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Characterization of the Complete Mitochondrial Genome of the Hybrid *Epinephelus moara*♀×*Epinephelus lanceolatus*♂, and Phylogenetic Analysis in Subfamily Epinephelinae

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Abstract This study presents the complete mitochondrial genome of the hybrid *Epinephelus moara*♀× *Epinephelus lanceolatus*♂. The genome is 16886 bp in length, and contains 13 protein-coding genes, 2 rRNA genes, 22 tRNA genes, a light-strand replication origin and a control region. Additionally, phylogenetic analysis based on the nucleotide sequences of 13 conserved protein-coding genes using the maximum likelihood method indicated that the mitochondrial genome is maternally inherited. This study presents genomic data for studying phylogenetic relationships and breeding of hybrid Epinephelinae.

Key words *Epinephelus moara*♀× *Epinephelus lanceolatus*♂; mitochondrial genome; phylogenetic analysis

1 Introduction

Hybridization is a technique commonly used in fish breeding, as it allows for a combination of advantageous traits from different species,which can improve the viability of the hybrid offspring and provide heterosis of hybrid progeny in the short term (Feliner and Aguilar, 1998; Urbanelli *et al*., 2014). An example of hybrid breeding is the economically important grouper and active hybrid breeding which has been performed for many years (Glamuzina *et al*., 2001; James *et al*., 1999; Liu *et al*., 2007; Sun *et al*., 2016a; Tseng and Poon, 1983). There are many advantages to grouper contributing to its economic success, including the large size of individuals, desired flavor, nutrient density and wide distribution throughout tropical and subtropical waters along the coast of continents (Craig *et al*., 2001; Ding *et al*., 2006; Heemstra and Randall, 1993). Grouper are a diverse group of fish, belonging to the subfamily Epinephelinae. There are 15 genera containing 159 species in Epinephelinae and it is this diversity that provides substantial genetic variability for hybridization (Heemstra and Randall, 1993). *Epinephelus moara* and *Epinephelus lanceolatus* are considered as gourmet grouper and each has unique advantageous characteristics. *E. moara* has strong tolerance to temperature and salt concentrations (Okada *et al*., 1996) and fast growth, while *E. lanceol* is the largest species in the group and has a rapid growth rate and desirable taste (Li *et al*., 2008). As expected, hybrid progeny of *E. moara* (♀) × *E. lanceol* (♂) have shown advantageous characteristics of each of the individual species, including rapid growth rate, disease resistance and other growth advantages.

Mitochondrial genomes (mitogenomes) are commonly used for studying phylogenetic relationships (Boore *et al*., 2005). Compared to nuclear DNA, mitogenomes are small and relatively simple and have a higher mutation rate and evolve more rapidly (Hamers, 2016). These characteristics of mitogenomes have obvious advantages in the identification of interspecies hybridization and genetic infiltration. The study of mitogenome characteristics, such as the mode of inheritance in hybrids provides guiding principles for the development of hybrid breeding.

We present here the complete sequence of the hybrid, *E. moara*♀× *E. lanceol*♂ (EMEL)mitogenome. The mitogenome was annotated and used for phylogenetic analysis. The data reported here indicate that the overall organization and composition of the EMEL mitogenome is similar to those of other vertebrates' although there are some anomalies. Importantly, the phylogenetic data indicates that the mitogenome is inherited from the mother, and,

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from a broader perspective, this mitogenome could provide useful genomic data for studying phylogenetic relationships and hybrid breeding in Epinephelinae.

2 Materials and Methods

2.1 Sample and DNA Extraction

Hybrid *E. moara*♀×*E. lanceolatus*♂ (designated EMEL) specimens were sampled from Laizhou Mingbo Aquatic Co., Ltd., Shandong province, China. Total genomic DNA was extracted from EMEL fins using a tissue DNA extraction kit (OMEGA, Georgia, USA) according to the manufacturer's instructions. All experiments were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

2.2 PCR Amplification and Sequencing

The published mitogenome of *E. moara* (GenBank: JQ518290) was used as a reference sequence to design 17 primer pairs that were used to amplify the complete mitogenome of EMEL (Table 1). PCR reactions were conducted using a total volume of 20 μL consisting of 14.8 μL deionized H_2O, 0.1 μL ExTaq DNA polymerase (5 U μL^{-1}, Takara), 1.6 μL dNTPs (2.5 mmol L^{-1} each), 2 μL 10 × PCR Buffer (15 mmol L^{-1} Mg^{2+}), 0.5 μL each primer (10 μmol L^{-1}) and 0.5 μL DNA template (40 ng μL^{-1}). Thermocycling conditions were: initial denaturation 94℃ for 4 min, followed by 30 cycles of 94℃ for 40 s, 55–60℃ for 35 s, 72℃ for 90 s, followed by final extension 72℃ for 10 min and 4℃ until use. The PCR products (5 μL) were separated by gel electrophoresis (0.8% agarose) and visualized under UV light by a gel imaging and analysis system (BIO- RAD, Segrate, Italy). Sequencing was performed by Genewiz Biotechnology (Beijing, China). Finally, the circular mitogenome of EMEL was obtained by assembling the overlapping contiguous fragments.

