Molecular Biology Reports (2024) 51:823
https://doi.org/10.1007/s11033-024-09767-0

ORIGINAL ARTICLE

Comparative mitochondrial genome analysis provides new insights into the classification of Modiolinae

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Received: 7 May 2024 / Accepted: 1 July 2024

Abstract

Background Mitochondrial genomes have become a powerful tool for studying molecular genetics and phylogeny of mollusks. Currently, the position of Modiolinae within Mytilidae and the taxonomic and phylogenetic relationships within Modiolinae were still controversial. This study focuses on the complete mitochondrial genomes of two species: *Modiolus modulaides* (Röding, 1798) and *Modiolus auriculatus* Krauss, 1848, which have not been sequenced before.

Methods and results We assembled and characterized the mitochondrial genomes of *M. modulaides* and *M. auriculatus* and then analyzed the phylogenetic relationships. The mitochondrial genomes of *M. modulaides* and *M. auriculatus* were 15,422 bp and 16,027 bp, respectively. Both of them were composed of 36 functional genes, including 12 protein-coding genes, 22 transfer RNAs, and 2 ribosomal RNAs. All protein-coding genes showed A+T bias, positive GC skews, and negative AT skews in nucleotide composition. Phylogenetic analysis based on the mitochondrial genomes showed that Modiolinae and Bathymodiolinae clustered together to form a sister relationship. Seven Modiolinae species were divided into two clades: L1 (*M. modulaides*, *M. auriculatus* and *Modiolus philippinarum* Hanley, 1843) and L2 [*Modiolus modiolus* (Linnaeus, 1758), *Modiolus kurilensis* Bernard, 1983, *Modiolus nipponicus* (Oyama, 1950), and *Modiolus comptus* (Sowerby III, 1915)]. The divergence time of the two clades was approximately 105.75 Ma. Furthermore, the transfer RNA gene rearrangement, longer genetic distance, and greater genetic differentiation were confirmed between the L1 and L2 clades, as well as differences in the external characteristics of the shells of the two clades.

Conclusions Based on the molecular data, it was speculated that species from the L1 clade might belong to other genera or new genera. This study provides molecular information for further taxonomic and phylogenetic studies of Mytilidae.

Keywords Mytilidae · Modiolinae · Mitochondrial genome · Phylogeny · Genetic distance

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Abbreviations

PCG	Protein-coding gene
cox1	Cytochrome c oxidase subunit I
nad3	NADH dehydrogenase subunit 3
atp6	ATP synthase F0 subunit 6
nad4	NADH dehydrogenase subunit 4
cox3	Cytochrome c oxidase subunit III
nad6	NADH dehydrogenase subunit 6
nad2	NADH dehydrogenase subunit 2
cytb	Cytochrome b
nad4l	NADH dehydrogenase subunit 4 L
nad5	NADH dehydrogenase subunit 5
cox2	Cytochrome c oxidase subunit II
nad1	NADH dehydrogenase subunit 1
atp8	ATP synthase F0 subunit 8
rrnS	Small subunit ribosomal RNA
rrnL	Large subunit ribosomal RNA

Published online: 18 July 2024

ORF Open Reading Frame
RSCU Relative Synonymous Codon Usage

Introduction

The Mytilidae family includes a large number of morphologically diverse mollusks worldwide, which live in estuary region, open coastal zone, deep-sea hydrothermal vents, and cold spring ecosystems [1]. Despite the rapid development of molecular techniques and the increasing implications in taxonomy, there are still many problems in the classification of mussels, which lead to the obstacles of ecological conservation, resource survey, and germplasm improvement [2, 3]. Morphological identification typically relies on features like shell shape, sculpture, hinge, and mussel scars [4]. However, their morphological characteristics are greatly influenced by environmental conditions and developmental stages [4]. Therefore, the classification results based solely on traditional morphology are somewhat inaccurate and need mutual support and evidence from molecular data.

The mitochondrial genome is an important tool for studying phylogeny because of its high mutation rate and parental simplicity of maternal inheritance [5]. In metazoans, the mitochondrial genome is a circular DNA molecule that includes 13 protein-coding genes (PCGs), 22 transcriptional RNA-coding genes (tRNAs), two ribosomal ribonucleic acid genes (rRNAs), and a control region [6–8]. The gene order within mitochondrial genomes constitutes a robust foundation for phylogenetic studies [9], and distinct structures within these genomes may also reflect specific phylogenetic connections [10]. In the mitochondrial genome of bivalves, coding genes with identical functions often show homology [11]. Unlike the nuclear genome, the mitochondrial genome has a relatively conserved gene composition and structure and possesses a higher nucleotide substitution rate [6, 12]. Wang et al. [13] conducted a phylogenetic analysis of species within the family Veneridae based on mitochondrial genomic data. Similarly, Lee et al. [14] performed a comprehensive phylogenetic analysis of Mytilidae species using mitochondrial genomic data, which covered almost all subfamilies of Mytilidae. However, for a wide variety of mollusks, the number of mitochondrial genomes available for taxonomy research is still too small.

Modiolinae Termier & Termier, 1950 is a common subfamily in Mytilidae [15]. According to the phylogenetic tree constructed from 18 S ribosomal RNA (rRNA) sequences, Modiolinae is positioned basally within Mytilidae [4]. This placement is consistent with the findings derived from early morphogenetic studies, which suggested that the subfamilies of Mytilidae evolved along four phylogenetic routes, initiating with Modiolinae [16]. Both insights affirm the fundamental position of Modiolinae within Mytilidae. However, research based on multiple gene fragments has shown that Modiolinae was distant from the stem base. It not only clustered with Bathymodiolinae, but also had a close relationship with Mytilinae [17]. Yet, analyses based on mitochondrial genomes suggested a sister relationship between Modiolinae and Bathymodiolinae [14]. These conflicting findings indicate challenges in determining the relationships of Modiolinae and its related species.

