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Mitochondrial genome of *Chthamalus challengeri* (Crustacea: Sessilia): gene order comparison within Chthamalidae and phylogenetic consideration within Balanomorpha

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Abstract

Acorn barnacles are important model species in researches on intertidal ecology, larval development and biofouling. At present, with the development of mitochondrial genomics, it is helpful to understand the phylogenetic relationship from the mitogenomic level. The complete mitochondrial genome of *Chthamalus challengeri* was presented. The genome is a circular molecule of 15 358 bp. Compared with other species in Balanomorpha, the non-coding region is longer, while the length of the genes is similar to the other species. The overall A+T content of the mitochondrial genome of *C. challengeri* is 70.5%. There are variations of initiation and stop codons in the known Balanomorpha mitochondrial genomes. The *C. challengeri* and *C. antennatus* within the same genus share the identical gene arrangement. However, the gene arrangement of different genera in Chthamalidae is different, as there is a translocation between two tRNA genes and an inversion involving a large gene block. In particular, both *srRNA* and *lrRNA* of the two species in *Chthamalus* are encoded in the heavy strand, differing from the former Balanomorpha species. The topology and gene arrangement in Chthamalidae support each other. Phylogenetic analysis indicates that the Chthamalidae is monophyletic, while the Balanidae and Archaeobalanidae are polyphyletic.

Key words: Balanomorpha, *Chthamalus challengeri*, mitochondrial genome, gene rearrangement, phylogeny

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1 Introduction

Acorn barnacles (Crustacea: Balanomorpha) are important model species in invertebrate larval biology, intertidal ecology, and anti-fouling research (Shen et al., 2017; Tsang et al., 2017). However, despite their important ecological and evolutionary role, there is a lack of thorough understanding on the phylogeny of this group (Chan et al., 2017). Over the past decade, the use of molecular markers and techniques (nuclear gene fragments, mitochondrial genes, etc.) leads to rapid development of barnacle research. As a genetic material outside the nucleus, the mitochondrial genome is relatively compact and easy to be sequenced, and thus is regarded as an important source of information for resolving metazoan phylogeny (Boore, 1999; Shen et al., 2016c). In the present study, the mitochondrial genome is a molecular marker which is helpful to analyze the phylogeny of metazoa from the genome level, and can be used for multi-directional molecular evolution studies from mitochondrial sequence information, gene composition and gene arrangement (Corradi and Bonen, 2012). Thus the complete mitochondrial genome sequence is a powerful tool for the study of animal molecular phylogeny (Boore, 1999; Zhang and Zhang, 2012).

Chthamalus challengeri is one of the common barnacles along the coast of Yellow Sea and Bohai Sea in northern China, distributed in supratidal zone and the intertidal rocky shore. It can withstand long-term periodic drying and has strong adaptability (Liu and Ren, 2007). Here we present the complete mitochondrial genome of the *C. challengeri*, including its gene content, protein-coding genes, codon usage, gene arrangement and phylogenetic relationship with other Balanomorpha species.

2 Materials and methods

2.1 *Sample collection and DNA extraction*

The specimen of *C. challengeri* was obtained from the intertidal of Ganyu District (34°35′49.05″N, 119°12′17.45″E), Lianyungang, Jiangsu Province, China. Total genomic DNA was extracted from the muscle tissue, using TIANamp Marine Animal DNA Kit (TIANGEN) following the manufacturer's protocol.

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2.2 *PCR amplification and sequencing*

Initially, we used universal primers and specific primers to amplify the *cox1* and *lrRNA* gene segments (Table 1). The reaction mixture for amplifying *cox1* and *lrRNA* gene segments, in a total volume of 20 μL, contained 7.4 μL sterile distilled H_2O, 1 μL each primer, 10 μL *Extaq* polymerase and 0.6 μL DNA template. The PCR amplifications were performed with the following cycling parameters: initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s, and a final extension at 72°C for 10 min.

