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Mitochondrial Genome of the Hermit Crab *Coenobita lila* (Anomura: Paguroidea) and Insights into Gene Rearrangements and Phylogeny of Anomura

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All authors contributed to the study concept and design. Conceptualization: DZZ, BPT, and GD. Methodology: GW. Formal analysis and investigation: JY, YZ, and YHM. Writing and editing: THY and XLS and DZZ. All authors read and approved the final manuscript.

Key words
***Coenobita lila*, Gene rearrangement, Hermit crab, Mitogenome, Paguroidea, Phylogenetic analysis**

ABSTRACT

The complete mitochondrial genome of *Coenobite lila* was sequenced and annotated. It was 16,396 bp in length and contained 13 protein-coding genes, 2 ribosomal RNA genes, 22 transfer RNA genes and a control region. Mitochondrial genome of *C. lila*, was with negative AT skew and positive GC skew. Ka / Ks of the 13 protein-coding genes indicated purifying selection. The replication-random loss and recombination model was used to explain the mechanism of gene rearrangement of 11 tRNAs and 2 PCGs in *C. lila* relative to pancrustaceans. Phylogenetic analysis using 13 protein-coding genes including 23 species of Anomura, 7 species of Brachyura and 1 outgroup showed that *C. lila* was in Coenobitidae family. The gene order of *C. lila* mitogenome underwent a large rearrangement.

INTRODUCTION

Mitochondrial genome is double-stranded closed-loop structure independent of nuclear chromosomes. In contrast to the light strand, the heavy strand of the mitochondrial genome has more G and less C (Simon *et al*., 1994). There is no intron sequence in mitochondrial genome with overlapped coding genes. The mitochondrial genome of metazoan is usually 14-20kb in length, which is mainly divided into four parts, including 13 PCGs (protein-coding genes), 22 tRNAs, 2 rRNAs and a control region (Boore, 1999). Mitochondrial genome has the characteristics of simple structure, maternal inheritance, rapid variation and gene rearrangement, which provides useful information for phylogenetic analysis (Fritzsch *et al*., 2006). Several models has been proposed to explain mitochondrial gene rearrangement, including replication-random loss, replication-non-random loss, recombination and tRNA mismatch mode (Lunt and Hyman, 1997; Moritz and Brown, 1987). Nowadays, complete mitochondrial genomes are increasingly used in population genetics, species identification, molecular evolution and phylogenetic research (Nie *et al*., 2022; Sun *et al*., 2022; Reding *et al*., 2021).

Paguroidea is one of the most abundant species groups in Anomura with more than 72 genuera and 1,100 species. Due to the high morphological and ecological diversity, the classification and phylogenetic relationship of Paguroidea have been controversial for a long time (Lemaitre and Mclaughlin, 2009). *Coenobita lila* was

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found to be a new species by morphological difference analysis and molecular data identification (Colín *et al.*, 2023). It is widely distributed in the coastal waters of Singapore, Indonesia and Malaysia, and usually inhabits 100 meters from the coast, hiding between grasses or rocks, edge of rocks, mangroves or estuaries.

Preliminary studies focused on the morphological characteristics of *C. lila*, but a few focused on its molecular, biological characteristics and phylogenetic status. Besides, the classification of Paguroidea where *C. lila* is located has always been ambiguous (Tan *et al.*, 2018; Li *et al.*, 2020). Therefore, the mitochondrial genome of *C. lila* was sequenced, and the basic structure and phylogenetic tree of mitochondrial genome were deeply analysed in this study to explore the evolution of mitochondrial genome.

MATERIALS AND METHODS

Sample collection, DNA extraction and sequencing

A sample of *C. lila* was collected from sand flat and stored at -80 ℃. Total DNA was extracted from the muscles using SQ Tissue DNA kit (Omega). The quality of the separated DNA was detected by 1% agarose gel electrophoresis, and sequenced by next-generation sequencing paired reads (Illumina Novaseq™; Shanghai Origingene Bio-pharmTechnology Co. Ltd. China).

