 platform. Both library construction and sequencing were performed at Novogene Bioinformatics Technology Co., Ltd (Beijing, China). New sequences were deposited in the GenBank (Table 1). Publicly available transcriptomes were downloaded from 21 gastropods, including 3 outgroups. Finally, all new data and selected published sequences were included in the subsequent workflow bring the total number of terminals to 35.

Table 1
Species used in present analyses with GenBank accession numbers and collection sites of specimens. Accession numbers of newly obtained sequences are given in bold.

Taxon	Family	Collection locality	Accession number
Barleeia angustata	Barleeiidae	Qingdao, China	SRR28040012
Echinolittorina cecillei	Littorinidae	Hainan, China	SRR28040011
Echinolittorina melanacme	Littorinidae	Hainan, China	SRR28040006
Echinolittorina radiata	Littorinidae	Hainan, China	SRR28040005
Echinolittorina vidua	Littorinidae	Hainan, China	SRR28040004
Lacuna carinifera	Littorinidae	Qingdao, China	SRR28040003
Littoraria ardouiniana	Littorinidae	Hainan, China	SRR28040002
Littoraria intermedia	Littorinidae	Guangxi, China	SRR28040001
Littoraria melanostoma	Littorinidae	Guangxi, China	SRR28040000
Littoraria sinensis	Littorinidae	Guangxi, China	SRR28039999
Littoraria undulata	Littorinidae	Hainan, China	SRR28040010
Littorina brevicula	Littorinidae	Qingdao, China	SRR28040009
Mainwaringia leithii	Littorinidae	Fujian, China	SRR28040008
Peasiella habei	Littorinidae	Qingdao, China	SRR28040007
Echinolittorina malaccana	Littorinidae	Xianen, China	SRR3214642 SRR3214669
Littoraria flava	Littorinidae	Espirito Santo, Anchieta, Brazil	SRR12708750 SRR12708751 SRR12708752 SRR12708753
Littorina arcana	Littorinidae	Ravenscar, England, United Kingdom	SRR11570922
Littorina fabalis	Littorinidae	Samil, Spain	SRR9849871
Littorina littorea	Littorinidae	Innsbruck, Austria	SRR11015452
Littorina obtusata	Littorinidae	Mindelo, Portugal	SRR9849872
Littorina saxatilis	Littorinidae	Holyhead,Wales, United Kingdom	SRR11570942
Pomatias elegans	Pomatiidae	Field site near Sumartin, Brac, Croatia	SRR7662989
Atlanta ariejansseni	Atlantidae	Atlantic Ocean	SRR14999142
Bithynia siamensis goniomphalos	Bithyniidae	Thailand	SRR1046838
Charonia sauliae	Ranellidae	Jeju island, Korea	SRR11069700
Charonia tritonis	Ranellidae	Sunshine Coast, Australia	SRR13643480
Crepidula fornicata	Calyptraeidae	MA, USA	SRR14267608
Crepidula navicella	Calyptraeidae	Veracruz, Playa Venado, Panama	SRR3168546
Euspira heros	Naticidae	Rhode Island, Jamestown, USA	SRR1505131
Neverita didyma	Naticidae	Weifang, China	SRR8472156
Marseniopsis mollis	Velutinidae	Adelaide Island, Adelaide Island	SRR3205287
Strombus gigas	Strombidae	–	SRR8490883
Thalassonerita naticoidea	Phenacolepadidae	Gulf of Mexico, Atlantic Ocean	SRR8318347
Monodonta labio	Trochinae	–	SRR20746351
Trochus nigropunctatus	Trochinae	KwaZulu-Natal, Widenham, South Africa	SRR19577564