Table 1. Common and specific primers used to amplify the mitochondrial genes

Primer name	Sequence (5′–3′)
DFXTH-COX1-1F	GATACTCGGGCTTATTTCACTTCTG
DFXTH-COX1-1R	CGACGGGGTATCCCTGCTA
DFXTH-lrRNA-1F	AGGAGGTAAAGCCTGCTCACTG
DFXTH-lrRNA-1R	AAAAAACCAGAAGATAGAAACCAACC
DFXTH-COX1-2F	GATTAGGAACACTTCACGGAACTCAA
DFXTH-COX1-2R	CCAAAAACAGCCCCCATAGATAGT
DFXTH-lrRNA -2R	GAAACCAACCTGGCTCACGC
DFXTH-lrRNA -2F	TGGTATGAATGGCTAAACGAGAAATC

The reaction mixture for amplifying *cox1-lrRNA*/*lrRNA-cox1* gene segments, in a total volume of 20 μL, contained 11.7 μL sterile distilled H_2O, 2.0 μL 10× PCR buffer, 3.2 μL dNTP mix (2.5 mmol/L), 1 μL each primer, 0.1 μL TaKaRa LA *Taq* polymerase, and 1 μL DNA template. The cycling parameters of PCR were as follows: initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, elongation at 68°C for 8 min, and a final extension at 68°C for 10 min. PCR products were purified (GeneMark), cloned (pGEMT easy, Promega) and sequenced (MAP BIOTECH in Shanghai).

2.3 *Gene identification and genome analysis*

In this study, the MITOS Webserver (http://mitos.bioinf.uni-leipzig.de/index.py) online software (Bernt et al., 2013) was used to predict the mitochondrial genes of *C. challengeri*, including 13 PCGs, 2 ribosomal RNAs and 22 transfer RNAs, which was a convenient, rapid and accurate way of annotating mitochondrial genomes (Shen et al., 2015b). Gene map of *C. challengeri* mitochondrial genome was drawn by OGDraw 1.2 (Lohse et al., 2013). In addition, codon usage in the 13 PCGs of the mitochondrial genome was estimated with DnaSP 5.10.01 (Librado and Rozas, 2009). The genomic analysis included general features, base composition and skew, gene arrangement, and protein-coding genes.

2.4 *Phylogenetic analysis*

Along with newly obtained mitochondrial genome sequence of *C. challengeri*, the 21 mitochondrial genomes currently available from Balanomorpha and Pendunculata were used in phylogenetic analysis, including 17 genomes from Balanomorpha: *Acasta sulcata*, *Striatobalanus amaryllis* (Tsang et al., 2015), *Armatobalanus allium*, *Amphibalanus amphitrite* (Shen et al., 2015a), *Megabalanus volcano*, *Megabalanus ajax* (Shen et al., 2016b), *Balanus balanus* (Shen et al., 2016d), *Chelonibiate studinuria*, *Nobia grandis* (Shen et al., 2016a), *Chthamalus antennatus*, *Notochthamalus scabrosus* (Wares, 2015), *Octomeris* sp. BKKC-2014, *Tetraclita japonica*, *Tetraclita serrata* (Shen et al., 2015b), *Tetraclitella divisa*, *Epopella plicata* (Shen et al., 2017) and *Savignium* sp. BKKC-2014, and four from Pedunculata including *Capitulum mitella* (Lim and Hwang, 2006), *Pollicipes polymerus* (Lavrov et al., 2004), *Lepas anserifera* and *Lepas australis*.

Nucleotide sequences of the 13 PCGs from these mitochondrial genomes were aligned using MEGA 7.0.25 (Kumar et al., 2016) with the default settings. To determine the best fitting mode, maximum likelihood (ML) method was performed using PhyML 3.0 (Guindon et al., 2010) and MEGA 7.0.25 (Kumar et al., 2016) with 100 bootstrap replicates, respectively.