Sequence assembly, gene annotation and analysis

Getorganells (http://github.com/Kinggerm/GetOrganelle) was used to splice the read sequence for multiple iterations to obtain the preliminary assembly results. Clean data without sequencing adapters were adjusted with Pilon v1.23 and de novo assembled by the NOVOPlasty 2.7.2 software. Finally, the mitochondrial genome sequence was obtained based on the reference genome mitochondrial scaffold start position and direction. In total, 37 genes were annotated using MITOS WebServer (http://mitos.bioinf.uni-leipzig.de/index.py) and the codon usage of PCGs was computed using the MEGA 7.0 software (Kumar *et al.*, 2016; Bernt *et al.*, 2013). We used OGDRAW (https://chlorobox.mpimp-golm.mpg.de/OGDraw.html) to draw the genome map. The skewness of nucleotide composition was analysed according to the following formulas: AT skew [(A-T)/(A + T)] and GC skew [(G-C)/(G + C)] (Perna and Kocher, 1995). The Ka / Ks values of each pair of homologous genes were calculated using KaKs_Calculator 2.0 through ParaAT and Mafft (Wang *et al.*, 2010; Rozewicki *et al.*, 2019).

Phylogenetic analysis and gene rearrangement

The phylogenetic relationship was reconstructed using 13 PCGs of 31 species (23 Anomura species, 7 Brachyura species and 1 outgroup) with *Pteronarcys princeps* as outgroup. Gblocks was used to multiple sequence alignment (Gerard and Jose, 2007). Maximum likelihood (ML) analysis was performed using RAxML 8.2.12, and the best amino acid substitution model selected by Prottest 3.4.2 (Posada, 2011). Bootstrap analysis (1000 replicates) was used to evaluate the relative support level for ML analysis (Sitnikova, 1996). Gene rearrangment was analysed with replication-random loss and recombination models.

RESULTS AND DISCUSSION

Genome composition and structure

The complete mitochondrial genome of *C. lila* was 16,396 bp in length (Fig. 1). Mitochondrial data was deposited in GenBank with the accession No. OP645220. It comprised 13 PCGs, 22 tRNAs, 2 rRNAs and a control region (Table 1). The overall nucleotide composition was A (26.6%), T (36.5%), G (21.7%) and C (15.2%). The GC content was 36.9 %, which was similar to that of *C. brevimanus* (35.0%) and other hermit crabs (Zhang *et al.*, 2021; Hickerson and Cunningham, 2000). Additionally, the AT-skew was appreciably negative (-0.157), reflecting a higher occurrence of Ts to As, and its GC-skew (0.176) was positive indicating a higher content of Gs than Cs.