2.3. Transcriptome assembly

Our approach refers to the bioinformatic pipeline of Cunha et al. (2019) and Kocot et al. (2019), which are detailed in Supplementary Materials. Briefly, we utilized RCorrector (Song and Florea, 2015) to correct random sequencing errors in raw reads. Subsequently, Fastp (version 0.19.7) (Chen et al., 2018) was employed to discard reads containing adapter contamination, low-quality nucleotides, and unrecognizable nucleotides. The above-filtered reads were compared with ribosomal RNA and mitochondrial DNA of closely related molluscs and removed with Bowtie2 v. 2.2.9 (Langmead and Salzberg, 2012). *De novo* transcriptome assembly for each species was performed using Trinity v2.9.1 (Haas et al., 2013) with default parameters. Then, a second run of Bowtie2 was performed on the transcriptome assemblies, followed by removing redundant sequences with CD-HIT-EST v. 4.6.4 (Fu et al., 2012; Li and Godzik, 2006). Contigs from all taxa were translated to amino acids using TRANSDECODER v. 3.0 (Haas et al., 2013), and the longest isoform of each gene was extracted with a custom Python script (extract_longest_pep_id.py). The completeness of transcriptome assemblies was evaluated by BUSCO v3.0.2 against the metazoa_odb9 data set (supplementary material, table S1) (Simão et al., 2015).

2.4. Matrix construction

A series of data processing procedures were executed on the orthologous groups, as implemented by Kocot et al. (2019). Orthologous sequences were identified using OMA V. 2.5.0 (Altenhoff et al., 2018). Original sequences with a length of less than 50 amino acids (AAs) were removed, and then orthogroups with $\geq$ 80 % taxon occupancy were selected. If one of the first or last 20 characters of an AA sequence was an X, all characters between the X and the end of the sequence will be deleted and treated as missing data. Each orthogroup was then aligned with MAFFT v.7.4.07 (Katoh et al., 2005), and alignments were trimmed to remove ambiguously aligned regions with ALISCORE (Mlsof and Katharina, 2009) and ALICUT (Kuck, 2009). Highly divergent sequences with a value greater than 75 % were removed using the EMBOSS program infoalign (Rice et al., 2000). Sequence regions containing 20 or fewer AAs in length, surrounded by 10 or more gaps on either side, were removed. Sequences not overlapping with all other sequences by at least 20 AAs were deleted.

To obtain a single gene representation for each taxon within every orthogroup, Maximum Likelihood (ML) trees were inferred using FastTree v. 2.17 (Price et al., 2010), with the '-slow' and '-gamma' options, and then PhyloTreePruner (Kocot et al., 2013) was used to select the optimal sequence for each taxon. Finally, matrix1 was constructed from all orthogroups obtained above for which at least 80 % of the taxon were represented, including a total of 1021 genes (Fig. 1). Systematic errors are one of the contributing factors to incongruence in systematic inference, such as evolutionary rates and heterogeneity. To mitigate potential biases saturation and long-branch attraction, matrix2 was generated by excluding the 20 % of genes with the slowest and fastest rates of evolution from matrix1, as determined using TRIMAl (Capella-Gutiérrez et al., 2009). This refinement resulted in a final dataset comprising 614 genes (Fig. 1). We used the Python package P4 (Foster, 2004) to evaluate compositional homogeneity for each gene from matrix1, which considered the datasets to be compositional homogeneity when the *p-value* was < 0.1 (matrix3), otherwise it is heterogeneous (matrix4) (Fig. 1).

2.5. Phylogenetic analyses

Three distinct phylogenetic approaches were implemented to elucidate the evolutionary relationships within Littorinimorpha using our concatenated datasets (Fig. 2): coalescent-based in ASTRAL-II v.4.10.12 (Mirarab and Warnow, 2015); maximum likelihood (ML) in IQ-TREE