3 Results and discussion

3.1 *General features*

The mitochondrial genome of *C. challengeri* is a circular molecule of 15 358 bp, similar to the sizes for the other acorn barnacles (Liu et al., 2016). It encodes 13 PCGs, 2 rRNA genes, and 22 tRNA genes. The heavy and light strands contain 30 and 7 genes, respectively (Fig. 1, Table 2). Five instances of gene overlaps are found in mitochondrial genome of *C. challengeri*. One 7-bp overlap is found between *nad4L* and *nad4* gene, and there are four other overlaps ranging from 1 to 5 bp. Non-coding regions make up 836 bp, with the longest one speculated as the control region (321 bp), which is located between *srRNA* and *trnK* (Table 2). Some non-coding regions of the *C. challengeri* genes are relatively longer than the other 21 species mentioned in this paper. The A+T content on the heavy strand of the mitochondrial genome of *C. challengeri* is 70.5%, which is the comparable values reported for the other 17 sessile barnacles (ranging from 65.4% to 73.4%). The entire *C. challengeri* mitochondrial genome sequence was deposited in GenBank with accession number KJ865097.

3.2 *Protein-coding genes and codon usage*

In *C. challengeri* mitochondrial genome, two PCGs (*cob* and *nad6*) are encoded on the light strand while the other 11 PCGs are located on the heavy strand. In the 18 mitochondrial genomes of the Sessilia species, the number of amino acids in the three PCGs (*cox2*, *cox3* and *atp8*) is the same, respectively. However, there are differences in gene length among the remaining 10 PCGs. In addition, among the four species of Chthamalidae (*C. challengeri*, *C. antennatus*, *Octomeris* sp. BKKC-2014 and *N. scabrosus*), the number of amino acids in four PCGs (*atp6*, *nad4*, *cob* and *nad2*) is also the same. As previously reported, metazoan mitochondrial PCGs often use several ATN alternatives as start codons (Shen et al., 2009, 2012, 2015b). All 13 PCGs in *C. challengeri* start with ATD (ATA, ATG or ATT) (Table 2). In the 18 mitochondrial genomes of the Balanomorpha species, *cox2*, *cox3*, *cob* and *nad4* (except for *B. balanus*) genes start with "ATG". The start codons of *nad4L*, *nad5*, *nad6*, *nad1* and *nad2* genes start with "GTG" or "TTG". The initiation codons of the *cox1* gene are diverse. Three protein-coding genes (*nad3*, *nad1* and *nad4*) in *C. challengeri* end with incomplete stop codon (T-), and the remaining PCGs use stop codons (TAA or TAG). Meanwhile, all the complete stop codons of *A. sulcata*, *A. allium*, *N. grandis*, *E. plicata* and *N. scabrosus* are "TAA".

The pattern of codon usage in the *C. challengeri* mitochondrial genome was studied with DnaSP 5.10.01 (Librado and Rozas, 2009). There are 3 681 codons in all 13 PCGs (excluding the incomplete termination). The most frequently used amino acids were Leu (14.47%), Ser (10.28%), Phe (9.72%) and Ile (9.21%), while Gln and Arg were used least, at 1.72% and 1.55%, respectively (Table 3). The A+T composition of the first and second codon in the 13 PCGs of *C. challengeri* is 63.0% and 64.4%, re-

