Brief Report

The Mitochondrial Genome of Littoraria melanostoma Reveals Phylogenetic Relationship of Littorinimorpha

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Brief Report

The Mitochondrial Genome of *Littoraria melanostoma* Reveals Phylogenetic Relationship of Littorinimorpha

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Abstract: *Littoraria melanostoma* (Gray, 1839) is a most common species of gastropods in mangroves. They quickly respond during the early stage of mangrove restoration and usually form a dominant community within a certain period. We characterized the complete mitochondrial genome of the species. The whole mitogenome of *L. melanostoma* was 16,149 bp in length and its nucleotide composition showed high AT-content of 64.16%. It had 37 genes, including 13 protein-coding genes, two ribosomal RNA genes, and 22 transfer RNA genes, one control region between tRNA-Phe and *COX3*. The A/T compositions in the control region is 74.7%, respectively, and are much higher than the overall A/T composition of the mitochondrial genomes. The amino acid composition and codon usage of the mitochondrial genomes of from eight superfamilies of Littorinimorpha were analyzed, and the results showed that CUU (Leu), GCU (Ala), AUU (Ile), UCU (Ser), UUA (Leu), GUU (Gly) and UUU (Phe) are the commonly used codons in this order. The results of phylogenetic analysis were roughly consistent with morphological classification, which showed that *L. melanostoma* is closely related to *L. sinensis*, a rock-dwelling species that is widespread in the coastal intertidal zone of China. These results may provide a basis to understand the phylogeny and evolution of the Littorinimorpha.

Keywords: mangrove; mitochondrial genome; *Littoraria melanostoma*; phylogeny; Littorinimorpha

1. Introduction

Mangroves are woody plant communities that become established on the intertidal flats of tropical and subtropical coasts. The harsh natural conditions of the coastal intertidal zone and the unique advantages of the transition zone between land and sea have produced the unique biome of the mangrove ecosystem. In addition, the resulting extremely rich biodiversity also plays a crucial role in the ecosystem. As one mollusk group in mangroves, gastropods play an important role in the detritus cycle and consume a large amount of plant tissue and humus [1].

Littorinidae, one of the major groups of arboreal gastropods in mangrove forests, adapt to the special environment of the coastal intertidal zone through numerous physiological and ecological manners, including their multiple reproductive means, which are known as a variable reproductive strategy [2]; complex food composition [3]; vertical climbing ability [4-6]; and variable shell color and shape [7]. Because gastropods have a limited ability to move, they generally only move on the same tree without external interference [8], and it is difficult for them to migrate from the intertidal zone to land. Consequently, they have a relatively fixed pattern of spatial distribution [9,10]. Simultaneous studies have shown that species of Littorinidae respond quickly to the restoration of mangrove vegetation, and they can form a dominant community in the early stage of vegetation restoration as exemplified by *L. melanostoma* [11]. Most of the Littorinidae in mangroves live on mangrove and salt marsh plants, driftwood, and stakes.

The family Littorinidae (Children, 1834) comprises more than 200 species that are common members of marine intertidal communities around the world, and most of them only live on mangrove plants[12]. Species of Littorinidae that have been reported in China include *L. melanostoma*,

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L. ardouiniana, *L. intermedia*, *L. pallescens*, *L. articulata* and *L. brevicula* [13-15]. *L. melanostoma* is often found in the branches and leaves of mangroves (Figure 1).

Morphological differences make the identification of gastropods confused, and the classification information is constantly adjusted. In recent years, molecular systematic studies were used to analyze the evolution and phylogeny of gastropods. Reid et al used nuclear 28S rRNA, mitochondrial 12S rRNA and COI to construct the phylogeny of family Littorininae [12]and genus *Littoraria* [16]. Li et al used complete mitochondrial genome sequences to analyze the phylogenic relationship between genus *Littorina* and *Littoraria*, but only four species were used in this study[17]. Phylogeny of Stromboidea, a superfamily in Littorinimorpha was studied based on 13 mitochondrial protein-coding genes[18]. By searching in Genbank, we found that the number of gastropods with available mitogenomes has increased, but there are few studies on the phylogenetic analyses that investigate relationships across the Littorinimorpha.