In parallel, the classification within Modiolinae is somewhat confusing, especially regarding *Modiolus* Lamarck, 1799, an important genus within the subfamily whose taxonomic position remains unclear. Initially, researchers firstly established subgenera in Mytilidae based on shell shape and divided *Modiolus* into 5 subgenera: *Eumytilus* Ihering, 1900, *Amygdalum* Megerle von Mühlfeld, 1811, *Gregariella* Monterosato, 1883, *Brachidontes* Swainson, 1840, and *Botula* Mörch, 1853 [18]. Subsequently, the subgenus *Brachidontes* was elevated to the genus *Brachidontes* Swainson, 1840 and the remaining subgenera were dropped and all were classified under *Modiolus* [19]. However, three subgenera [*Modiolus*, *Amygdalus* (*Amygdalum*), *and Limnoperna* Rochebrune, 1882] have replaced in *Modiolus* based on characteristics such as shell shape and the presence of yellow fur on the outer shell [20]. The three subgenera were then promoted to genus level, and the species with smooth shells and longer pipes were placed in the newly established genus *Lioberus* Dall, 1898 [21]. In the 1990s, Chinese scholars divided *Modiolus* into five subgenera: *Modiolus*, *Modiolatus* Jousseaume, 1893, *Lioberus*, *Modiolusia* Yamamoto & Habe, 1958, and *Fulfiga* Lamy, 1919 [15]. In Huber's study, *Modiolus*, *Modiolatus*, *Lioberus* were retained as genus taxa, and the genera *Gibbomodiola* Sacco, 1898, *Jolya* Bourguignat, 1877, *Benthomodiolus* Dell, 1987, *Idas* Jeffreys, 1876, *Adipicola* Dautzenberg, 1927, and *Amygdalum* were juxtaposed with them and classified in Modiolinae [22]. Recently, the genera *Benthomodiolus*, *Idas*, and *Adipicola* were classified into the subfamily Bathymodiolinae. Although the classification within Modiolinae has been more detailed, it is still changing and controversial. At present, only 5 species from *Modiolus* have their mitochondrial genomes displayed in NCBI [14, 23–25], which are *M. modiolus*, *M. comptus*, *M. nipponicus*, *M. kurilensis*, and *M. philippinarum*. The mitochondrial genomes of these species ranged from 15,591 bp to 16,389 bp and were composed of 12 or 13 PCGs, 22 tRNAs and 2 rRNAs [14, 23–25]. Therefore, more molecular data is needed to refine the controversy over the classification of *Modiolus*.

In this study, the mitochondrial genomes of *Modiolus modulaides* (Röding, 1798) *and Modiolus auriculatus* (Krauss, 1848) were characterized for the first time. This effort is aimed at providing a more molecular basis for

determining the taxonomic status of Modiolinae and the relationships among species within Modiolinae.

Materials and methods

Sample collection and total DNA extraction

In April 2023, one specimen of *Modiolus modulaides* and one specimen of *Modiolus auriculatus* were collected from Beihai, Guangxi Zhuang Autonomous Region (21.6110° N, 109.5687° E) and Wenchang, Hainan Province (19.4019° N, 110.7471° E), respectively. The specimens were identified as *M. modulaides* and *M. auriculatus* according to the morphological descriptions [15, 22]. The total DNA was extracted from the adductor muscle of each sample using a TIANamp Marine Animals DNA kit (DP324-03, Tiangen Biotech Co., Ltd, Beijing, China) according to the reagent instructions. The remaining samples were preserved in 95% alcohol and deposited in the Laboratory of Marine Organism Taxonomy & Phylogeny, Qingdao Key Laboratory of Marine Biodiversity and Conservation, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China.

Sequencing, assembly, and annotation of the mitochondrial genomes

For each species, the genomic library was constructed with the genome shotgun (WGS) strategy and sequenced on the Illumina NovaSeq platform (Illumina, San Diego, CA, USA) at Shanghai Personal Biotechnology Co., Ltd. The insert size was 2×150 bp and sequenced using the paired-end method. Then SPAdes v3.11.0 [26] was employed for the de novo assembly to construct contig and scaffold sequences. Mummer v3.1 [27] and Pilon v1.18 [28] were used to fill gaps between contigs and obtain the complete sequence. The complete mitogenome sequences were uploaded to the MITOS2 web server for functional annotation [29]. The genetic code selection was set to 5 Invertebrate and the other settings were adjusted from the default parameters. The boundaries of PCGs were determined by an online ORF finder (https://www.ncbi.nlm.nih.gov/orffinder) and manually corrected by comparison with genes from the same family [14]. Finally, Proksee (https://proksee.ca/) was used to visualize the mitochondrial genomes [30].

Bioinformatics analysis of mitochondrial genome

The skew values of the mitochondrial genomes were analyzed using PhyloSuite v1.2.2 [31] and calculated using the following formulas: AT skew = (A−T)/(A+T) and GC skew= (G−C)/(G+C). Nucleotide composition and relative synonymous codon use (RSCU) were performed using MEGA X [32]. Phylogenetic relationships within Mytilidae species were constructed based on 12 PCGs and 2 rRNAs sequences, with *Crassostrea gigas* (Thunberg, 1793) and *Atrina pectinata* (Linnaeus, 1767) used as the outgroup (Table 1). Firstly, the sequences of RNAs and PCGs were aligned using MAFFT in normal mode and codon mode, respectively [33]. The sequences of the 12 PCGs and 2 rRNAs from the former results were then concatenated into a data matrix. The AICc criterion and greedy algorithm in PartitionFinder2 [34] were used to select the optimal substitution models and partitioning schemes for the concatenated data. Finally, Bayesian inference (BI) and maximum likelihood analysis (ML) were used to reconstruct the phylogenetic tree. MrBayes v 3.2.7 [35] was employed to construct the BI tree under partition model, running four Markov chains for 2 million generations and the sampling frequency was 1000 generations. The initial 25% of the trees were omitted as burn-in fraction. IQ-TREE [36] was used to perform ML analyses with 5,000 ultrafast bootstraps [37] of the Shimodaira–Hasegawa–like approximate likelihood-ratio test (SH-aLRT) [38]. The phylogenetic tree showed the branch support values for Bayesian posterior probabilities (PP) and the maximum likelihood bootstrap support values (BS). Interactive Tree of Life (ITOL) was used to demonstrate the phylogenetic tree and gene arrangements [39]. In addition, pairwise comparisons of mitogenomes of *Modiolus modiolus* and *Modiolus auriculatus* were performed using the Common Interval Rearrangement Explorer (CREx) [40]. To evaluate the divergence times among Modiolinae and Bathymodiolinae species, a maximum likelihood tree based on 12 PCGs and 2 rRNAs was constructed using the same method above. The divergence time tree was built using RelTime in MEGA X [32] and the Kimura 2-parameter mode was selected. Two nodes were time-calibrated. The nodes were queried on TimeTree (http://www.timetree.org/) that the separation of genus *Bathymodiolus* from *Modiolus* occurred at about 132.00 Ma, and *Modiolus modiolus* (Linnaeus, 1758) differentiated from *Modiolus kurilensis* (Bernard, 1983) at about 30.01 Ma [41]. DnaSP v6.12.03 software [42] was then used to calculate the nonsynonymous substitution rate (Ka) and synonymous substitution rate (Ks) of each PCG from mitochondrial genomes of the two clades in Modiolinae. MEGA X [32] was used to align and evaluate the inter- and intra-clades genetic distances with the Maximum Composite Likelihood model based on the commonly used *cox1*, *rrnL*, and the PCGs with the highest Ka/Ks values for *Modiolus*. The genetic differentiation coefficient (F-statistics, F_{ST}) of between clades was calculated using Arlequin v3.5 [43].