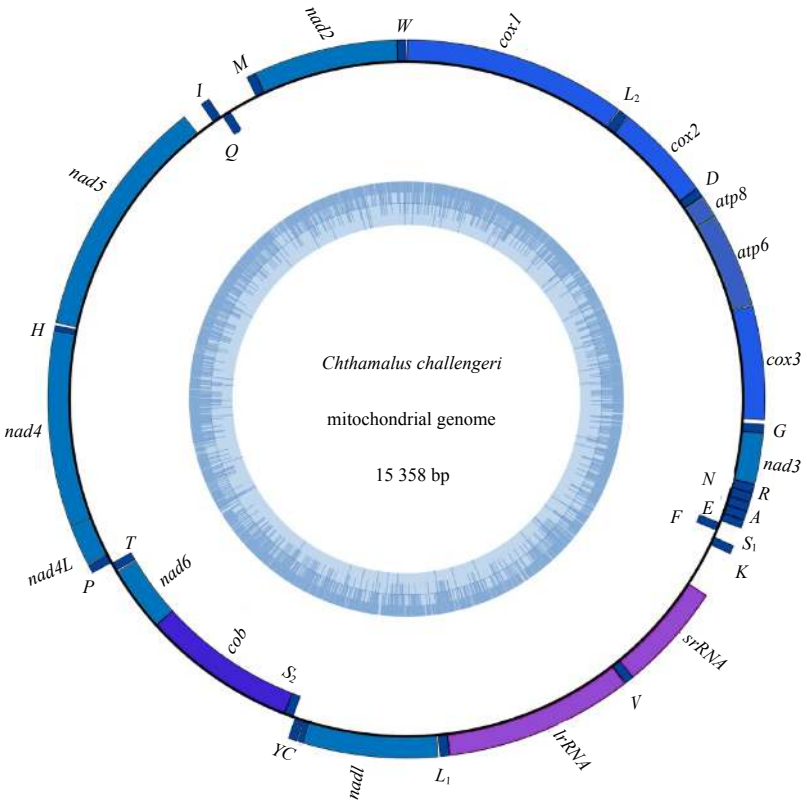

Fig. 1. Gene map of mitochondrial genome of *Chthamalus challengeri* (Crustacea: Balanomorpha). Transfer RNA genes are shown with single letter abbreviation for amino acids. Genes encoded on the heavy and light strands are shown outside and inside the circular gene map, respectively.

spectively. Yet the value for the third codon position elevates to 81.3%, which is within the range of 17 other Balanomorpha species (ranging from 62.0% to 87.9%).

3.3 *Base composition and skew*

The bias of the base composition in each gene can be described by skewness (Perna and Kocher, 1995) that measures the relative numbers of A to T (AT skew) and G to C (GC skew), and is calculated as (A%–T%)/(A%+T%) and (G%–C%)/(C%+G%), respectively. The heavy strand in the *C. challengeri* mitochondrial genome consists of 34.9% A, 22.2% C, 12.5% G and 30.5% T. AT and GC skews of the whole genome are –0.130 and 0.092, respectively. The A+T contents of 13 PCGs range from 65.6% (*cox1*) to 79.2% (*atp8*), and those of *srRNA* and *lrRNA* are 68.1% and 75.0%, respectively. All 13 PCGs consist of 27.4% A, 14.7% C, 15.8% G, and 42.2% T bases. AT skew and GC skew are –0.214 and 0.036, respectively. All 13 PCGs have skews of T vs. A (AT skew between –0.046 and –0.288). However, both RNAs have A skew of 0.027 and 0.022, respectively. On the other hand, there are six PCGs have skews of G vs. C (ranging from 0.020 to 0.241), and the other 7 PCGs have skews of C vs. G (between –0.025 and –0.255). In addition, both rRNAs have skew of G vs. C for *srRNA* and *lrRNA* (0.190 and 0.220, respectively; Table 4).

3.4 *Ribosomal and transfer RNA genes*

The length of *srRNA* and *lrRNA* is 758 and 1 310 bp, respectively, which are similar to length in the other barnacle mitochondrial genomes (*srRNA* ranges from 751 to 825 bp; *lrRNA* ranges from 1 290 to 1 374 bp) (Shen et al., 2015b; Wares, 2015). The two *rRNA*s, located between $trnL_1$ and the control region, are separated by one tRNA gene (*trnV*). The length of 22 tRNA genes in the *C. challengeri* is similar to other Balanomorpha species, ranging from 58 ($trnS_1$) to 70 ($trnS_2$) nucleotides (Shen et al., 2015b).