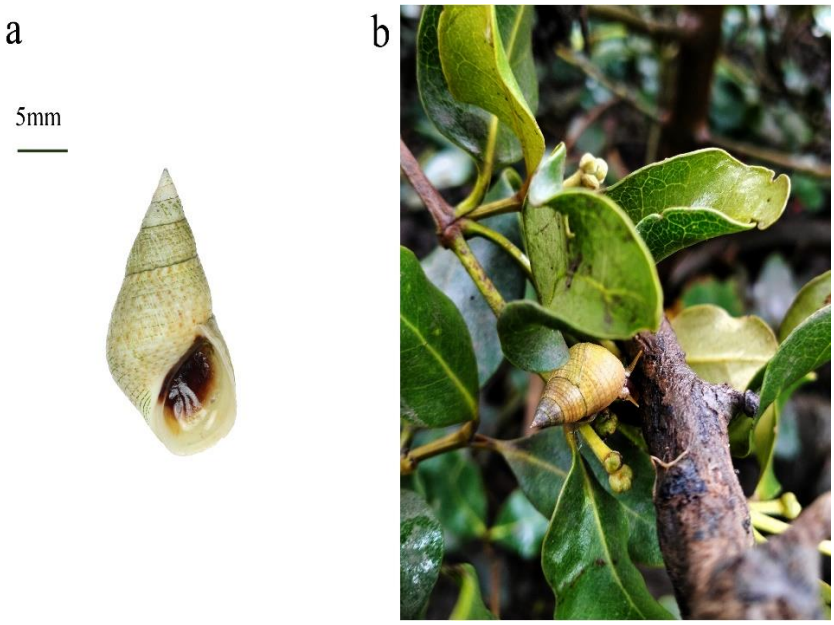

Figure 1. a. Specimen image of *L. melanostoma*; b. *L. melanostoma* inhabited on the leave of *Avicennia marina* (a common species of mangrove in China).

In this study, *L. melanostoma*, one of the most common gastropoda species in the mangrove wetlands of China, was studied for its molecular evolution and phylogeny. The mitochondrial genomes of *L. melanostoma* was sequenced, and the genome structure, base composition, codon usage, intergenic region, and codon preference of the mitochondrial genomes were analyzed. In addition, a phylogenetic tree of 30 species from 8 superfamilies of Littorinimorpha based on 13 PCGs was constructed using the maximum likelihood (ML) method. This study may increase our understanding of the phylogeny and evolution of Littorinimorpha.

2. Materials and methods

2.1. Sample collection and DNA extraction

Specimens of *L. melanostoma* was obtained from mangrove wetlands in Beihai, Guangxi, China (21.57°N, 109.16°E) and vouchered in the specimen room of the Guangxi Mangrove Research Center (Accession numbers LM#11-20, respectively). Since this species is unprotected invertebrates, no specific permission was required to collect samples from these locations. Total genomic DNA was obtained from the muscles of individuals using a QIAamp DNA Micro Kit (Qiagen, Hilden, Germany).

2.2. Sequencing, assembling and analysis

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The gene *cox1* was amplified using the universal primers LCO1490 and HCO2198 by standard PCR method [19]. The extracted DNA was sequenced using a NovaSeq 6000 platform (Illumina, San Diego, CA), and the mitogenome was assembled with NOVOPlasty v2.7.0 [20] and annotated with MitoZ v2.4 [21]. Protein-coding genes (PCGs) were determined by determining the open reading frames (ORFs) based on the invertebrate mitochondrial genetic code, and rRNAs and tRNAs were identified using the MITOS Web Server (http://mitos2.bioinf.uni-leipzig.de/index.py) [22]. The codon usage was calculated using MEGA 7.0 [23] . Strand bias was calculated using the following formulae: AT-skew = (A−T)/(A+T) and GC-skew = (G−C)/(G+C) [24]. The circular maps of the mitochondrial genomes were drawn using the online mitochondrial visualization tool Organellar Genome DRAW [25]. The nucleotide composition, codon usage, and comparative mitogenomic architecture tables for the two mitogenomes and data that were used to plot the relative synonymous codon usage (RSCU) figures were all calculated/created using PhyloSuite [26].