Table 1 Taxonomic information and GenBank accession numbers of mitochondrial genome in this study. Sequences obtained in this study are marked in bold

Family	Subfamily	Species	GenBank accession nos.
Mytilidae	Arcuatulinae	*Arcuatula senhousia*	GU001954
	Xenostrobinae	*Xenostrobus securis*	ON128254
	Limnoperninae	*Limnoperna fortunei*	KP756905
	Bathymodiolinae	*Bathymodiolus japonicus*	AP014560
		Bathymodiolus septemdierum	AP014562
		Bathymodiolus azoricus	MT916742
		Bathymodiolus brooksi	MT916743
		Gigantidas haimaensis	MT916746
		Gigantidas vrijenhoeki	ON128253
	Brachidontinae	*Perumytilus purpuratus*	MH330331
		Mytilisepta keenae	MK721542
	Crenellinae	*Gregariella coralliophaga*	MK721545
	Septiferinae	*Septifer bilocularis*	MK721549
	Lithophaginae	*Lithophaga curta*	MK721546
	Modiolinae	*Modiolus modiolus*	KX821782
		Modiolus kurilensis	KY242717
		Modiolus nipponicus	MK721547
		Modiolus comptus	MN602036
		Modiolus philippinarum	KY705073
		Modiolus modulaides	**PP135062**
		Modiolus auriculatus	**PP135063**
	Mytilinae	*Semimytilus algosus*	MT026712
		Crenomytilus grayanus	MK721543
		Mytella strigata	OR666116
		Mytilus galloprovincialis	AY497292
		Mytilus trossulus	AY823625
		Mytilus californianus	GQ527172
		Mytilus coruscus	KJ577549
		Perna viridis	JQ970425
		Perna canaliculus	MK775557
		Perna perna	OK576479
		Perna perna	KM655841
Pinnidae		*Atrina pectinata*	KC153059
Ostreidae		*Crassostrea gigas*	AF177226

Results

The features of mitochondrial genomes

The mitochondrial genomes of *M. modulaides* and *M. auriculatus* were 15,422 bp and 16,027 bp, respectively (GenBank accession nos. PP135062 and PP135063, respectively). Both were structured as double-stranded circular molecules (Fig. 1) and consisted of 36 functional genes, including 12 PCGs (*cox1*, *nad3*, *atp6*, *nad4*, *cox3*, *nad6*, *nad2*, *cytb*, *nad4l*, *nad5*, *cox2*, and *nad1*), 22 transfer RNAs (tRNAs), and 2 rRNAs (Table 2). The contents of A, T, C and G bases in the mitochondrial genomes of *M. modulaides* were 23.1%, 39.4%, 12.0%, and 25.4%, and those of *M. auriculatus* were 25.0%, 39.9%, 12.5%, and 22.7%. Both species showed obvious (A+T) bias (62.5% and 64.9%). All the functional genes of both mitochondrial genomes were encoded on the heavy strand.

Protein-coding genes, ribosomal RNAs and transfer RNAs

All PCGs in the two mitochondrial genomes showed obvious A+T bias, ranging from 58.8% (*nad3*, *M. modulaides*) to 66.7% (*nad6*, *M. auriculatus*). Additionally, all PCGs also showed a negative AT skew, ranging from −0.392 (*cox3*, *M. modulaides*) to -0.140 (*cox2*, *M. auriculatus*) and a positive CG skew, ranging from 0.166 (*cytb*, *M. modulaides*) to 0.510 (*nad3*, *M. auriculatus*). The start codons of the PCGs included ATG, ATA, ATT, TTG, and GTG, while the stop codons included TAG, TAA, and the incomplete stop codon T– (Table 2). With regards to the start codons, all the PCGs used the typical codon ATN except for *atp6* and *nad6* in *M. modulaides*. Most of the stop codons were the typical TAA or TAG, with the exception of incomplete stop codons in *cox3* of *M. modulaides* and *nad3*, *nad4*, *nad4l* of *M. auriculatus*.

The amino acid count analysis revealed that Phe, Val, Leu2, and Gly were the most commonly occurring amino acids in both mitochondrial genomes. Among the 22 amino acids encoded, nine amino acids (Ala, Arg, Gly, Leu1, Pro, Ser1, Ser2, Thr, and Val) used four codons, and the rest used two codons. The codons CCU (Pro) and GUU (Val), with RSCU values above 2, were the most frequently used in the *M. modulaides* and *M. auriculatus* mitochondrial genomes, respectively (Fig. 2).

In both species, the *rrnS*s were flanked by $tRNA^{S1}$ and $tRNA^{M}$, and the *rrnL*s were flanked by $tRNA^{F}$ and $tRNA^{S2}$. In addition, the AT contents of both rRNAs showed a negative AT skew and a positive GC skew. Similarly, most tRNAs displayed a negative AT skew and a positive GC skew.

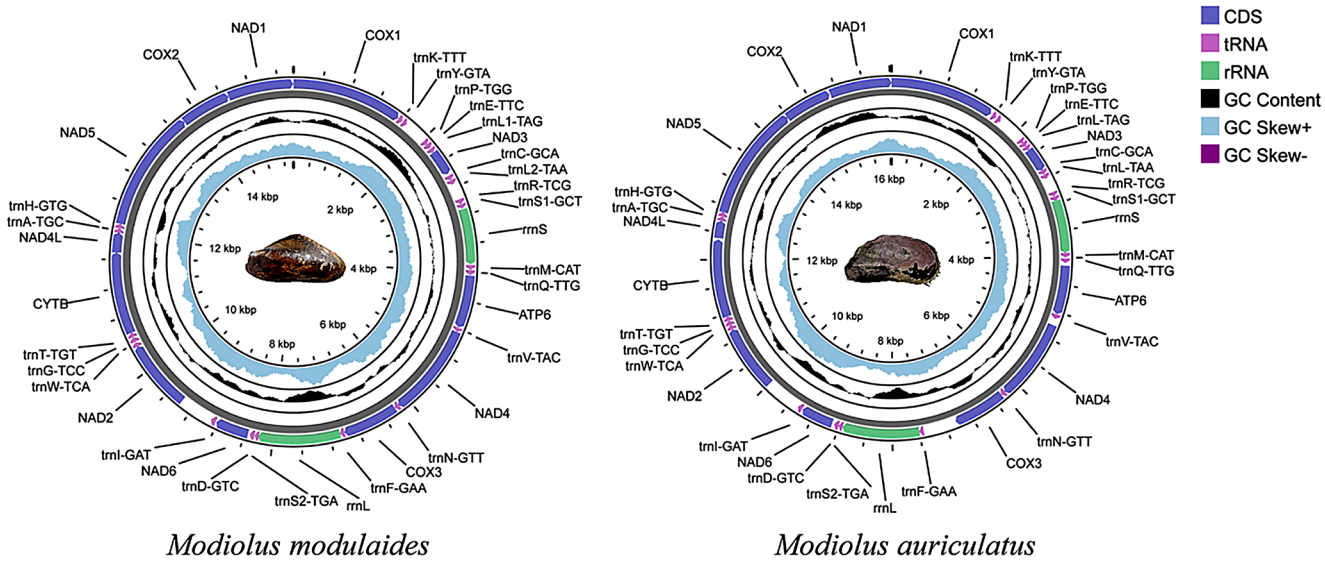

Fig. 1 Complete mitochondrial genome maps of *Modiolus modulaides* and *Modiolus auriculatus*

Phylogenetic relationships and gene arrangement

Phylogenetic relationships were inferred using two analytical methods (BI and ML) based on 12 PCGs and 2 rRNAs from 34 taxa. The resulting phylogenetic trees, constructed using both BI and ML methods, exhibited consistent topologies and the majority of the nodes in these trees were characterized by high support values (Fig. 3).