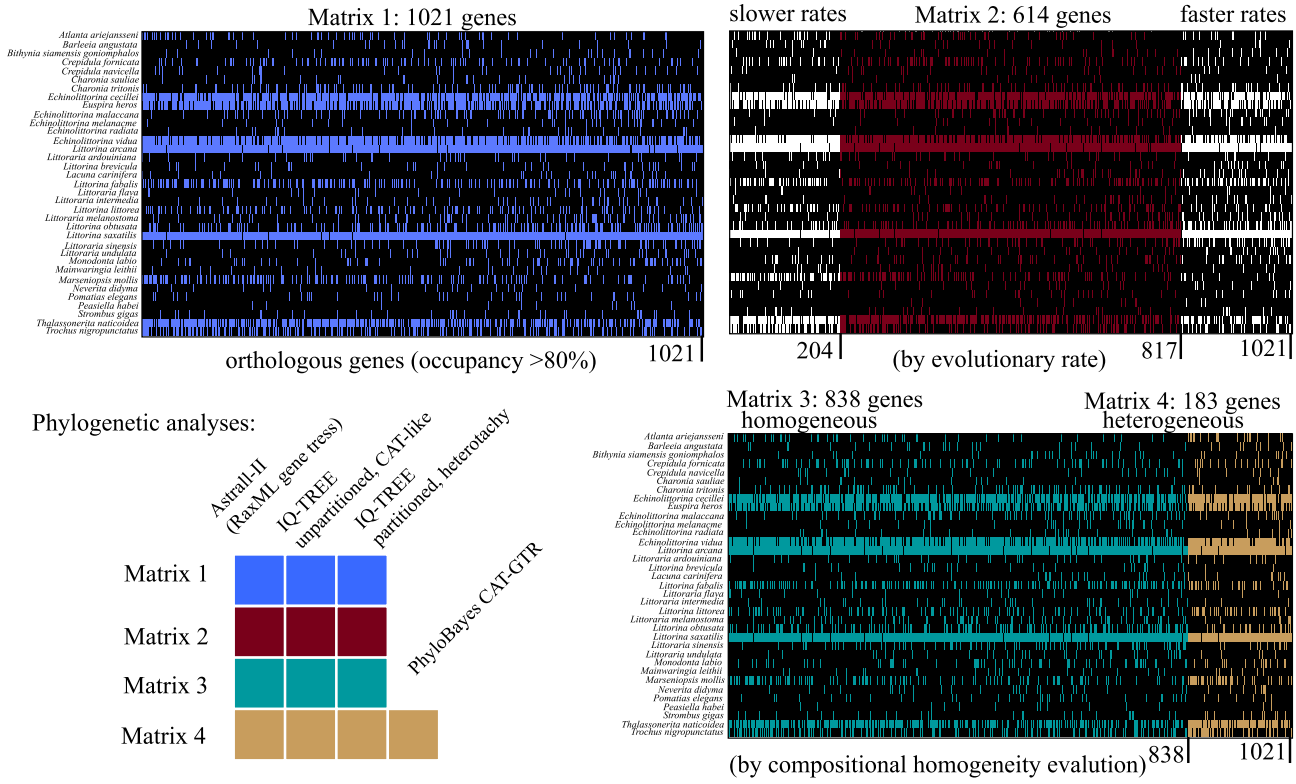

Fig. 1. Matrices and phylogenetic methods used to infer Littorinoidea phylogenetic relationships. With 80% taxon occupancy, matrix 1 is the largest, with 1021 genes. Matrix 2 is the subset of 614 genes after ordering all genes by evolutionary rate and removing the 20% slowest and 20% fastest evolving genes. Matrix 3 includes the 838 genes that are homogeneous in amino acid composition; genes are ordered by p-value of the homogeneity test. Matrix 4 includes the 183 genes that are heterogeneous in amino acid composition.