2.3. Phylogenetic analysis

The nucleotide sequences of the complete mt genomes from 30 species (Table 1), including five species from Littorinoidea, 24 species from other superfamilies of Littorinimorpha, and *Ovatella vulcani* as an outgroup, were downloaded from GenBank. A total of 30 amino acid sequences were aligned using MAFFT v.7.215 [27] and trimmed with trimAl v.1.4.1 [28] with the heuristic method 'automated1.' The phylogenetic tree was reconstructed using IQ-TREE v2 [29] based on ML with the partitioning method [30], and Branch support analysis was conducted using 10,000 ultrafast bootstrap replicates.

Table 1. Classification and origins of the mitogenomic sequences used in this study.

Taxonomy	Species	bp	Accession number
Littorinoidea	***Littoraria melanostoma***	16,149	NC064398
	Littorina brevicula	16,356	MT362562
	Littorina saxatilis	16,887	KU952094
	Littoraria sinensis	16,420	MN496138
	Melarhaphe neritoides	15,676	MH119311
Stromboidea	*Harpago chiragra*	16,404	MN885884
	Lambis lambis	15,481	MH115428
	Conomurex luhuanus	15,799	KY853669
	Strombus gigas	15,461	KM245630
Cypraeoidea	*Cypraea tigris*	16,177	MK783263
	Monetaria annulus	16,087	LC469295
Naticoidea	*Lunatia gilva*	16,139	MK395168
	Euspira gilva	16,119	MN419026
	Neverita didyma	15,252	MK548644
	Glossaulax reiniana	15,254	MH543334
Xenophoroidea	*Onustus exutus*	16,043	MK327366
Vermetoidea	*Dendropoma gregarium*	15,641	HM174252
	Ceraesignum maximum	15,578	HM174253
	Thylacodes squamigerus	15,544	HM174255
	Eualetes tulipa	15,078	NC_014585

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Tonnoidea	*Charonia lampas*	15,405	MG181942
	Monoplex parthenopeus	15,270	EU827200
Truncatelloidea	*Oncomelania hupensis*	15,191	EU079378
	Oncomelania quadrasi	15,184	LC276227
	Tricula hortensis	15,179	EU440735
Rissooidea	*Godlewskia godlewskia*	15,224	KY697387
	Baicalia turriformis	15,127	KY697386
	Korotnewia korotnewi	15,171	KY697389
	Maackia herderiana	15,154	KY697388
Outgroup	*Ovatella vulcani*	14,274	JN615139

3. Results and discussion

3.1. Genome structure and organization

The complete *L. melanostoma* mitochondrial genome was 16,149 bp long, and it was uploaded to GenBank after annotation (ACCESSION ID: NC064398). The gene compositions of *L. melanostoma* was the same as most of the mitochondrial genomes of gastropods that have been published. Contained 37 genes, including 13 protein-coding, two rRNA and 22 tRNA genes [21,31] (Figure 2). According to the difference in G+T content, the two strands of mitochondrial DNA could be separated into a heavy strand (H strand) and a light strand (L strand). The 13 protein-coding genes in the mitochondrial genomes of *L. melanostoma* is located on the H strand, which is consistent with the findings of previous studies that showed that the mitochondrial genomes of Littorinidae, such as *Littorina. fabalis*, *Littorina. obtusata* and *Littorina. saxatilis*, harbor protein-encoding genes on the H strand [31]. Most genes are on the H strand except the eight tRNAs that are located on the L strand, and include *trnM* (CAU), *trnY* (GUA), *trnC* (GCA), *trnW* (UCA), *trnQ* (UUG), *trnG* (UCC), *trnE* (UUC), and *trnT* (UGU). The 13 PCGs include seven NADH dehydrogenase genes (complex I)—*ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, and *ND6*; three cytochrome c oxidase genes (complex IV)—*COX1*, *COX2*, and *COX3*; two ATPase subunits (*ATP6* and *ATP8*); and one cytochrome b gene.