Our results revealed the monophyly of the family Mytilidae, as well as subfamilies Modiolinae and Bathymodiolinae, while indicating the polyphyly of the subfamilies Brachidontinae and Mytilinae. The family Mytilidae consisted of two clades and one of them was [(Lithophaginae + Limnoperninae) + (Xenostrobinae + Modiolinae + Bathymodiolinae)]. Within the subfamily Modiolinae, 7 species were divided into two clades (L1 and L2). The clade L1 included three *Modiolus* species: *M. philippinarum* Hanley, 1843 and the two newly sequenced species, *M. modulaides* and *M. auriculatus*, while clade L2 included the other four *Modiolus* species. Notably, the newly sequenced *M. auriculatus* initially clustered with *M. philippinarum*, establishing a sister relationship. Subsequently, they clustered together with *M. modulaides* (PP = 1, BS = 100). The molecular dating analysis estimated that clades L1 and L2 diverged at approximately 105.75 Ma (Fig. 4). The L1 and L2 clades diverged at about 99.47 Ma and 89.15 Ma, respectively.

Although all PCGs and rRNAs shared the same arrangement, the tRNA arrangements between the L1 and L2 clades were quite different (Fig. 5). CREx analysis indicated that one transposition, one tandem duplication random loss (TDRL), and three inversions might have occurred from *M. auriculatus* (L1 clade) to *M. modiolus* (L2 clade) (Fig. 6). In Modiolinae, the *atp8* gene is absent except for *M. modiolus*. Besides, it is worth noting that there is a duplication of $tRNA^{Q}$ in *M. comptus*.

Genetic analysis within Modiolinae

Selection pressure and genetic distance analysis were used to identify the relationships between the two clades within Modiolinae. The results of selection pressure analysis showed that all the PCGs had Ka/Ks < 1 (Table 3), indicating purifying selection. In both L1 and L2 clades, *cox1* had the smallest Ka/Ks values (0.10486 and 0.04232), while *nad6* (0.50292) and *nad3* (0.36665) exhibited the highest values, respectively. *Nad6* and *nad3*, as well as *cox1* and *rrnL*, were selected for genetic distance analysis. The results demonstrated that the genetic distance between L1 and L2 clades ranged from 0.23297 (based on *cox1*) to 0.38398 (based on *nad6*). Furthermore, the *Fst* values ranged from 0.17129 (based on *rrnL*) to 0.29839 (based on *nad3*) (Table 4).

Discussion

Genomic characteristics of *M. modulaides* and *M. Auriculatus*

The gene composition of the mitochondrial genome of *M. modulaides* and *M. auriculatus* (36 functional genes, including 12 PCGs, 22 tRNAs, and 2 rRNAs) fits the typical composition pattern of mitochondrial genomes in mollusks [44]. It is common for bivalves to lack the *atp8* gene in their mitochondrial genomes [5, 45, 46], and some studies have suggested that the absence of the *atp8* gene might be associated with its transfer to the nuclear genome or that the

Table 2 Mitochondrial genome organizations of *Modiolus modulaides* and *Modiolus Auriculatus*

Feature	Position		Codon				Anticodon	Skew values				Strand
			Start	Stop	Start	Stop		AT skew	GC skew	AT skew	GC skew	
	M. modulaides	*M. auriculatus*	*M. modulaides*		*M. auriculatus*			*M. modulaides*		*M. auriculatus*		
cox1	1–1548	1–1548	ATG	TAA	ATG	TAA		−0.303	0.294	−0.321	0.219	+
trnK	1557–1623	1577–1641					TTT	−0.200	0.250	0.056	0.103	+
trnY	1633–1696	1661–1726					GTA	−0.081	0.259	−0.143	0.083	+
trnP	2028–2091	2121–2180					TGG	−0.429	0.448	−0.243	0.304	+
trnE	2092–2158	2181–2247					TTC	−0.317	0.385	−0.021	0.200	+
trnL1	2167–2232	2250–2312					TAG	0.026	0.407	−0.405	0.308	+
nad3	2234–2590	2316–2670	ATG	TAA	ATG	T--		−0.390	0.510	−0.360	0.446	+
trnC	2617–2681	2671–2734					GCA	−0.045	0.143	−0.100	0.250	+
trnL2	2684–2750	2740–2805					TAA	−0.136	0.478	−0.070	0.304	+
trnR	2974–3040	3022–3085					TCG	−0.189	0.067	−0.444	0.214	+
trnS1	3044–3107	3090–3155					GCT	−0.179	0.120	−0.220	0.120	+
rrnS	3118–3888	3156–3931						−0.087	0.363	−0.030	0.311	+
trnM	3900–3964	3932–3998					CAT	−0.042	0.053	−0.091	0.048	+
trnQ	3969–4038	4009–4076					TTG	−0.250	0.333	−0.209	0.360	+
atp6	4047–4778	4117–4830	GTG	TAG	ATA	TAA		−0.369	0.396	−0.332	0.352	+
trnV	4785–4845	4837–4904					TAC	−0.077	0.182	−0.042	0.300	+
nad4	4848–6152	5001–6207	ATT	TAG	ATT	T--		−0.368	0.448	−0.361	0.327	+
trnN	6151–6216	6208–6272					GTT	−0.061	0.529	−0.018	0.600	+
cox3	6220–6994	6275–7051	ATT	T--	ATT	TAA		−0.392	0.299	−0.343	0.258	+
trnF	6996–7060	7539–7604					GAA	0.067	0.200	0.081	0.310	+
rrnL	7061–8204	7605–8740						−0.088	0.349	−0.046	0.233	+
trnS2	8197–8256	8741–8800					TGA	−0.171	0.474	−0.209	0.294	+
trnD	8259–8325	8802–8869					GTC	−0.184	0.333	0.000	−0.222	+
nad6	8350–8817	8902–9378	TTG	TAA	ATG	TAA		−0.375	0.453	−0.365	0.434	+
trnI	8828–8897	9389–9457					GAT	−0.179	0.290	−0.256	0.385	+
nad2	9376–10,350	9995–10,957	ATT	TAG	ATG	TAA		−0.341	0.408	−0.283	0.370	+
trnW	10,351–10,416	10,959–11,025					TCA	0.020	0.294	0.038	0.200	+
trnG	10,425–10,491	11,026–11,091					TCC	−0.087	0.048	0.050	0.154	+
trnT	10,496–10,558	11,094–11,156					TGT	−0.095	−0.143	−0.081	−0.154	+
cytb	10,560–11,696	11,158–12,294	ATG	TAA	ATG	TAA		−0.320	0.208	−0.289	0.166	+
nad4l	11,696–11,971	12,315–12,567	ATG	TAG	ATT	T--		−0.313	0.364	−0.317	0.370	+
trnA	11,970–12,033	12,568–12,629					TGC	−0.119	0.235	−0.143	0.300	+
trnH	12,034–12,096	12,630–12,695					GTG	−0.163	0.300	−0.087	0.300	+
nad5	12,097–13,791	12,696–14,393	ATG	TAA	ATG	TAA		−0.242	0.347	−0.225	0.246	+
cox2	13,793–14,497	14,394–15,098	ATG	TAA	ATG	TAG		−0.182	0.331	−0.140	0.249	+
nad1	14,501–15,418	15,110–16,027	ATG	TAG	ATG	TAA		−0.248	0.335	−0.238	0.242	+