3.5 *Gene arrangement*

Gene arrangement is often used as a supplementary means to help us understand the evolutionary and phylogenetic relationships among species. In recent years, more and more data show that although pancrustaceans generally exhibit ancestral mitochondrial gene arrangement, the mitochondrial gene order in Balanomorpha species is not conserved, with differences in gene blocks or individual genes.

The two species (*C. challengeri* and *C. antennatus*) in *Chthamalus* share the identical gene order. However, the gene orders of different genera in Chthamalidae are different and there are translocation and inversion in genes or gene blocks (Fig. 2). Between *N. scabrosus* and *Octomeris* sp. BKKC-2014, there is translocation between *trnI* and *trnQ*. However, comparing with the two species in *Chthamalus*, there is an inversion of a large gene block (*nad5-H-nad4-nad4L-P-T-nad6-cob-*S_2*-Y-C-nad1-*L_1*-lrRNA-V-srRNA-K*), which is a considerable rearrangement among different genera in Chthamalidae. In addition, both *lrRNA* and *srRNA* in *Chthamalus* are encoded on the heavy strand, which is different from the other 16 Balanomorpha species (mentioned above), in which both *lrRNA* and *srRNA* are encoded on the light strand. This is the biggest difference found on the rRNA so far, and the reason needs further analysis and research. Gene rearrangement appears to have occurred frequently in Chthamalidae.

According to the above comparison and analysis, it is found

Table 2. Mitochondrial genomic profile of *Chthamalus challengeri* (Crustacea: Balanomorpha)

Gene	Strand	Position		Nucleotides	Codons		Anti-codon	Intergenic sequence*
		Start	Stop		Start	Stop		
cox1	H	4	1 551	1 548	ATA	TAA		4
trnL$_2$	H	1 556	1 623	68			TAA	2
cox2	H	1 626	2 309	684	ATG	TAA		0
trnD	H	2 310	2 372	63			GTC	0
atp8	H	2 373	2 531	159	ATT	TAA		3
atp6	H	2 525	3 190	666	ATG	TAA		4
cox3	H	3 195	3 983	789	ATG	TAA		27
trnG	H	4 011	4 073	63			TCC	0
nad3	H	4 074	4 425	352	ATT	T-		0
trnR	H	4 426	4 487	62			TCG	0
trnN	H	4 488	4 551	64			GTT	0
trnA	H	4 552	4 617	66			TGC	0
trnE	H	4 618	4 682	65			TTC	0
trnS$_1$	H	4 683	4 740	58			GCT	15
trnF	L	4 756	4 819	64			GAA	47
trnK	L	4 867	4 930	64			TTT	0
Control region		4 931	5 251	321				0
srRNA	H	5 252	6 009	758				–5
trnV	H	6 005	6 071	67			TAC	0
lrRNA	H	6 072	7 381	1 310				0
trnL$_1$	H	7 382	7 449	68			TAG	9
nad1	H	7 459	8 371	913	ATG	T-		0
trnC	H	8 372	8 432	61			GCA	3
trnY	H	8 436	8 500	65			GTA	15
trnS$_2$	L	8 516	8 585	70			TGA	–2
cob	L	8 584	9 723	1 140	ATG	TAA		–1
nad6	L	9 723	10 205	483	ATG	TAA		6
trnT	L	10 212	10 277	66			TGT	4
trnP	H	10 282	10 344	63			TGG	3
nad4L	H	10 348	10 638	291	ATA	TAA		–7
nad4	H	10 632	11 961	1 330	ATG	T-		0
trnH	H	11 962	12 025	64			GTG	15
nad5	H	12 041	13 729	1 689	ATT	TAG		139
trnI	L	13 869	13 935	67			GAT	11
trnQ	H	13 947	14 014	68			TTG	215
trnM	H	14 230	14 295	66			CAT	0
nad2	H	14 296	15 294	999	ATG	TAA		–1
trnW	H	15 294	15 358	65			TCA	3

Note: * Negative numbers indicate overlapping nucleotides between adjacent genes.

that the gene arrangement between each of the two species pairs (*C. challengeri* and *C. antennatus*; *Octomeris* sp. BKKC-2014 and *N. scabrosus*) are similar. The gene arrangement of *N. scabrosus* is found to be closest to the pancrustacean ground pattern, so that this species can be presumed to close to basal in Chthamalidae. The gene arrangement of *C. challengeri* appears to be most derived. Yet, the pattern of the evolutionary changes in the gene arrangement of Chthamalidae requires data based on extensive taxon coverage from the family.