Table 2 summarized the proportions of gene bases and protein-coding gene sequence bases in the complete mitochondrial genome sequences of *L. melanostoma.* The base composition of the *L. melanostoma* mitochondrial genome was 29.79% A, 34.37% T, 14.66% G and 21.18% C. The A+T content (64.16%) of its mitochondrial genes was higher than the G+C content (35.84%), and the A+T content of protein-coding genes was 62.39%. These results show *L. melanostoma* genomes display an obvious nucleotide composition that is biased to A+T, which is consistent with the other genomes of Littorinidae species that have been reported [31]. The base composition bias is usually reflected by AT skew and GC skew. The calculated AT skew and GC skew of the *L. melanostoma* mitochondrial genome were 0.071 and -0.182, respectively. These data indicate that the bases T and C appear more frequently than A and G in the mitochondrial genomes of *L. melanostoma*.

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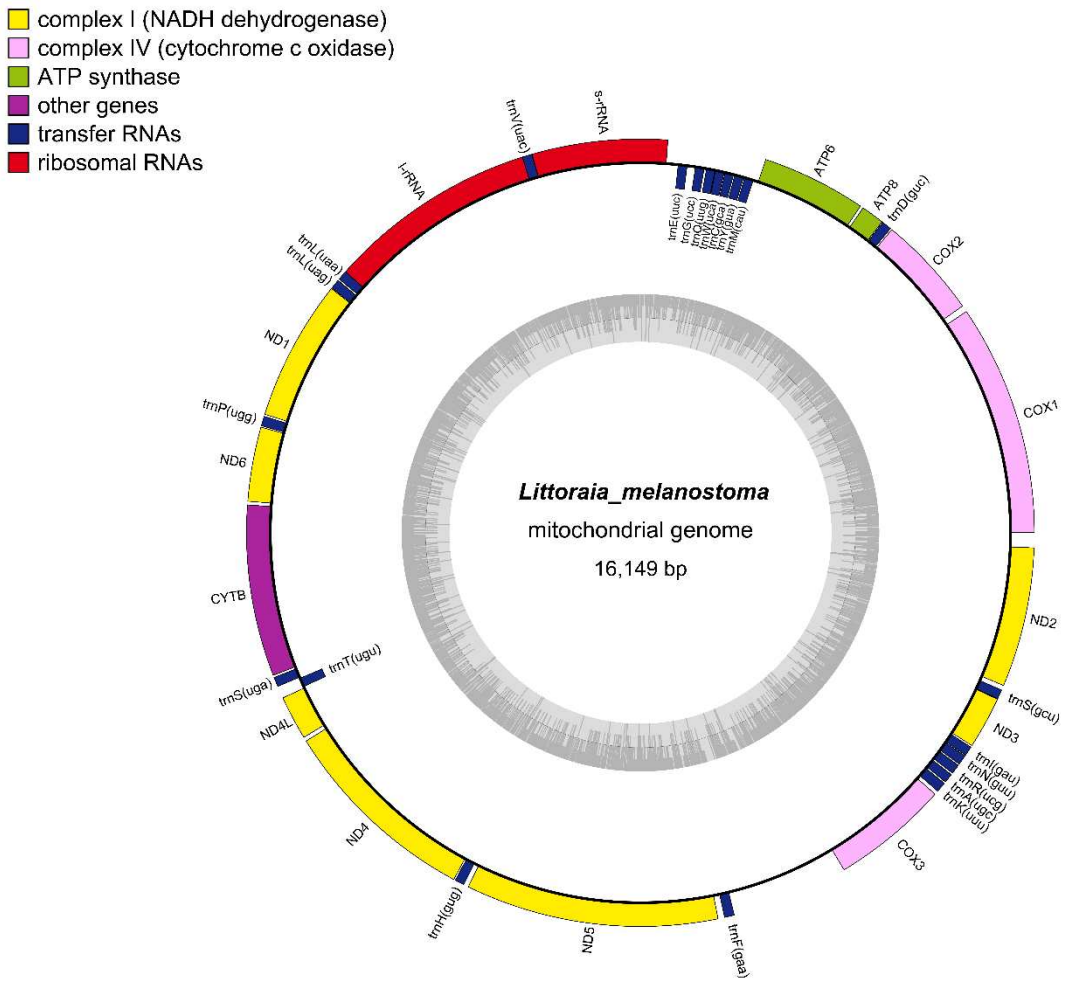

Figure 2. Circular maps of the mitogenomes of *L. melanostoma*.