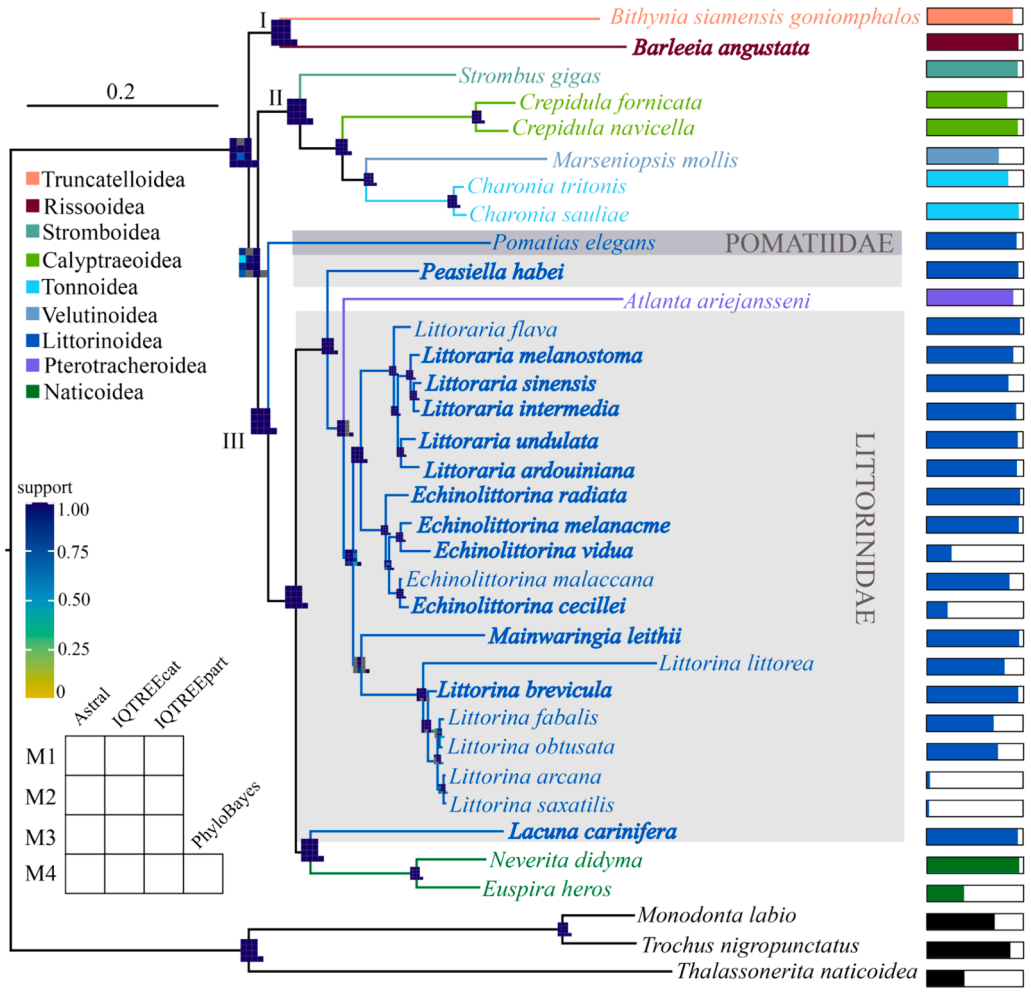

Fig. 2. Littorinoidea phylogeny inferred from matrix (M2) with ML and a profile mixture model (IQTREEcat). Node support are marked with a plot consisting of 13 squares, coloured in a continuous scale according to support value, from 0 to 1. Grey squares in the plots represent inconsistencies in the branch topology. New transcriptomes are represented in bold. M1–M4, matrices 1–4. Coloured bars show the proportion of genes sampled for each taxon.

v.2.1.2; and Bayesian inference (BI) in PhyloBayes MPI v.1.7a (Lartillot et al., 2013). For the coalescent-based method, gene trees were initially inferred using RAxML v.8.2.12 (Stamatakis, 2014) (−N10 −m PROTGAMMALG4X), followed by concatenation in Astral-II for species tree estimation. ML trees were constructed using IQ-TREE for both non-partitioned and partitioned datasets in each matrix. The non-partitioned analysis was performed for homogeneity with 1500 ultrafast bootstrap, employing model search parameters that encompassed both the LG4 and the C10 to C60 −profile mixture models (−B 1500 −m MFP + MERGE −rcluster 10 −mad LG4M, LG4X, LG + C10, LG + C20, LG + C30, LG + C40, LG + C50, LG + C60 −mrate G, R, E). The partitioned analysis was performed for heterogeneity also including 1500 ultrafast bootstrap, employing model search parameters the LG4 mixture models (−B 1500 −m MFP + MERGE −rcluster 10 −madd LG4M, LG4X −mrate G, R, E). Due to the computational demands of PhyloBayes, Bayesian analyses were only run on Matrix4 using the site-heterogeneous CAT + GTR model with constant sites being discarded (−dc) to speed up computation. Two independent MCMC chains were run, each sampled every cycle for greater than 10,000 cycles each, with a majority-rule consensus tree obtained after a burnin of 10 % of the number of cycles. Convergence was checked using tools implemented in PhyloBayes.