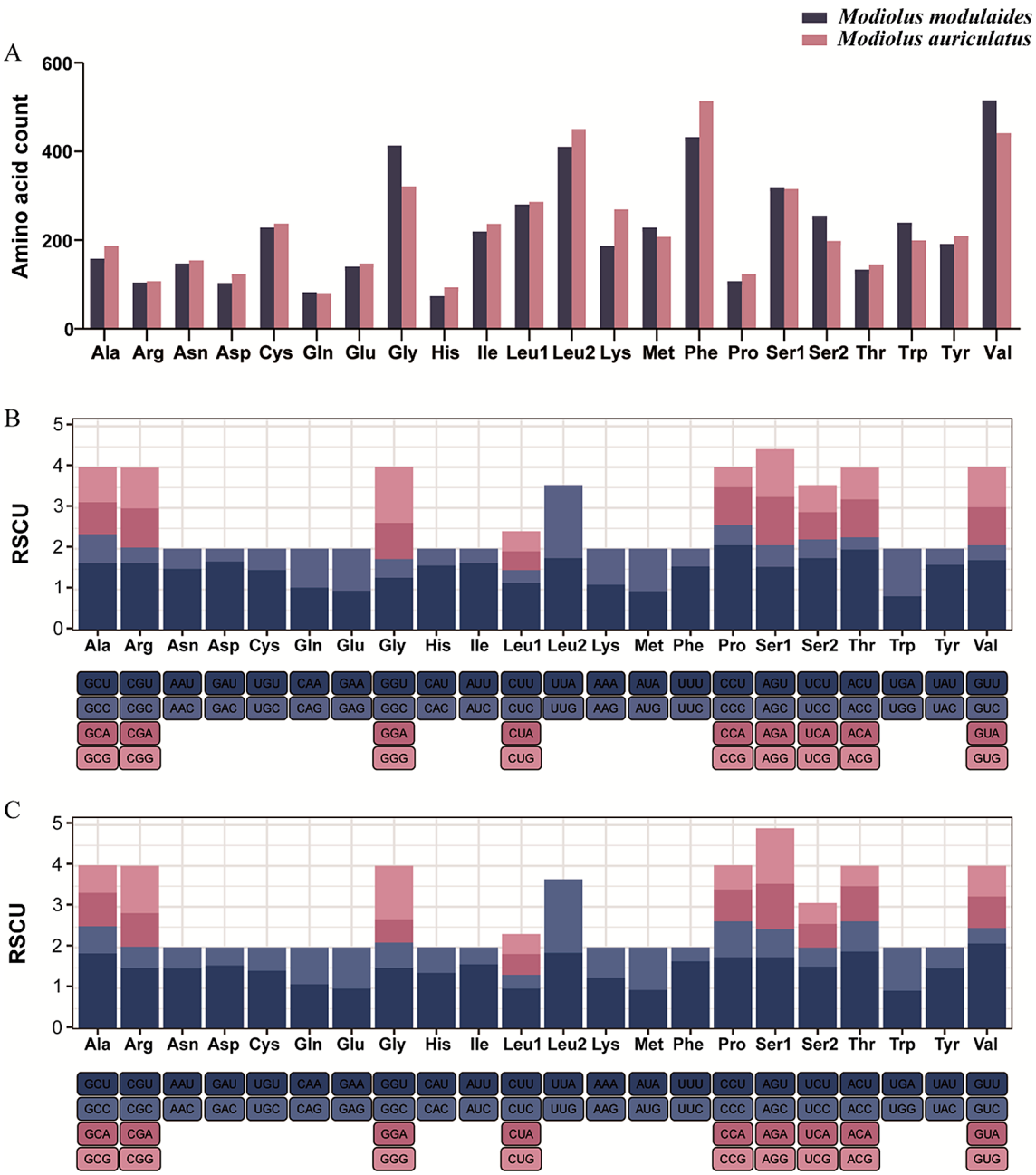

Fig. 2 Amino acid counts (**A**) and relative synonymous codon usage (RSCU) of *Modiolus modulaides* (**B**) and *Modiolus auriculatus* (**C**) mitochondrial genome

atp8 gene sequence was too short and variable to annotate [47–49]. Our ORF search and sequence alignment results revealed that no *atp8* gene was found in the mitochondrial genes of *M. modulaides* and *M. auriculatus*. The base distribution of the mitochondrial genomes of bivalves is commonly not balanced and tends to show significant A + T bias, which is consistent with our results [50]. In addition, the mitochondrial genomes of *M. modulaides* and *M. auriculatus* displayed obvious base skew characteristics, which is thought to be caused by the initiation of mismatch repair due to the different probabilities of spontaneous mutations occurring in uncoiled single-stranded DNA during transcription or replication [51–53]. The incomplete stop codon T– of *M. modulaides* and *M. auriculatus* in the mitochondrial genome is expected to form a complete UAA stop codon by polyadenylation of the 3' end of the transcript during post-transcriptional processing [54].

The mitochondrial genome exhibits a clear preference in the selection of the third nucleotide of synonymous codons [55]. Our findings revealed that amino acids with high use