Table 2. Composition and base content of *L. melanostoma* protein coding genes.

	Littoraria melanostoma							
	A %	T %	G %	C %	AT %	GC %	AT-Skew	GC-Skew
Mitogenome	29.79	34.37	14.66	21.18	64.16	35.84	-0.071	-0.182
All PCGS	27.77	34.62	15.15	22.46	62.39	37.61	-0.110	-0.194
COX1	26.43	34.18	17.71	21.68	60.61	39.39	-0.128	-0.101
COX2	28.97	30.57	17.76	22.71	59.53	40.47	-0.027	-0.122
ATP8	32.70	36.48	10.69	20.13	69.18	30.82	-0.055	-0.306
ATP6	26.44	36.06	13.22	24.28	62.50	37.50	-0.154	-0.295
ND1	26.09	34.50	15.23	24.17	60.60	39.40	-0.139	-0.227
ND6	27.31	34.94	13.05	24.70	62.25	37.75	-0.123	-0.309
CYTB	25.70	33.07	15.09	26.14	58.77	41.23	-0.125	-0.268
ND4L	25.70	33.07	15.09	26.14	68.01	31.99	-0.125	-0.268
ND4	28.56	37.21	13.57	20.66	65.77	34.23	-0.132	-0.207
ND5	30.15	33.13	13.35	23.36	63.29	36.71	-0.047	-0.273

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COX3	25.90	32.18	19.36	22.56	58.08	41.92	-0.108	-0.076
ND3	27.35	39.32	15.10	18.23	66.67	33.33	-0.180	-0.094
ND2	28.89	37.38	14.39	19.33	66.27	33.73	-0.128	-0.147

3.2. PCGs and codon usage

The nucleotide lengths of the 13 protein-coding genes of *L. melanostoma* is 11,034 bp, which encode 3,678 amino acid residues, respectively. Most protein-coding genes start with ATN and end with TAA or TAG codons (Table 3).

The codon usage of 13 protein-coding genes in the *L. melanostoma* mitochondrial genomes is shown in Table 3. In the *L. melanostoma* genome, only one gene (*ND3*) used ATA as the start codon, and two used ATT as the start codon, namely *ND4* and *ND5*. The remaining 10 genes (*COX1*, *COX2*, *ATP8*, *ATP6*, *ND1*, *ND6*, *CYTB*, *ND4L*, *COX3*, and *ND2*) all used ATG as the start codon. *ND4L*, *ND5* and *ND2* used TAG, CTT and AAT as the stop codon, respectively, and these codons were each used by one gene only. There were 10 genes (*COX1*, *COX2*, *ATP8*, *ATP6*, *ND1*, *ND6*, *CYTB*, *ND4*, *COX3*, and *ND3*) that used TAA as the stop codon.

Table 3. Mitogenomic organization of *L. melanostoma*.