3. Results

To elucidate the stable phylogenetic relationships within Littorinoidea, we generated 13 phylogenomic trees using ML, BI, and coalescent based analyses across four datasets (Matrix1-Matrix4). These datasets were constructed to mitigate the impact of evolutionary rate and heterogeneity on phylogenetic trees, from de novo assembling 35 transcriptomes representing nine superfamilies of Littorinimorpha. The topology of all main but two clades recovered highly congruent across all matrices and inference methods (Fig. 2). The subclade III (Naticoidea + Littorinoidea + Atlantidae) and subclade II (Velutinoidea + Tonnoidea + Calyptraeoidea + Stromboidea) were resolved as the sister group to subclade I (Truncatelloidea + Rissooidea) with maximal support among Littorinimorpha. The first exception involved the subclade I being the sister group with the subclade III with strong support, and then together with subclade II as the sister group with low support in the unpartitioned IQ-TREE analyses of Matrix1(BP = 41) (supplementary material, figure S3), as it did not account for the influence of site heterogeneity and variation in evolutionary rates, both of which can have significant impacts on the accuracy and reliability of results in the context of phylogenetic inference (Steenwyk et al., 2023). This inconsistency was further verified by partitioned IQ-TREE analyses using a heterogeneous model on Matrix1 and evolutionary rate analyses on Matrix2 (supplementary material). The second exception is the phylogenetic position of *Mainwaringia leithii*, exhibiting variation across

different analytical approaches. In the unpartitioned IQ-TREE and BI analyses and partitioned IQ-TREE analyses, *M. leithii* was respectively recovered as a sister group of *Littorina* with high support and *Atlanta ariejansseni* with low support. In contrast, in the coalescent based analyses, it was recovered as the sister group of the clade consisting *Littoraria*, *Echinolittorina*, and *Littorina*, receiving full support. In this study, we propose that *Mainwaringia* is the sister group of *Littorina*, which is supported by high support and previous anatomy research (Reid, 1986).

Within subclade III, surprisingly, we found that Naticidae and Atlantidae are nested within Littorinoidea, especially with the inclusion of microgastropoda (*Lacuna carinifera*, *Peasiella habei*, *M. leithii*, and *A. ariejansseni*) and terrestrial species (*Pomatias elegans*). *A. ariejansseni* from Atlantidae consistently nested within the subfamily littorininae, being recovered as sister to the group including the *Littoraria* + *Echinolittorina* and the *Mainwaringia* + *Littorina* clade with strong support. Each genus within Littorininae is monophyletic, and the clade arrangement, with *Littoraria* and *Echinolittorina* as sister groups to *Mainwaringia* and *Littorina*, resulted in a paraphyletic grouping with *P. habei*. Naticidae, represented by *Euspira heros* and *Neverita didyma*, was consistently recovered as the sister group to *L. carinifera* of the subfamily Lacuninae in all analyses with full support, which not documented in previous studies. Finally, the terrestrial species *P. elegans* from Pomatiidae is recovered as the most basal branch within subclade III.

In the subclade II, Calyptraeoidea, exhibiting protandrous hermaphroditism, formed a sister group with the clade comprising Tonnoidea and Velutinoidea, and together they constituted a sister group with Stromboidea. The phylogenetic relationships among them vary significantly in previous studies due to differences in the scope of sample collection (Cunha and Giribet, 2019; Irwin et al., 2021; Jiang et al., 2019).