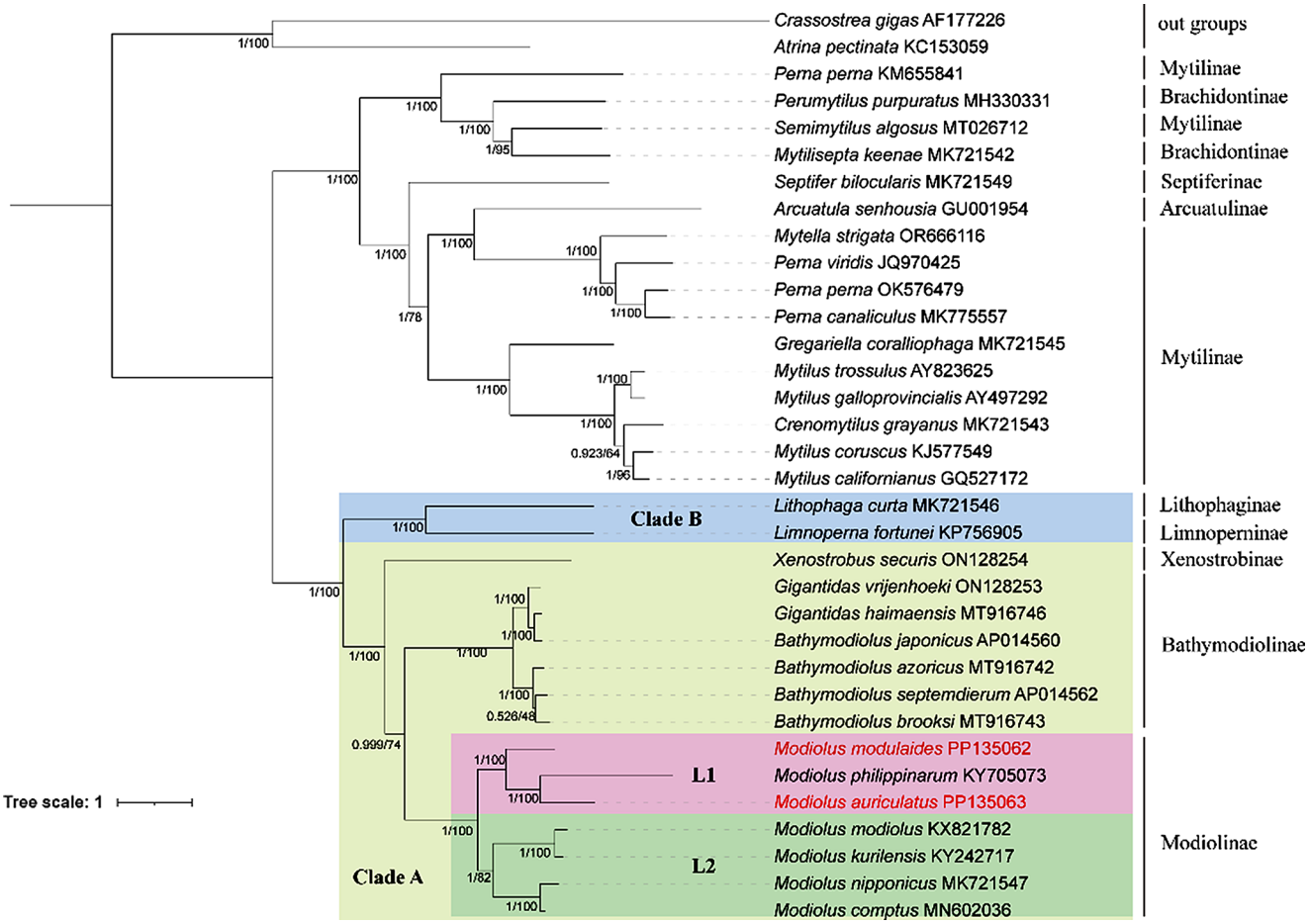

Fig. 3 Phylogenetic tree of the Mytilidae family based on 12 PCGs and 2 rRNAs. *Atrina pectinata* and *Crassostrea gigas* were used as outgroups. Bayesian posterior probabilities followed by maximum likelihood bootstrap support values are shown for each node. Species names and GenBank accession numbers of the newly determined mitochondrial genome species in this study were marked in red

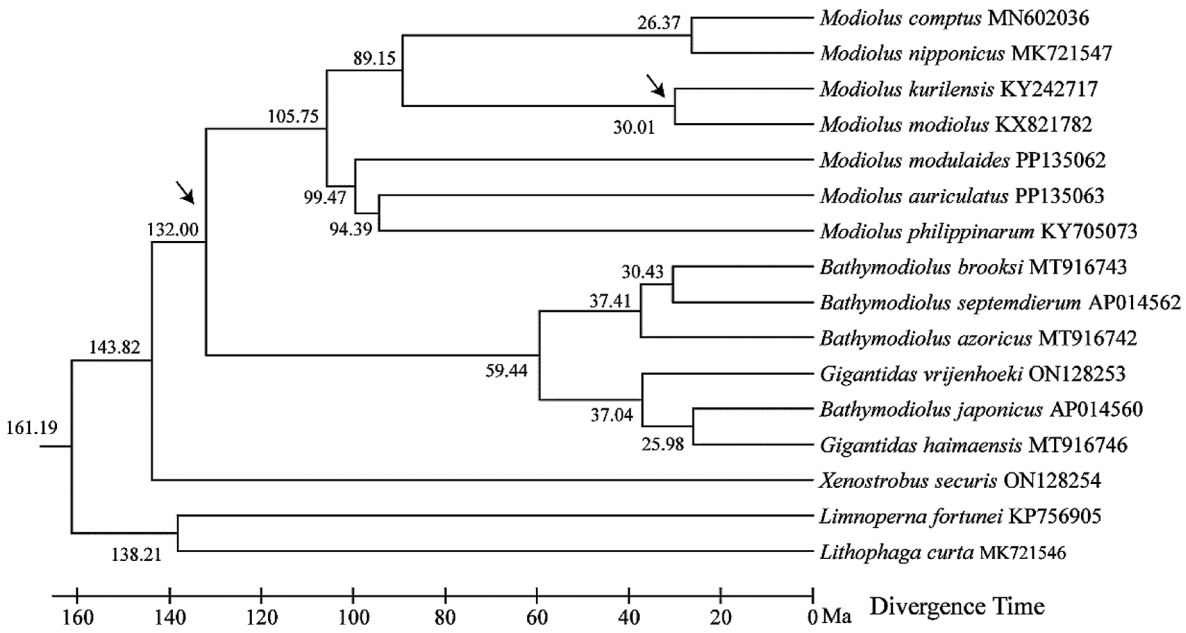

Fig. 4 Divergence time tree in Modiolinae and Bathymodiolinae. Correction points are indicated by arrows