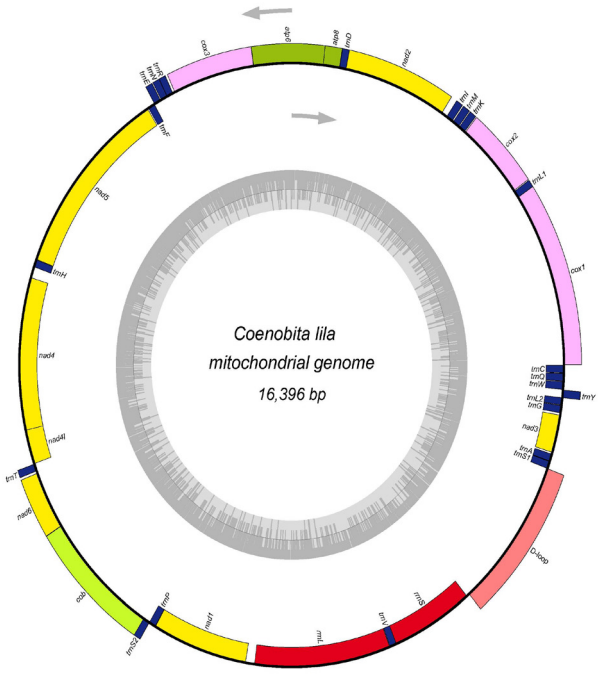

Fig. 1. Gene map of the mitogenome of *Coenobita lila.*

Table I. The mitochondrial genome features of *Coenobita lila.*

Genes	Position		Length (bp)	Amino acid	Start/Stop condon	Anticodon	Intergenic region	Strand
	From	To						
cox1	1	1539	1539	512	ATG/TAA		-	H
trnL1	1535	1598	64			CTA	-5	H
cox2	1606	2295	690	229	ATG/TAG		7	H
trnK	2304	2368	65			AAA	8	H
trnM	2376	2443	68			ATG	7	H
trnI	2454	2519	66			ATC	10	H
nad2	2574	3587	1014	337	ATT/TAA		54	H
trnD	3574	3638	65			GAC	-14	H
atp8	3639	3797	159	52	ATT/TAG		0	H
atp6	3794	4465	672	223	ATA/TAA		-4	H
cox3	4465	5256	792	263	ATG/TAG		-1	H
trnR	5276	5338	63			CGA	19	H
trnN	5338	5402	65			AAC	-1	H
trnE	5410	5475	66			GAA	7	H
trnF	5478	5543	66			TTC	2	L
nad5	5553	7266	1714	571	ATT/T		9	L
trnH	7270	7335	66			CAC	3	L
nad4	7432	8772	1341	446	ATG/TAA		96	L
nad4l	8766	9068	303	100	ATG/TAA		-7	L
trnT	9071	9135	65			ACA	2	H
nad6	9155	9665	510	169	ATA/TGA		19	H
cob	9666	10799	1134	377	ATA/TGA		0	H
trnS2	10798	10862	65			TCA	-2	H
trnP	10862	10928	67			CCA	-1	L
nad1	10930	11853	924	307	ATA/TAG		1	L
16S	11939	13258	1320				85	L
trnV	13262	13328	67			GTA	3	L
12S	13326	14120	795				-3	L
trnS1	15481	15546	66			AGA	1360	L
trnA	15549	15613	65			GCA	2	L
nad3	15628	15960	333	110	ATT/TAA		14	L
trnG	15979	16044	66			GGA	18	L
trnL2	16048	16112	65			TTA	3	L
trnY	16112	16178	67			TAC	-1	H
trnW	16183	16251	69			TGA	4	L
trnQ	16256	16323	68			CAA	4	L
trnC	16327	16394	68			TGC	3	L

PCGs and codon usage

The total length of PCGs in mitogenome of *C. lila* was 11,113 bp. Eight PCGs (*nad6, cob, cox1, cox2, nad2, atp8, atp6* and *cox3*) were encoded on the H-strand, while the remaining five PCGs (*nad3, nad1, nad4l, nad4* and *nad5*) were encoded on the L-strand (Table I). The start codons were similar to invertebrate mitochondrial genomes (Xu *et al*., 2016), and five PCGs (*cox1, cox2, cox3, nad4* and *nad4l*) started from the ATG, four PCGs (*nad2, atp8, nad5* and *nad3*) started from the ATT and four PCGs (*atp6, nad6, cob,* and *nad1*) started from the ATA. Six PCGs (*cox1, nad2, atp6, nad4, nad4l* and *nad3*) were terminated with TAA, four PCGs (*cox2, atp8, cox3* and *nad1*) with TAG, two (*cob* and *nad6*) with TGA and one (*nad5*) with a incomplete termination codon T (Table I). The existence of incomplete termination codons was a common phenomenon in mitochondrial genes (Gong *et al*., 2017; Hamasaki *et al*., 2017). One explanation for this phenomenon was that the TAA end was produced by post-transcriptional polyadenylation (Honarmand and Shoubridge, 2020).