The subclade I consisted of the microgastropoda superfamily Truncatelloidea and Rissooidea, formed sister groups with full support. This topology was consistent with previously published phylogeny (Criscione and Ponder, 2013). However, when expanding the sampling range, Rissooidea was paraphyletic to the Vanikoroidea, albeit with insignificant support values. Nevertheless, the two superfamilies constituted a robust clade with the Truncatelloidea (Takano and Kano, 2014). Possible explanations for the incongruence are mainly due to differences in the number of species.

4. Discussion

The phylogenetic relationships within the superfamily Littorinoidea have long been a subject of uncertainty. Our study addresses this gap by constructing the phylogenetic framework for Littorinoidea using transcriptomic data with a particular emphasis on enhancing species sampling density, especially including microgastropoda. Surprisingly, our findings reveal that Naticidae is nested within Littorinoidea with strong support (Fig. 2), which contrasts with established knowledge, as prior research has consistently highlighted distinct morphological and anatomical differences between Naticoidea and Littorinoidea (Alejandra et al., 2009; Azuma, 1961; Barker, 2001). Naticoidea has conventionally been regarded as a monophyletic group, whether based on transcriptomic or mitochondrial genetic data (Irwin et al., 2021; Jiang et al., 2019; Machkour-M'Rabet et al., 2021). Therefore, our study suggests potential challenges in the systematic classification within Littorinoidea, highlighting the need for further investigation into these intriguing phylogenetic relationships.

Based on morphological and anatomical studies, the terrestrial Pomatiidae, representing by the species *P. elegans*, has traditionally been considered closely related to Littorinidae, with both families falling under the superfamily Littorinoidea (Garnault, 1887; Creek, 1951; Barker, 2001). However, due to the long-standing challenges of uneven sampling across various taxa in resolving the phylogeny within Littorinoidea, the systematic classification of Pomatiidae has not been confirmed by the phylogenetic studies based on DNA/RNA sequences (Williams et al., 2003; Reid et al., 2012). The present study suggests that *Pomatias* is distantly related to Littorinidae species in the phylogenetic tree, which is incongruent with previous studies (Reid, 1989; Barker, 2001). In terms of anatomy, some taxonomists suggested that the close relationship between *Pomatias* and Littorinidae is attributed to the homology of their penial nerve, both supposedly originating from the right pedal ganglion (Garnault, 1887; Creek, 1951; Ponder, 1988), but Linke (1935) and Reid (1989) proposed *Pomatias* arises at the base of the pleuropedal connective. Additionally, the penial nerve of *Rapana venosa* (Muricoidea) is also found to originate from the right pedal ganglion (Li., 1990). As such, we question the homologous nature of the penial nerve origin from the right pedal ganglion as representative of Littorinoidea, while it may be a common trait in gastropods. Notably, *Pomatias* and Littorinidae exhibit substantial morphological differences in the structure of the opercula. *Pomatias* operculum typically comprises two calcified layers with a network of minute canals between them, contrasting with the single corneous layer without minute canal structures seen in Littorinidae opercula. (Fig. 3). Our findings indicate that *Pomatias* may not belong to Littorinoidea but likely originated from a more ancient ancestor.

Our study proposes a novel insight into the phylogenetic positioning of *L. carinifera*, which was recovered as the sister taxon to the Naticidae with full support, in contrast to its current position in the subfamily Lacuninae within Littorinidae (Bouchet et al., 2017; WoRMS). The taxonomic placement of *Lacuna* has been controversial as the classification of *Lacuna* has long relied on morphological and anatomical characteristics. *Lacuna* has been frequently placed into the family Lacunidae rather than Littorinidae (Gray, 1857; Winckworth, 1932; Habe, 1953; Golikov et al., 1975; Boss, 1982). However, some taxonomists have merged the two families because of the lack of significant differences in either radular (Arnaud et al., 1978) or anatomical characters (Ponder, 1976; Reid, 1988). Our present phylogenetic findings is consistent with a previous study (Takano and Kano, 2014) that divided Littorinoidea into two distant lineages, with the branch containing only *Lacuna pallidula* as the sister group to Naticidae. Anatomically, our understanding is also supported by the widespread presence of the foot in two halves and the penial glands in most Littorinidae species, features not documented in *Lacuna* (Marcus et al., 1963; Reid, 1989). Therefore, we propose a reevaluation of *Lacuna*'s classification, suggesting its potential removal from Littorinidae. However, it is worth noting that our study lacks other representative species of Lacuninae, which may slightly constrain the broad applicability of the conclusions regarding the positioning of *Lacuna*. We look forward to incorporating more representative groups of Lacuninae in future studies to better understand the phylogenetic placement of *Lacuna*.