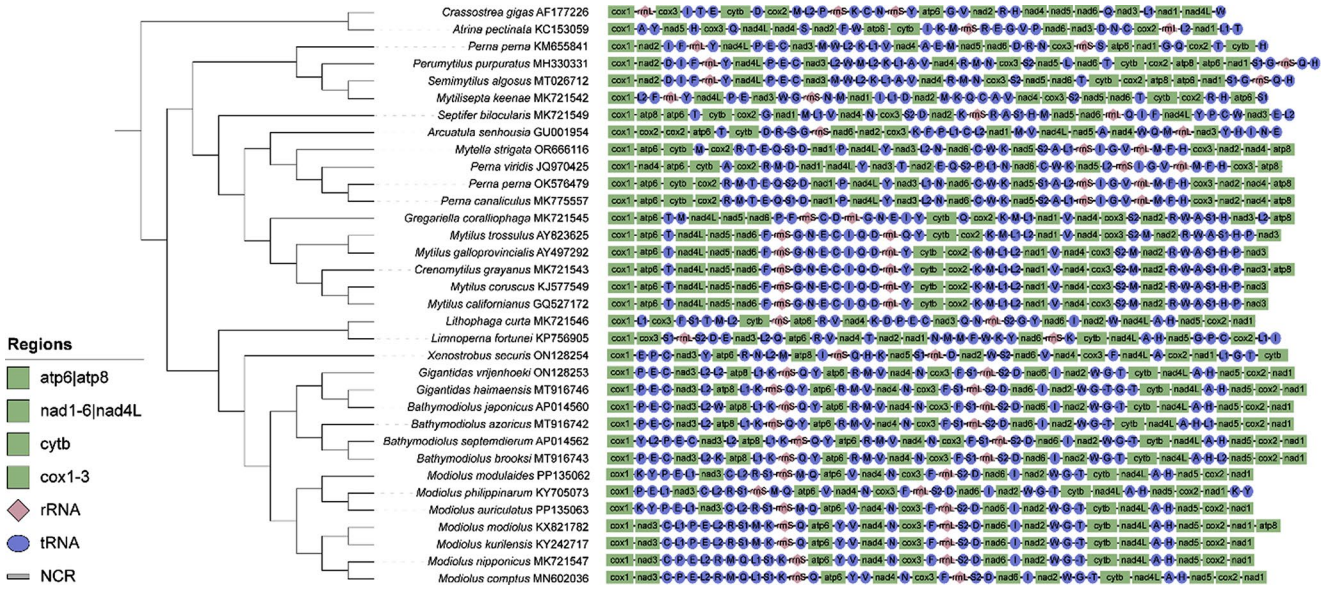

Fig. 5 Gene arrangements of mitogenomes in the Mytilidae family

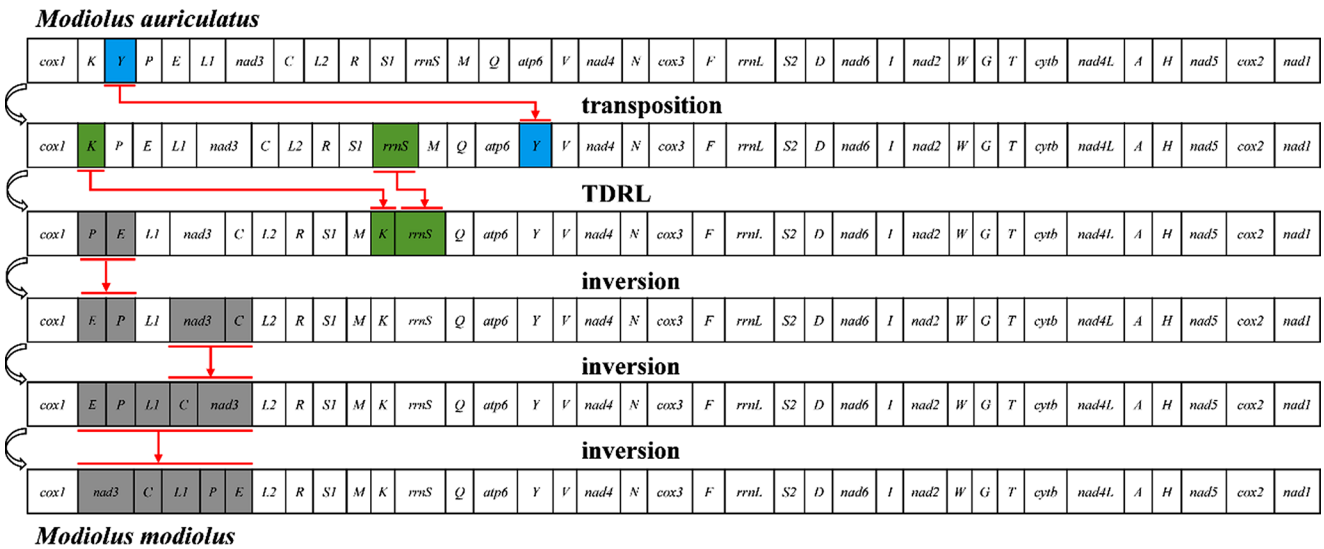

Fig. 6 Presumed gene rearrangement events from *Modiolus auriculatus* to *Modiolus modiolus*. Red lines and colored blocks represent rearrangement processes and genes

frequencies and RSCU values above 2 had codon bias with U (T) and C endings. This preference may be related to natural selection and mutation, which is believed to be a mechanism for mitochondria to maintain the structural and functional stability of proteins during rapid evolution [56]. Although codon usage varies slightly across species, mitochondrial genome codon usage remains similar among closely related species [57]. This might account for the similarity in amino acid count and codon use bias between the mitochondrial genomes of *M. modulaides* and *M. auriculatus*.

Classification and phylogenetic relationship of Modiolinae

The monophyly of subfamilies Septiferinae, Arcuatulinae, Limnoperninae, Lithophaginae, and Xenostrobinae could not be determined since only one species from each subfamily participated in the establishment of the phylogenetic tree. However, Mytilinae and Brachidontinae are polyphyletic groups. Modiolinae and Bathymodiolinae clustered together to form a sister group relationship, which is consistent with previous studies [14, 41]. *X. securis* is clustered with the taxa Modiolinae and Bathymodiolinae, followed by clade A. The inclusion of Xenostrobinae species in

Table 3 The evolutionary constraint (Ka/Ks) analyses of 13 mitochondrial protein coding genes in three separate clades of the genus *Modiolus*. Ka: nonsynonymous substitution rate, Ks: synonymous substitution rate calculations

Genes	L1				L2			
	bp	Ka	Ks	Ka/Ks	bp	Ka	Ks	Ka/Ks
atp6	711	0.25871	0.65733	0.39358	699	0.20027	0.62413	0.32088
cox1	1542	0.07657	0.73022	0.10486	1509	0.02449	0.57867	0.04232
cox2	702	0.09906	0.71015	0.13949	699	0.03645	0.51805	0.07036
cox3	774	0.11680	0.65913	0.17720	774	0.06744	0.58289	0.11570
cytb	1134	0.11900	0.69954	0.17011	1131	0.05489	0.51188	0.10723
nad1	915	0.16124	0.77524	0.20799	792	0.05902	0.55899	0.10558
nad2	948	0.27976	0.67591	0.41390	963	0.14347	0.55037	0.26068
nad3	348	0.21180	0.65658	0.32258	354	0.17716	0.48318	0.36665
nad4	1302	0.25802	0.71989	0.35842	1302	0.16402	0.66816	0.24548
nad4L	264	0.22015	0.68392	0.32189	273	0.11545	0.56861	0.20304
nad5	1677	0.27546	0.67604	0.40746	1674	0.14479	0.53800	0.26913
nad6	465	0.31403	0.62441	0.50292	441	0.11658	0.48594	0.23991

Table 4 Genetic distance and genetic differentiation coefficient (F-statistics, F_{ST}) between the L1 and L2 clades of Modiolinae based on mitochondrial *cox1*, *rrnL*, *nad6*, and *nad3*. GD: genetic distance

Clades	*cox1*		*rrnL*		*nad6*		*nad3*	
	GD	F_{ST}	GD	F_{ST}	GD	F_{ST}	GD	F_{ST}
L1-L2	0.23297	0.20705	0.24637	0.17129	0.38398	0.28195	0.37775	0.29839

our study may have influenced this result, which was not observed in other studies [41, 58]. The freshwater mussel member *Limnoperna fortunei* (Dunker, 1856) is a sister of *Lithophaga curta* (Lischke, 1874), which is placed as a sister of Xenostrobinae, Modiolinae, and Bathymodiolinae (clade A). A closer relationship between clade B (Limnoperninae and Lithophaginae) and clade A had also been reported in previous studies [14, 41]. Moreover, the finding that *Semimytilus algosus* (Gould, 1850) in Mytilinae clustered together with Brachidontinae was also supported by other studies [59]. Our results showed that the taxonomic status of Modiolinae is relatively far from the base of the phylogenetic tree, which supports the taxonomic result of Modiolinae based on multiple gene fragments [17]. Notably, *Perna perna* (Linnaeus, 1758) (KM655841) is located at the base of Brachidontinae and away from the other *Perna* sequences. The mitochondrial genome sequence length of *P. perna* (KM655841) is 2,350 bp longer than that of another *P. perna* sequence (OK576479). By comparison, *P. perna* (KM655841) has three control regions and its PCGs arrangement is quite different from that of the genus *Perna*. We propose that the identification of *P. perna* (KM655841) may be incorrect, and further research is needed to determine the specific reasons.