Totally, 13 PCGs of the mitogenome totally encoded 3696 amino acids. The number of codons varied from 52 (*atp8*) to 571 (*nad5*) (Table I). The most frequently used amino acids were Leu (15.8%) and Ile (11.0%), and the least common amino acids were Cys (1.1%), Trp (1.6%), and Met (2.2%). The RSCU (relative synonymous codon usage) value of the 13 PCGs for the third positions was shown in Figure 2. The usage of the two most frequent amino acids (Ile and Leu) were ATT and TTA, biased toward in A and T, while Cys, Trp and Met with low frequency were rich in G and T. The AT content of 13 PCGs was 61.2 %, and the AT skewness and GC skewness were -0.219 and 0.014, indicating that the species preferred T to A and G to C (Table II).

Transfer RNAs and ribosomal RNAs

The *C. lila* mitogenome contained 22 tRNAs genes, and the length of tRNA genes ranged from 63 (*trn-Arg*) to 69 bp (*trn-Trp*) with the total length 1452 bp (Tables I, II). Similar to the AT skew and GC skew of PCGs, its AT skew was negative (-0.014) and GC skew was positive (0.119), respectively (Table II). The two rRNA genes were identified on the L-strand in *C. lila* mitogenome, with the *12S rRNA* located between *D-loop* and *trn-Val,* and the *16S rRNA* located between *trn-Val* and *nad1*. The length of *12S rRNA* was 795 bp and the *16S rRNA* was 1320 bp. The AT-skew and GC-skew of rRNAs were 0.110 and -0.039, indicating that more As and Cs than Ts and Gs in rRNAs (Table II).

Table II. Composition and skewness of *Coenobita lila* mitogenome.

	Length (bp)	A (%)	T (%)	G (%)	C (%)	A+T (%)	AT-skew	GC-skew
Mitog-enome	16396	26.6	36.5	21.7	15.2	63.1	-0.157	0.176
cox1	1539	20.7	40.5	23.9	14.9	61.2	-0.323	0.229
cox2	690	21.7	39.7	24.5	14.1	61.5	-0.292	0.271
atp8	159	28.9	39.0	22.0	10.1	67.9	-0.148	0.373
atp6	672	20.1	41.8	21.0	17.1	61.9	-0.351	0.102
cox3	792	20.2	40.3	22.9	16.7	60.5	-0.332	0.157
nad3	333	29.7	30.6	14.4	25.2	60.4	-0.015	-0.272
nad1	924	26.8	32.1	16.9	24.1	59.0	-0.089	-0.177
nad5	1714	31.0	30.5	14.3	24.2	61.5	0.008	-0.258
nad4	1341	29.0	30.4	16.3	24.3	59.4	-0.024	-0.199
nad4l	303	26.7	32.3	15.5	25.4	59.1	-0.095	-0.242
nad6	498	20.1	44.4	22.9	12.7	64.5	-0.377	0.288
cob	1134	19.4	41.3	22.0	17.4	60.7	-0.360	0.117
nad2	1014	17.5	46.3	21.4	14.9	63.7	-0.452	0.179
tRNAs	1452	32.4	33.3	19.2	15.1	65.8	-0.014	0.119
rRNAs	2115	37.9	30.4	15.3	16.5	68.2	0.110	-0.039
PCGs	11113	23.9	37.3	19.7	19.1	61.2	-0.219	0.014

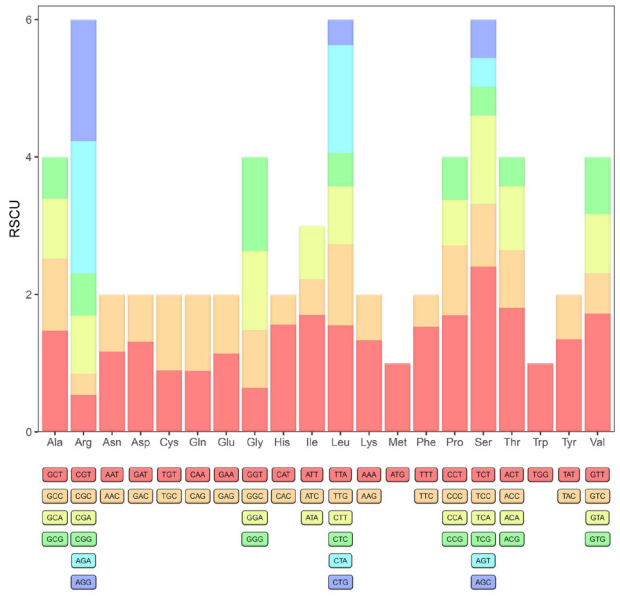

Fig. 2. Relative synonymous codon usage in *Coenobita lila* mitogenome.