		Position		Size(bp)	Intergenic nucleotides	Codon		Strand
	gene	From	To			Start	Stop	
	Littoraia melanostoma							
1	COX1	1	1536	1536		ATG	TAA	H
2	COX2	1575	2261	687	38	ATG	TAA	H
3	trnD(guc)	2268	2336	69	6			H
4	ATP8	2338	2496	159	1	ATG	TAA	H
5	ATP6	2512	3207	696	15	ATG	TAA	H
6	trnM(cau)	3240	3306	67	32			L
7	trnY(gua)	3310	3377	68	3			L
8	trnC(gca)	3382	3446	65	4			L
9	trnW(uca)	3448	3514	67	1			L
10	trnQ(uug)	3514	3578	65	-1			L
11	trnG(ucc)	3590	3656	67	11			L
12	trnE(uuc)	3710	3777	68	53			L
13	s-rRNA	3856	4756	901	78			H
14	trnV(uac)	4754	4822	69	-3			H
15	l-rRNA	4801	6219	1419	-22			H
16	trnL(uaa)	6210	6277	68	-10			H
17	trnL(uag)	6284	6352	69	6			H
18	ND1	6353	7291	939	0	ATG	TAA	H
19	trnP(ugg)	7301	7369	69	9			H
20	ND6	7374	7871	498	4	ATG	TAA	H
21	CYTB	7890	9029	1140	18	ATG	TAA	H
22	trnS(uga)	9040	9107	68	10			H

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23	trnT(ugu)	9111	9178	68	3			L
24	ND4L	9185	9481	297	6	ATG	TAG	H
25	ND4	9505	10845	1341	23	ATT	TAA	H
26	trnH(gug)	10852	10918	67	6			H
27	ND5	10947	12624	1678	28	ATT	CTT	H
28	trnF(gaa)	12663	12732	70	38			H
	CR	12733	13505	773	0			
29	COX3	13506	14285	780	773	ATG	TAA	H
30	trnK(uuu)	14307	14378	72	21			H
31	trnA(ugc)	14385	14451	67	6			H
32	trnR(ucg)	14459	14527	69	7			H
33	trnN(guu)	14533	14602	70	5			H
34	trnI(gau)	14604	14671	68	1			H
35	ND3	14679	15029	351	7	ATA	TAA	H
36	trnS(gcu)	15029	15095	67	-1			H
37	ND2	15123	16053	931	27	ATG	AAT	H

Figure 3 shows the amino acid composition and codon usage of the mitochondrial genomes of eight species from eight superfamilies. The results showed that CUU (Leu), GCU (Ala), AUU (Ile), UCU (Ser), UUA (Leu), GUU (Gly) and UUU (Phe) were the most commonly used codons. These observations suggest that there is a strong AT bias for protein-coding genes in the mitochondrial genomes of Littorinidae animals.