3.6 *Phylogeny analysis*

A ML tree was constructed using amino acid sequences of 13 PCGs from 22 complete mitochondrial genomes (Fig. 3). Within Balanomorpha, members from Archaeobalanidae, Balanidae and Pyrgomatidae are grouped together (Bootstrap, BP=100), but their interrelationships are poorly resolved other than the groupings of the two *Megabalanus* species (BP=100) and the two species from Pyrgomatidae (BP=90). As the existing data and documents, with the increase and supplement of data, it is supposed that Balanidae and Archaeobalanidae are non-monophyly (Shen et al., 2015b; Tsang et al., 2014, 2017). Four species of Tetraclitidae cluster with *C. testudinaria* of Coronulidae (BP=100), inferring their close relationship. The *N. scabrosus* is located at the basal of Chthamalidae, clustering with *Octomeris* sp. BKKC-2014 and the two species (*C. challengeri* and *C. antennatus*) in *Chthamalus*, and the topology is the same with the relationship inferred based on gene arrangement. In conclusion, the topology and gene arrangement in Chthamalidae support each other. Phylogenetic analysis indicates that the Balanidae and Archaeobalanidae are polyphyletic. From the topology of the current phylogenetic tree, Chthamalidae is monophyletic. Yet, more data and research will be needed to reveal the phylogeny within Balanomorpha and its constituent families, including Chthamalidae.

Table 3. Codon usage in 13 mitochondrial PCGs of *Chthamalus challengeri* (Crustacea: Balanomorpha)

Codon	Number	Percent	Codon	Number	Percent	Codon	Number	Percent	Codon	Number	Percent
UUU-F	292	7.93	UCU-S	161	4.37	UAU-Y	115	3.12	UGU-C	31	0.84
UUC-F	66	1.79	UCC-S	22	0.60	UAC-Y	37	1.01	UGC-C	2	0.05
UUA-L	276	7.50	UCA-S	51	1.39	UAA-*	9	0.24	UGA-W	86	2.34
UUG-L	65	1.77	UCG-S	14	0.38	UAG-*	1	0.03	UGG-W	15	0.41
CUU-L	96	2.61	CCU-P	90	2.44	CAU-H	51	1.39	CGU-R	24	0.65
CUC-L	12	0.33	CCC-P	18	0.49	CAC-H	24	0.65	CGC-R	0	0
CUA-L	71	1.93	CCA-P	28	0.76	CAA-Q	44	1.20	CGA-R	29	0.79
CUG-L	12	0.33	CCG-P	4	0.11	CAG-Q	19	0.52	CGG-R	4	0.11
AUU-I	291	7.91	ACU-T	93	2.53	AAU-N	101	2.74	AGU-S	51	1.39
AUC-I	48	1.30	ACC-T	14	0.38	AAC-N	27	0.73	AGC-S	10	0.27
AUA-M	163	4.43	ACA-T	57	1.55	AAA-K	79	2.15	AGA-S	68	1.85
AUG-M	50	1.36	ACG-T	6	0.16	AAG-K	21	0.57	AGG-S	1	0.03
GUU-V	97	2.64	GCU-A	108	2.93	GAU-D	63	1.71	GGU-G	73	1.98
GUC-V	13	0.35	GCC-A	19	0.52	GAC-D	11	0.30	GGC-G	16	0.43
GUA-V	90	2.44	GCA-A	45	1.22	GAA-E	60	1.63	GGA-G	100	2.72
GUG-V	36	0.98	GCG-A	18	0.49	GAG-E	33	0.90	GGG-G	50	1.36

Note: * indicates stop codon.