Gene order of mitogenome

The gene order of *C. lila* mitogenome underwent

a large rearrangement compared with its ancestor (pancontinental crustaceans) (Boore *et al*. 1998) (Fig. 3). In summary, six gene clusters dramatically differed from the typical order, involving eleven tRNAs (*L1*, *L2*, *G*, *A*, *S1*, *P*, *I*, *Q*, *M*, *W* and *Y*), and two PCGs (*nad2* and *nad3*). The gene *cox1- cox2- K- D- atp8- atp6- cox3- R- N- E- F- nad5- H- nad4- nad4l- T- nad6- cob- S2- nad1- 16S- V- 12S- C* were not rearrangement, which was the same as that of the ancestral crustaceans. In these six gene clusters, the *I- Q- M- nad2* cluster was split into two parts, with *Q* being transferred to the end of the linear mitochondrial genome. Another (*I*, *M* and *nad2*) was transferred downstream of *K*. The *W- C- Y* cluster order became the *Y- W- C* order, and *L1* moved between *cox1* and *cox2*. When a single *P* moved downstream from *T* to *S2*, the *G- nad3- A- S1* cluster moved from the *cox3* of the heavy strand to the *CR* downstream of the heavy strand. A single *L2* moved to the position between the *S1- A- nad3- G* cluster and the *Y- W- Q- C* cluster downstream of *CR* forming a large-scale rearrangement region.

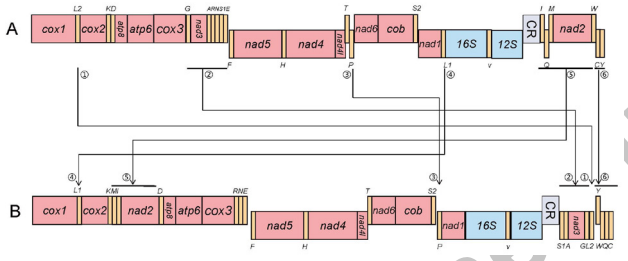

Fig. 3. Gene rearrangements in *Coenobita lila* mitogenome. A, The ancestral gene arrangement of crustaceans. B, The gene order in the *Coenobita lila* mitogenome.

Here, we used replication-random loss and recombination models to explain the mitochondrial genome rearrangement of *C. lila*. First, one gene cluster underwent a complete copy to form one dimer block (*I- Q- M*). Continuous copies were followed by random loss of duplicate genes, *I- Q- M- I- Q- M* (underline represents the deleted gene), and then a new gene block (*Q- M- I*) was formed. Tandem repeats followed by random loss have been widely used to explain this type of translocation of mitochondrial genes (Gong *et al*., 2019; Shi *et al*., 2015; Chai *et al*., 2017). Therefore, we determined that the repeat-random loss model was the most likely explanation for the rearrangement of this gene block. Subsequently, the *M- I- nad2* block moved to the junction of *K* and *D*. In the second step, seven genes or gene blocks were translocated. *L1* was moved to the junction of *cox1* and *cox2*, *L2* was moved to the middle of *nad2* and *W*, and then *Y* was moved to the downstream of *L2*. The gene cluster *G- nad3- A* and gene *S1* moved to between *CR* and *Q* and changed to *S1-A-nad3-G*. At the same time, *P* moved to the middle of *S2* and *nad1*, and *Q* moved to the middle of *W* and *C*. Moreover, restructuring events seemed to explain these translocations and the final gene arrangement of mitochondrial genome in *C. lila*.