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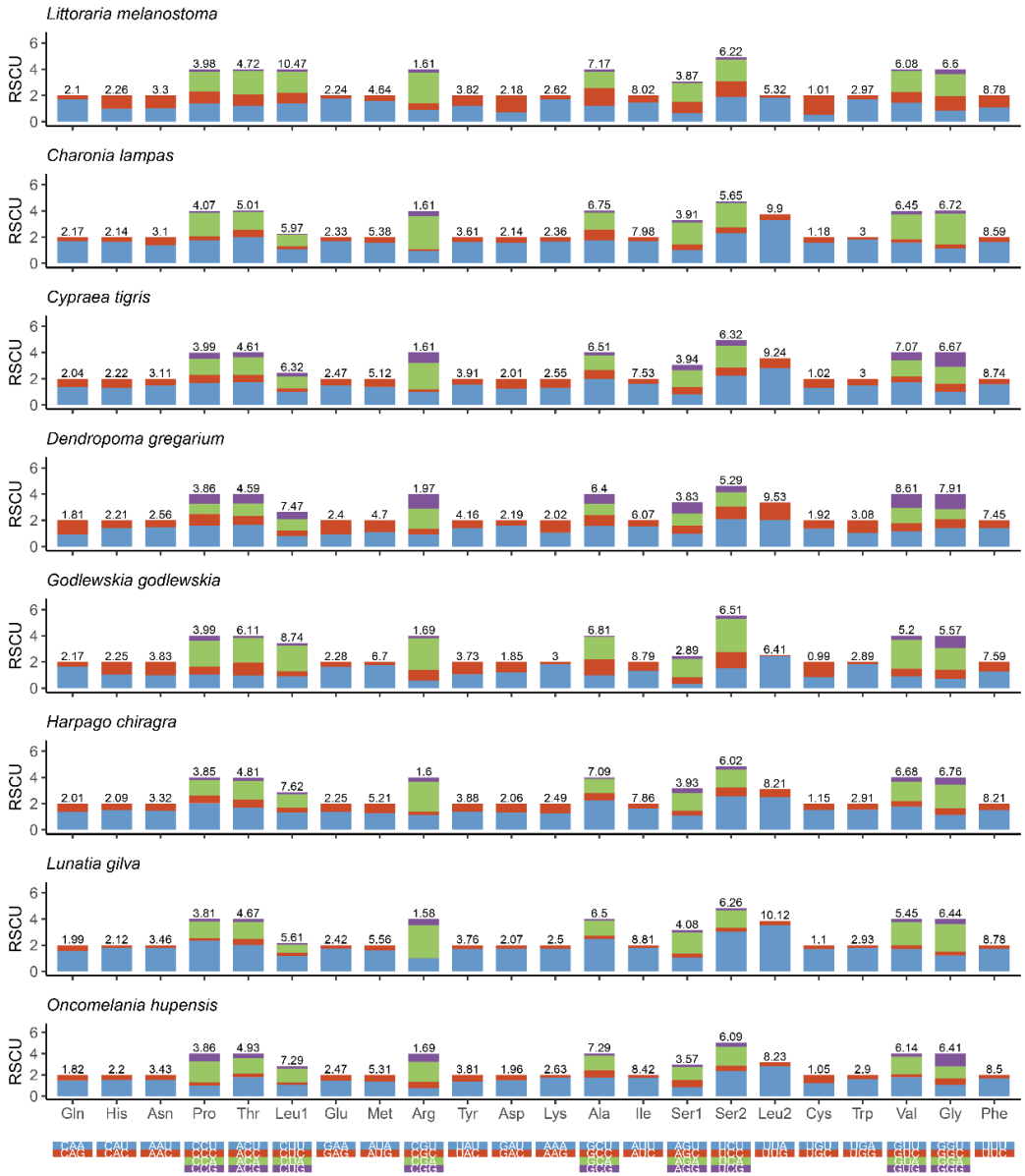

Figure 3. Relative synonymous codon usage (RSCU) in the mitogenomes of eight species of Littorinimorpha.

3.3. Ribosomal and transfer RNA genes

Two rRNA genes, l-rRNA and s-rRNA, are located between *trnL* (UAA) and *trnV* (UAA) and between *trnV* (UAA) and *trE* (UUC), respectively.

In the mitochondrial genome of *L. melanostoma*, l-rRNA was 1,419 bp, and s-rRNA was 901 bp (Table 3). A total of 22 tRNA genes was found in *L. melanostoma*, and its cloverleaf structures was 65–72 bp.

3.4. Intergenic spaces and overlapping sequences

There were five overlapping gene regions in the mitochondrial genome of *L. melanostoma*, which ranged from 1 to 22 bp in length, and 30 intergenic regions, which ranged from 1 to 773 bp long. The longest intergenic region was located between *trnF* (GAA) and *COX3* (Table 3).

3.5. Control regions

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The control region (CR) of mitochondrial DNA is the primary non-coding region of the mitochondrial genome of animals, also known as the D-loop region, which is a key part for the replication and transcription of the mitochondrial genome and regulates the replication and transcription of the mitochondrial genome. During the process of evolution, since the selection pressure that acts on this region is relatively non-intrusive, the CR usually displays the largest sequence and variation in length, the highest rate of evolution, and is the most polymorphic in the mitochondrial genome [32]. However, since the UTR sequences of invertebrates are poorly conserved, there is no defined CR in their mitochondrial genomes[33]. For example, Marques studied the genomes of *L. fabalis*, *L. obtusata*, and *L. saxatilis* and found a region that contained some unique features, such as a non-coding region with a hairpin structure and a tandem repeat sequence, located between tRNA-Phe (*trnF* [GAA]) and *COX3*, and an AT content that was higher than the overall AT content in the mitochondrial genome. This region was then predicted as the CR. Similar to those results, we found a non-coding sequence that contained some unique features in the *L. melanostoma* genomes. It was between tRNA-Phe and *COX3*, and the AT content was 74.7%, respectively. This is much higher than the AT content of the mitochondrial genomes (64.16%). Thus, we consider that this region is a unique non-coding region of the *Littoraria* genus, which may play a regulatory role in the replication and transcription of the mtDNA of this genus.