Table 1 Primer pairs used for mitogenome amplification

Primer label	Forwardprimer sequence (5'→3')	Primer label	Reverse primer sequence (5'→3')	Melting temperature
ML1F	AACAAGGAGCAGGTATCAGG	ML1R	TCTATCGCTCCGTTAGTTCC	56
ML2F	CCAAAGCATAGTTCAAAGCAG	ML2R	GTTATGCCATTCATACAGGTC	58
ML3F	ACGTGTATATCGGAAACGGAC	ML3R	AAGCGTTGAGATCATAGTTGG	60
ML4F	CAACGATTAAAGTCCTACGTG	ML4R	AGGAAGTGGTGTAATGGAAGC	58
ML5F	CCTATTACTTAGCCTTGGAC	ML5R	AGAGTTTGTAGGATCGAAGC	60
ML6F	AAGCCTGTACTTCTACTTACG	ML6R	AAGGTGTTTGATATTGAGAGG	60
ML7F	CCTCTAGCAGGAAACCTAGC	ML7R	GGTCATGGTTAGTTCTACTGC	58
ML8F	ATACTCAGACTACCCAGACGC	ML8R	ATGTTGTGCTTATAAACTGGTC	60
ML9F	GCTAGAAATTGGTGACTACCG	ML9R	TGAATGGCTTGTTTTCGTTCC	56
ML10F	TTAGGTACAATTCTACTCCTC	ML10R	AGCACTGTAGTAGGTTAAGG	58
ML11F	AATTTGATTCCACTTCCACTCC	ML11R	GTCAGGCAGTTGGGATAAGC	56
ML12F	ATTCCATAACCACCTAATGACC	ML12R	CAAGATCAATTAAATAAGGAGGC	58
ML13F	CGAGAACATCTACTTATAACCC	ML13R	TGTCTTGTTCGTCATTGAGGC	60
ML14F	TCAACATCAAGTCAACTAGGC	ML14R	TAATTTGTTGAATCGTTGGAGC	60
ML15F	AAATACGACCAATATACCACC	ML15R	AAGAGCGAGAAGTATAGGACG	60
ML16F	GTTCCATTACTTCACACCTCC	ML16R	ACGGATAAAAACCACCAGACC	58
ML17F	AATAGTTCAGAGACTAGAACGC	ML17R	CTTAATGTCTGTCACTGCTGG	60

2.3 Sequence Analysis

The complete mitogenome of EMEL was assembled using SeqMan (DNASTAR software package). Protein-coding genes were predicted using both ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The tRNA scan-SE 1.21 program (Lowe and Eddy, 1997) was used to screen for tRNA genes, except the $tRNA^{Ser(AGY)}$ gene which was predicted using comparative genomics. Complete mitogenomes of 36 Epinephelinae species and 3 hybrid offspring (*E. fuscoguttatus*♀×*E. lanceolatus*♂, *E. coioides*♀×*E. lanceolatus*♂, *E. moara*♀×*E. lanceolatus*♂) (Table 2) were used for the comparative genomic analysis. The EMEL mitogenome map was drawn with OGDRAW (http://ogdraw.mpimp-golm.mpg.de/). The structure of O_L (the stem and loop structure) was predicted using the mfold web server (Zuker, 2003). Additionally, base composition and codon usage were predicted using MEGA v6.0 (Tamura *et al*., 2013).

2.4 Phylogenetic Analysis

Phylogenetic analysis was performed using the complete mitogenomes of 36 Epinephelinae species and three hybrids (Table 2). *Hypoplectrus gemma* (GenBank: FJ848375) was used as an outgroup for phylogenetic analysis. Nucleotide sequences of the 13 conserved protein-coding genes (PCGs) were aligned using MEGA v6.0, and then concatenated after deleting ambiguous positions. The best partitioning scheme and nucleotide substitution models were selected using PartitionFinder v1.1.1 (Lanfear *et al*., 2014) (shown in Table 3). The phylogenetic tree was constructed using maximum likelihood (ML) analysis with the rapid bootstrap feature (random seed value 12345) of RAxML v7.2.6 (Stamatakis, 2006). Node support was calculated with 1000 bootstrap replicates.