Ka/ Ks ratio

The ratio of Ka/ Ks represents the ratio between non-synonymous mutations (Ka) and synonymous mutations (Ks) of the two protein-coding genes, which determines whether there is selective pressure on the protein-coding gene (Hurst, 2002). In the study, the calculated Ka/ Ks values of the 13 PCGs of the Anomura were all less than 1 (Fig. 4), suggesting the presence of purification selection. The ratio of *atp8* was the largest, ranging from 0.056 to 0.920, indicating that *atp8* faced the least pressure. This was consistent with the results of *C. clypeatus* (Colin *et al*., 2022). On the contrary, the evolutionary selection pressure on *atp6* was different from that on *atp8*, where the evolutionary pressure was high.

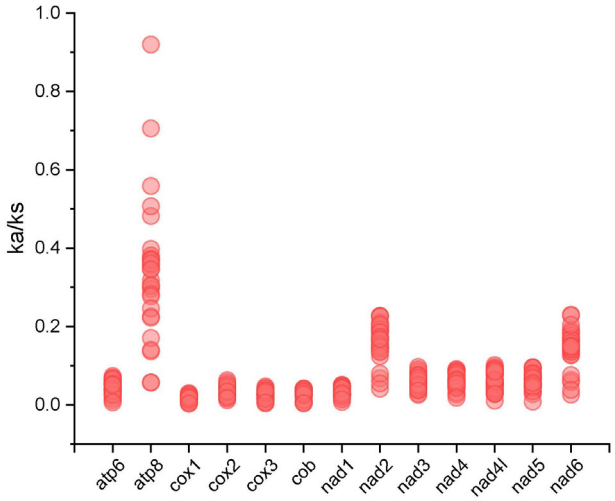

Fig. 4. Ka/ Ks ratios of 13 PCGs.

Phylogenetic analysis and gene rearrangement patterns

In order to analyse the phylogenetic status of *C. lila* in Anomura, we constructed a phylogenetic tree (ML) based on 13 PCGs of 30 species with *P. princeps* as the outgroup (Table III, Fig. 5). The results showed that all 23 species of Anomura and 7 species of Brachyura were clustered together separately. And 9 superfamily were monophyletic groups except for the Paguroidea. Paguroidea was paraphyletic with Paguroidae group and Coenobitidae + Diogenidae group, which was consistent with the previous research (Tan *et al*., 2018; Li *et al*., 2020). And *C. lila* was clustered in Coenobitidae family.

Table III. List of 31 mitogenome data in this paper.

Species	Length(bp)	Accession No.
Superfamily: Paguroidea		
Family: Diogenidae		
Dardanus arrosor	16,592	NC_060631
Dardanus aspersus	16,916	MW715812
Clibanarius infraspinatus	16,504	NC_025776
Family: Coenobitidae		
Birgus latro	16,411	NC_045091
Coenobita rugosus	16,433	MN030161
Coenobita lila	16,396	OP645220
Coenobita variabilis	16,421	KY352236
Coenobita brevimanus	16.393	MN030160
Superfamily: Hippoidea		
Family: Albuneidae		
Stemonopa insignis	15,596	KY352240
Superfamily: Galatheoidea		
Family: Porcellanidae		
Petrolisthes haswelli	15,348	NC_025572
Family: Galatheidae		
Munida gregaria	16,326	NC_030255
Family: Munidopsidae		
Munidopsis lauensis	17,483	MH717895
Munidopsis verrilli	17,636	MH717896
Superfamily: Paguroidea		
Family: Paguridae		
Pagurus longicarpus	15,630	AF150756
Pagurus similis	17,100	NC_057304
Pagurus nigrofascia	15,423	NC_042412
Superfamily: Lithodoidea		
Family: Lithodidae		
Paralithodes brevipes	16,303	NC_021458
Paralithodes platypus	16,883	NC_042240
Paralithodes camtschaticus	16,720	NC_020029
Superfamily: Lomoidea		
Family: Lomidae		
Lomis hirta	17,239	KY352239
Superfamily: Chirostyloidea		
Family: Kiwaidae		
Kiwa tyleri	16,865	NC_034927
Family: Chirostylidae		
Gastroptychus rogeri	16,504	KY352238
Gastroptychus investigatoris	16,423	KY352237
Superfamily: Xanthoidea		
Family: Oziidae		
Epixanthus frontalis	15,993	MF457404
Superfamily: Pilumnoidea		
Family: Pilumnidae		
Pilumnus vespertilio	16,222	MF457402
Table continued on next column.....		