3.6. Phylogenetic analyses

To further study the genetic background and taxonomic relationship of *L. melanostoma*, the complete mitochondrial genome sequences of *L. melanostoma* was compared with the complete mitochondrial genome sequences of 28 other species from 8 superfamily of the Littorinimorpha. *Ovatella vulcani* was utilized as an outgroup, and a phylogenetic tree was constructed based on 13 PCGs using IQ-TREE v2 with the ML method (Figure 4). It can be inferred from the phylogenetic tree that the rest of sequences were divided into four clades except for the outgroup. Among them, Littorinoidea and Naticoidea were grouped into one clade; nine species from Stromboidea, Tonnoidea and Cypraeoidea were grouped into one clade; the species from Rissooidea and Truncatelloidea were grouped into one clade, and the Vermetoidea species formed one separated clade. This phylogenetic relationship is consistent with the results using the traditional classification method. It is apparent from the phylogenetic tree that *L. melanostoma* is closely related to *L. sinensis*, which is a rock-dwelling species that is widespread in the coastal intertidal zone of China, just as the previous study[16].

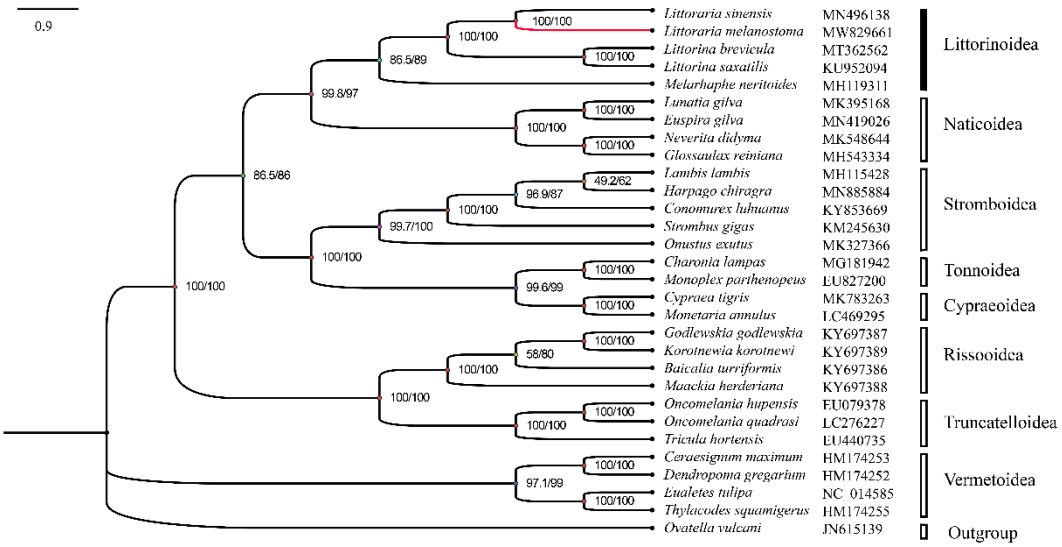

Figure 4. Maximum likelihood phylogenetic tree inferred from 13 PCGs. SH-aLRT and UFBoot support values are given on nodes.

4. Conclusions

In this study, the mitogenomes of *L. melanostoma* was sequenced, and 37 genes (13 PCGs, 22 tRNA genes and 2 rRNA genes) and one control region are located as typical of a Littorinoidea mitogenome. The ML phylogenetic relationships based on 13 PGs of the order Littorinimorpha were analyzed, indicating that the basis for the relationship based on a molecular analysis is consistent with that of the traditional morphological method.

Authors' Contributions: KC drafted the manuscript and performed data analysis. MLY collected and processed animal samples. XL designed and conceived the experiment and performed the data analysis. HSD and XL edited the manuscript. All authors read and approved the final manuscript.

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Conflict of interest: The authors declare there are no competing interests.

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