Previous studies have consistently placed *P. habei* within the monophyletic group Littorinae (Reid, 1989; WoRMS). However, our study challenges this classification, revealing that Littorinae is paraphyletic. Given the revision history of *Peasiella*, this is also not surprising. Kesteven (1903) proposed removing *Peasiella* from littorinids to the Modulidae due to the presence of a multispiral operculum (Fig. 3). Subsequently, Rosewater (1970) suggested placing *Peasiella* in a separate subfamily of Littorinidae. However, this is not supported by anatomical characteristics (Abbott, 1954; Rosewater, 1972; Reid, 1986). Finally, Reid (1989) classified *Peasiella* into Littorinae only based on the presence of the synapomorphy paraspermatic nurse cells, although variations in nurse cell characters were noted within the topology. Meanwhile, the distinctive combination of an open prostate, closed penial sperm duct, penial gland, and absence of the glandular disc is unique to the genus *Peasiella* (Reid, 1986). Consequently, we argue that relying on a single shared trait for placing *Peasiella* within Littorininae warrants discussion. One shared trait may not be sufficient evidence for a group having a common ancestor. We suggest a revision of the taxonomic classification of *Peasiella* based on multiple morphological, anatomical traits, and molecular phylogenetics. Based on our current research, *Peasiella* may belong to a distinct family separate from