Species	Length(bp)	Accession No.
Superfamily: Ocypodoidea		
Family: Ocypodidae		
Cranuca inversa	15,677	MF457405
Tubuca capricornis	15,629	MF457401
Tubuca polita	15,672	MF457400
Superfamily: Grapsoidea		
Family: Gecarcinidae		
Cardisoma carnifex	15,597	NC_039105
Family: Grapsidae		
Pachygrapsus marmoratus	15,406	MF457403
Superfamily: Plecoptera		
Family: Pteronarcyidae		
Pteronarcys princeps	16,004	NC_006133

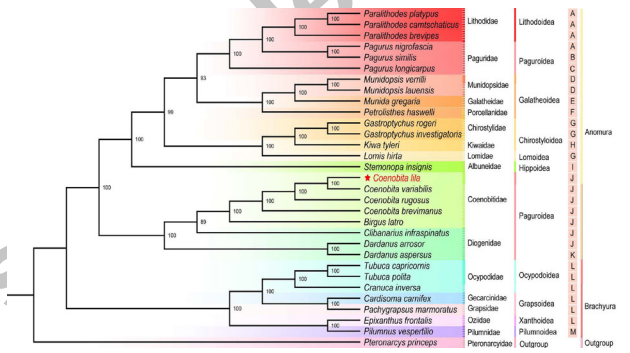

Fig. 5. Phylogenetic tree inferred from the 13 PCGs based on maximum likelihood (ML) analysis.

According to the gene rearragement of all the 30 species, 13 gene rearragement patterns (A-M) were defined. Mitochondrial gene rearrangements were mainly divided into three main forms: Shuffling, translocation and inversion. In general, every family of Anomura had its own unique arrangement type, such as Lithodidae (A), Munidopsidae (D), Galatheidae (E), Porcellanidae (F), Chirostylidae and Lomidae (G), Kiwaidae (H), Albuneidae (I), Coenobitidae (J) except for Paguridae (B and C) and Diogenidae (J and K). In all the gene rearrangement patterns of Anomura (A-K), we found that three gene clusters were conserved: *cox2-trnK, atp8-atp6-cox3-trnR-trnN, trnP-nad1*. Paguroidea was divided into two independent clades in the phylogenetic tree. In addition, these two clades had five different patterns in gene rearrangement patterns. Coenobitidae+Diogenidae group was clustered together, and except for *D. aspersus*, the gene rearrangement patterns of the other seven species were the same, while the gene rearrangement patterns of the three species in Paguridae were different. This indicated that gene rearrangement might be used in the study of systematic evolution of Anomura.

CONCLUSION

This study reported the complete mitochondrial genome of *C. lila*. It was 16,396 bp in length and contained 13 protein-coding genes, 2 ribosomal RNA genes, 22 transfer RNA genes and a control region. Mitochondrial genome of *C. lila* was with negative AT skew and positive GC skew. Ka/Ks of the 13 protein-coding genes indicated purifying selection. The phylogenetic tree provided a certain reference for the reclassification of Paguroidea. Mitochondrial genome characteristics and gene rearrangement patterns might be used in the study of systematic evolution of Anomura.

DECLARATIONS

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IRB approval

The research work was approved by Jiangsu Key Laboratory for Bioresources of Saline Soils, Jiangsu Provincial Key Laboratory of Coastal Wetland Bioresources and Environmental Protection, Jiangsu Synthetic Innovation Center for Coastal Bio-agriculture, Yancheng Teachers University, Yancheng, China.

Data availability statement

The data that support the findings of this study are openly available in NCBI (Accession number: OP645220).

Statement of conflict of interest

The authors have declared no conflicts of interest.

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