Table 4. Nucleotide composition and skews of *Chthamalus challengeri* mitochondrial protein-coding and ribosomal RNA genes

Gene	Proportion of nucleotides				A+T/%	AT Skew	GC Skew
	A	C	G	T			
atp6	0.296	0.150	0.126	0.428	72.4	–0.183	–0.087
atp8	0.346	0.119	0.088	0.447	79.2	–0.127	–0.152
cob	0.286	0.182	0.146	0.386	67.2	–0.149	–0.107
cox1	0.263	0.165	0.179	0.393	65.6	–0.198	0.039
cox2	0.306	0.155	0.145	0.395	70.0	–0.127	–0.034
cox3	0.237	0.177	0.157	0.428	66.5	–0.288	–0.061
nad1	0.265	0.118	0.176	0.440	70.5	–0.248	0.197
nad2	0.262	0.143	0.136	0.458	72.1	–0.272	–0.025
nad3	0.264	0.139	0.145	0.452	71.6	–0.262	0.020
nad4	0.266	0.127	0.174	0.433	69.8	–0.238	0.157
nad4L	0.268	0.103	0.168	0.460	72.9	–0.264	0.241
nad5	0.262	0.128	0.181	0.429	69.1	–0.243	0.169
nad6	0.369	0.143	0.085	0.404	77.2	–0.046	–0.255
srRNA	0.350	0.129	0.190	0.331	68.1	0.027	0.190
lrRNA	0.383	0.098	0.153	0.366	75.0	0.022	0.220
All PCGs	0.273	0.147	0.158	0.422	69.5	–0.214	0.036
H-Strand	0.307	0.134	0.161	0.398	70.5	–0.130	0.092

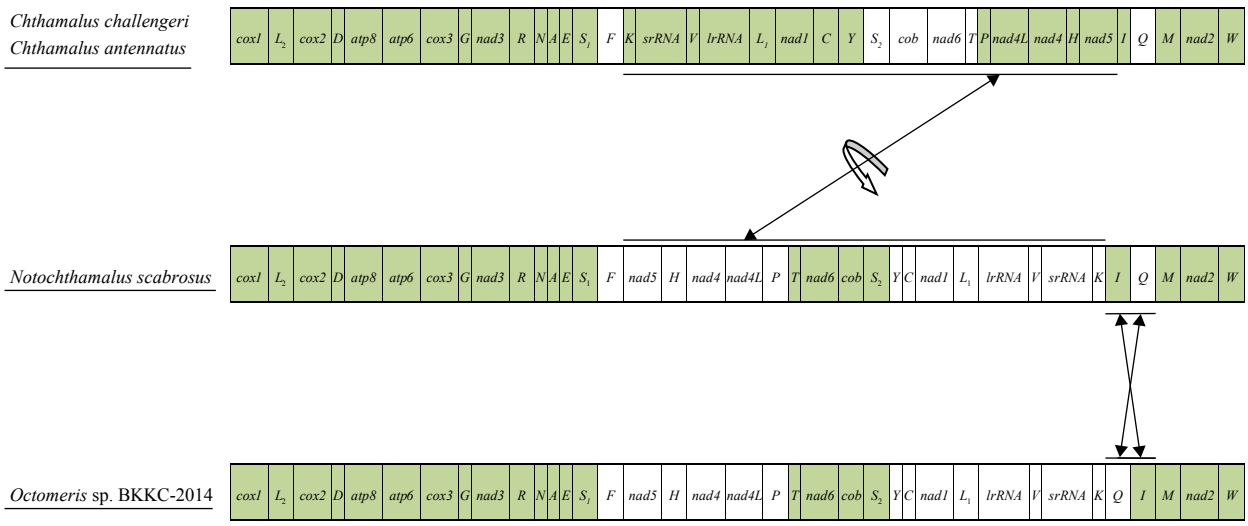

Fig. 2. Comparison of the gene arrangement in four mitochondrial genomes from the Chthamalidae (Crustacea: Balanomorpha). Gene segments are not drawn to scale. Mitochondrial genome gene arrangements of *C. antennatus*, *C. challengeri*, *N. scabrosus* and *Octomeris* sp. BKKC-2014 are shown. "Green" indicates heavy strand and "white" light strand. The double arrow shows translocation and the circling arrow inversion.