Researches have shown that the differentiation time range of Modiolinae is 43.6 ~ 120.5 Ma [17] or 52.3 ~ 119.9 Ma [14], which are similar to our results. The L1 clade differentiated at about 99.47 Ma and the L2 clade differentiated at about 89.15 Ma. Bathymodiolinae diverged into the *Gigantidas* clade and *Bathymodiolus* clade at about 59.44 Ma. However, it is noteworthy that *Bathymodiolus japonicus* (Hashimoto & Okutani, 1994) from *Bathymodiolus* clade appears in the *Gigantidas* clade. According to our results, the differentiation of Bathymodiolinae mainly occurred in the Cenozoic, which supports the hypothesis that Bathymodiolinae originated from shallow water and transitioned to deep water [17]. Molecular dating suggested that the lineage division of Modiolinae occurred approximately during the Mesozoic. This could be attributed to favorable Mesozoic conditions such as warm temperatures, high sea levels, and large continental shelf areas [14, 60], which may have promoted the diversification rate of Modiolinae [61].

The internal topology of Modiolinae is primarily divided into L1 and L2 clades. Among them, the sister relationship between *M. modiolus* and *M. kurilensis* is supported by previous studies [14]. Furthermore, the close relationship between *M. nipponicus* and *M. comptus* has also been reported in other studies [46]. Through gene rearrangement analysis, we found that while the order of PCGs in the L1 and L2 clades remain consistent, the arrangements of other genes vary. The gene order of the mitochondrial genome has been used to study the evolution of organisms and their genomes by providing information on the characteristics of ancient lineage phylogeny [62]. Our results reveal that there was one transposition, one TDRL, and three inversions from *M. auriculatus* (L1 clade) to *M. modiolus* (L2 clade). Most of these rearrangements occurred between tRNAs. Given that tRNA gene sequences are the basis of phylogenetic classification [62], the markedly distinct tRNA rearrangements in clades L1 and L2 cast uncertainty on their taxonomic relationships.

According to the evolutionary constraint analyses of the L1 and L2 clades, *cox1* had the smallest Ka/Ks value, while *nad6* and *nad3* had the largest Ka/Ks values. This indicates that the greatest variations in PCGs between the mitochondrial genomes of the two clades occurred in *nad6* and *nad3* [5, 63]. Moreover, *cox1* and *rrnL* are commonly used as molecular markers in taxonomic researches of mollusks [64, 65]. Therefore, *nad6*, *nad3*, *cox1*, and *rrnL* were used simultaneously for genetic distance and F_{ST} analysis. In our results, the genetic distance between the L1 and L2 clades ranged from 0.23297 to 0.38398, depending on the molecular marker used. Some researchers used a genetic distance of 0.02 or 0.12 as a threshold for species classification [66, 67]. Moreover, the genetic distance within genera was reported to range from 0.022 to 0.097 in a study involving the genus *Mytilus* [68]. Obviously, the genetic distances observed in our study surpass these species thresholds. It is generally believed that genetic differentiation between clades is small, moderate, or greater when F_{ST} is 0 ~ 0.05, 0.15 ~ 0.25, or 0.15 ~ 0.25, respectively [69]. Hence, there is also considerable genetic differentiation among the two clades. The L1 and L2 clades diverged at 105.75 Ma, which may also provide a time basis for the accumulation of genetic variation between the two clades. Morphologically, species in the L1 clade are characterized by short, thin shells with yellow fur, while those in the L2 clade are generally stout and long [15]. Considering the many differences between the L1 and L2 clades and that the typical species of the genus *Modiolus* was *M. modiolus* in clade L2, we hypothesized that species in the L1 clade may belong to other genera in Modiolinae or potentially a new genus. However, more data and detailed morphological observations are needed to confirm their taxonomic identification.

Conclusions

The mitochondrial genomes of *M. modulaides* and *M. auriculatus* are 15,422 bp and 16,027 bp, respectively. Both genomes consist of 36 functional genes. All protein-coding genes in the two mitochondrial genomes exhibited a consistent bias in nucleotide composition with an A+T bias, a positive GC skew, and a negative AT skew. The phylogenetic analysis based on mitochondrial genomes did not reveal the basal position of Modiolinae in Mytilidae. Instead, Modiolinae exhibited the closest affinity with Bathymodiolinae. Within Modiolinae, 7 *Modiolus* species could be divided into two clades, clade L1 (*M. modulaides*, *M. auriculatus* and *M. philippinarum*) and clade L2 (*M. modiolus*, *M. kurilensis*, *M. nipponicus*, and *M. comptus*). The divergence time for the two clades was estimated to be approximately 105.75 Ma. The genetic distance and genetic differentiation between the two clades exceeded the species threshold. Additionally, differences in the external characteristics of the shells and tRNA arrangements of the two clades were also confirmed. Therefore, we speculated that species in the L1 clade might belong to other genera or new genera of Modiolinae.

Acknowledgements We would like to thank the reviewers for their constructive comments and helpful suggestions that improved the manuscript.

Author contributions All authors contributed to the study conception and design. Y. Z. and S. Y. conceived the research and conducted experiments. P. M., Y. Z., Y. Z., C. Z. and X. M. helped collect samples and carry out the data analysis. The original draft was written by Y. Z. and S. Y. P. M. and Z. Z. reviewed and edited the manuscript and all authors commented on previous versions of the manuscript. Z.Z. and P. M. were in charge of the funding provision. All authors read and approved the final manuscript.

Funding This research was funded by the National Key R&D Program of China (NO. 2022YFD2401204) and the National Natural Science Foundation of China (NO. 42006080).

Data availability No datasets were generated or analysed during the current study.

Declarations

Ethical approval Ethical review and approval were waived for this study because the mussels in this study are invertebrates with no sense or subjective experience.

Sampling and field studies All necessary permits for sampling and observational field studies have been obtained by the authors from the competent authorities and are mentioned in the acknowledgements. The study is compliant with CBD and Nagoya protocols.

Competing interests The authors declare no competing interests.

Conflict of interest The authors have no conflicts of interest to declare.

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