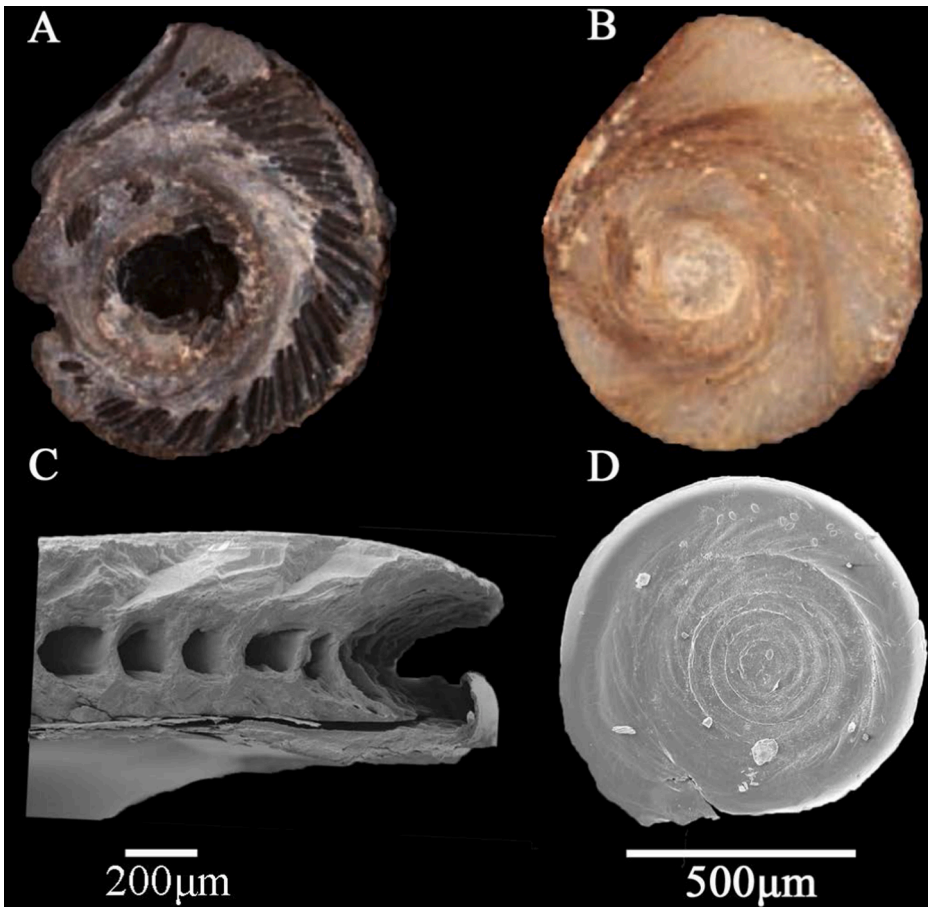

Fig. 3. Image of the operculum. A-C: Operculum of *Pomatias elegans* (O. F. MÜLLER 1774) (Wilmsmeier and Neubert, 2012). A: Operculum showing the microcanals; B: Outer surface of operculum; C: Cross section of a *Pomatias elegans* (O. F. MÜLLER 1774) operculum under the SEM. D: The operculum SEM of *Peasiella habei* D. Reid & Mak, 1998.

Littorinidae.

The present study also revealed that *A. ariejansseni* of the Atlantidae is situated phylogenetically between the families Littorinidae and Naticidae (Fig. 2). This discovery has not yet documented in most previous studies, primarily due to the challenge of sampling this group, which comprises small planktonic pteropods. Moreover, most previous research efforts have focused on taxonomic investigations and diversity (Vera and Seapy, 2006; Wall-Palmer et al., 2019, 2018). Only a few molecular phylogenetic studies based on COI data have focused on Pteropods (Wall-Palmer et al., 2016). Our study represents the first phylogenomics research on Pteropods at the order level of Littorinimorpha using transcriptomic data. The taxonomic classification of *A. ariejansseni* in our study is consistent with a previous phylogenomics study based on ultraconserved elements (UCEs) (Goulding et al., 2023), which supports the revision of placing Pteropods within the Littorinimorpha (Hausdorf and Bouchet, 2005; WoRMS).

5. Conclusion

This study highlights the significance of integrating microgastropoda taxa into molecular phylogenetic reconstructions of gastropod subgroups. By increasing the sampling density within the Littorinoidea, particularly focusing on micro-littorinids, and incorporating them into the framework of the Littorinimorpha, we have reassessed the phylogenetic relationships among clades within Littorinoidea. Our findings suggest that a major revision of Littorinoidea is warranted: 1) The relationship between *Pomatias* and Littorinidae appears distant, raising the possibility that *Pomatias* may not belong to Littorinoidea and might have originated from a more ancient ancestor. The homology of penial innervation within the Littorinoidea should be reexamined. 2) *Lacuna* is proposed to belong to a clade closely related to the Naticidae, prompting consideration for its removal from Littorinidae. 3) Based on our current phylogenetic research, *Peasiella* may belong to a distinct family separate from Littorinidae. 4) Our phylogenetic analyses support the revision of placing Pteropods within the Littorinimorpha, situated between Littorinoidea and Naticoidea. Additionally, our phylogenetic analysis also underscores that site heterogeneity and variation in evolutionary rates are important factors influencing the stability of phylogenetic inference.

6. Data accessibility

All newly generated transcriptomes were deposited in the NCBI under BioProject PRJNA1078436, and include accession numbers SRR28040000-SRR28040012. The necessary process data and Supplementary Materials were provided in the figshare: https://figshare.com/s/a1b6aefcd9f8ef7816f7.

CRediT authorship contribution statement

Lu Qi: Writing – original draft. **Ning Zhang:** Formal analysis, Conceptualization. **Biyang Xu:** Investigation. **Qinzeng Xu:** Resources, Formal analysis. **Xiao Han:** . **Lingfeng Kong:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Qi Li:** Writing – review & editing, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have shared my code at the Attach File step

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2024.108139.

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