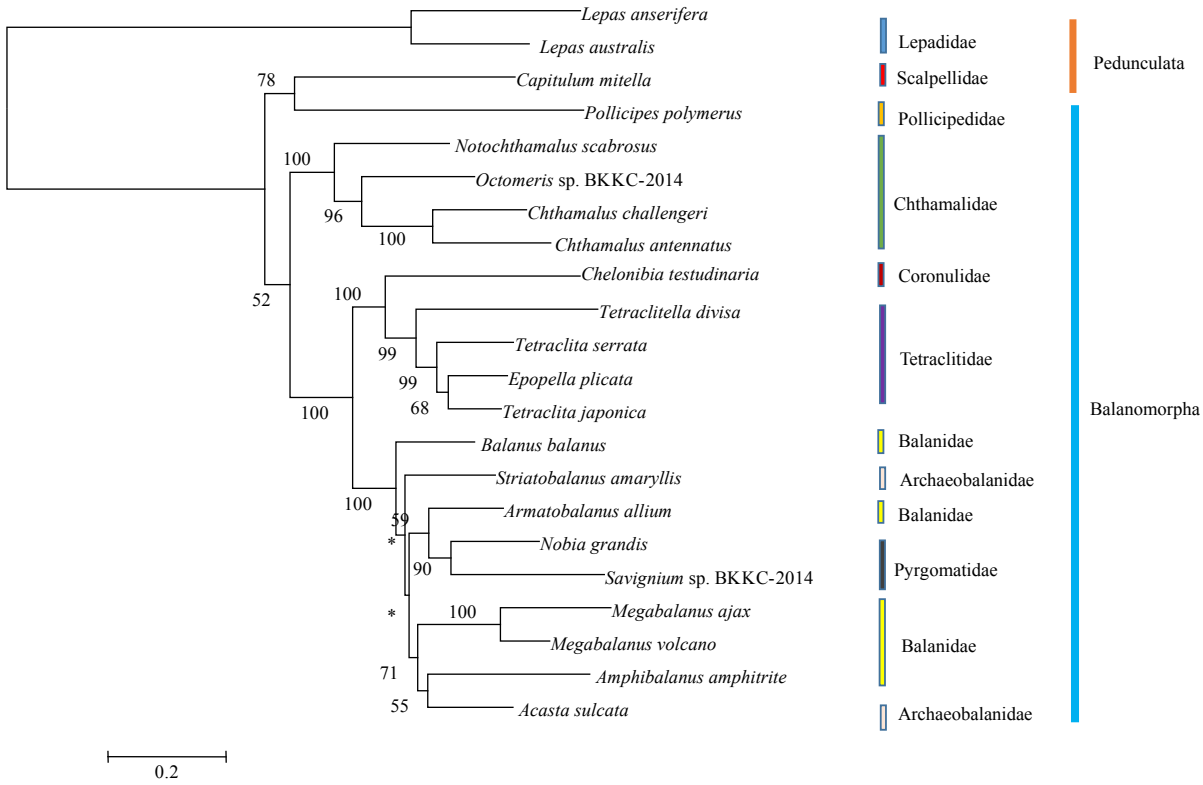

Fig. 3. Phylogenetic tree constructed from the ML analysis of 13 PCGs (amino acid data) from 22 mitochondrial genomes. The numbers at the nodes indicate the bootstrap values obtained from ML analysis. * indicates that the bootstrap values are below 50.

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