3 Results and Discussion

3.1 Genome Structure and Organization

Total mitogenome length in Epinephelinae species ranges from 16389 bp in *Epinephelus latifasciatus* to 17277 bp in *Epinephelus bleekeri*. Variability in the length

Table 2 List of species from the subfamily Epinephelinae used in this study

No	Species name	Genus	Accession ID	Reference
1	*Aethaloperca rogaa*	*Aethaloperca*	KC593376	Zhuang *et al*., 2013
2	*Anyperodon leucogrammicus*	*Anyperodon*	GQ131336	Unpublished
3	*Cephalopholis argus*	*Cephalopholis*	KC593377	Zhuang *et al*., 2013
4	*Cephalopholis boenak*	*Cephalopholis*	KC537759	Li *et al*., 2014
5	*Cephalopholis sexmaculata*	*Cephalopholis*	KJ469385	Hsiao *et al*., 2016
6	*Cephalopholis sonnerati*	*Cephalopholis*	KC593378	Zhuang *et al*., 2013
7	*Cromileptes altivelis*	*Cromileptes*	KC845547	Qin *et al*., 2014
8	*Epinephelus moara* × *Epinephelus lanceolatus*		KU881800	In this study
9	*Epinephelus akaara*	*Epinephelus*	EU043377	Zhuang *et al*., 2009
10	*Epinephelus areolatus*	*Epinephelus*	KC466080	He *et al*., 2013
11	*Epinephelus awoara*	*Epinephelus*	JX109835	Qu *et al*., 2012
12	*Epinephelus bleekeri*	*Epinephelus*	KF556648	Unpublished
13	*Epinephelus bontoides*	*Epinephelus*	KT619054	Hsiao *et al*., 2015
14	*Epinephelus coioides*	*Epinephelus*	EU043376	Zhuang *et al*., 2009
15	*Epinephelus coioides* × *Epinephelus lanceolatus*		KP257572	Wang *et al*., 2015a
16	*Epinephelus corallicola*	*Epinephelus*	KP072053	Zheng *et al*., 2014
17	*Epinephelus epistictus*	*Epinephelus*	KC816460	Peng *et al*., 2014a
18	*Epinephelus fasciatomaculosus*	*Epinephelus*	KC480085	Li *et al*., 2013
19	*Epinephelus fuscoguttatus*	*Epinephelus*	JX119192	Zhuang *et al*., 2013
20	*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*		KM605254	Zhu and Yu, 2014a
21	*Epinephelus lanceolatus*	*Epinephelus*	FJ472837	Wang *et al*., 2014
22	*Epinephelus latifasciatus*	*Epinephelus*	KC480177	Lai *et al*, 2013
23	*Epinephelus malabaricus*	*Epinephelus*	KM873711	Zhu and Yu, 2014b
24	*Epinephelus merra*	*Epinephelus*	AP005991	Miya *et al*., 2013
25	*Epinephelus moara*	*Epinephelus*	JQ518290	Liu *et al*., 2013
26	*Epinephelus quoyanus*	*Epinephelus*	KC790539	Peng *et al*., 2014b
27	*Epinephelus sexfasciatus*	*Epinephelus*	KC959953	Du *et al*., 2013
28	*Epinephelus stictus*	*Epinephelus*	KC527593	Ye *et al*., 2014a
29	*Epinephelus trimaculatus*	*Epinephelus*	KC847086	Ye *et al*., 2014b
30	*Epinephelus tukula*	*Epinephelus*	KJ414470	Yang *et al*., 2014
31	*Hyporthodus octofasciatus*	*Hyporthodus*	JX135579	Zhuang *et al*., 2013
32	*Hyporthodus septemfasciatus*	*Hyporthodus*	FJ594966	Unpublished
33	*Plectropomus areolatus*	*Plectropomus*	KC262636	Shen *et al*., 2013
34	*Plectropomus leopardus*	*Plectropomus*	DQ101270	Zhu *et al*., 2008
35	*Triso dermopterus*	*Triso*	KC593371	Zhuang *et al*., 2013
36	*Variola albimarginata*	*Variola*	KC593370	Zhuang *et al*., 2013
37	*Variola louti*	*Variola*	KC593369	Zhuang *et al*., 2013
38	*Grammistes sexlineatus*	*Grammistes*	KJ489014	Sun *et al*., 2016b
39	*Diploprion bifasciatum*	*Diploprion*	KP256530	Wang *et al*., 2015b

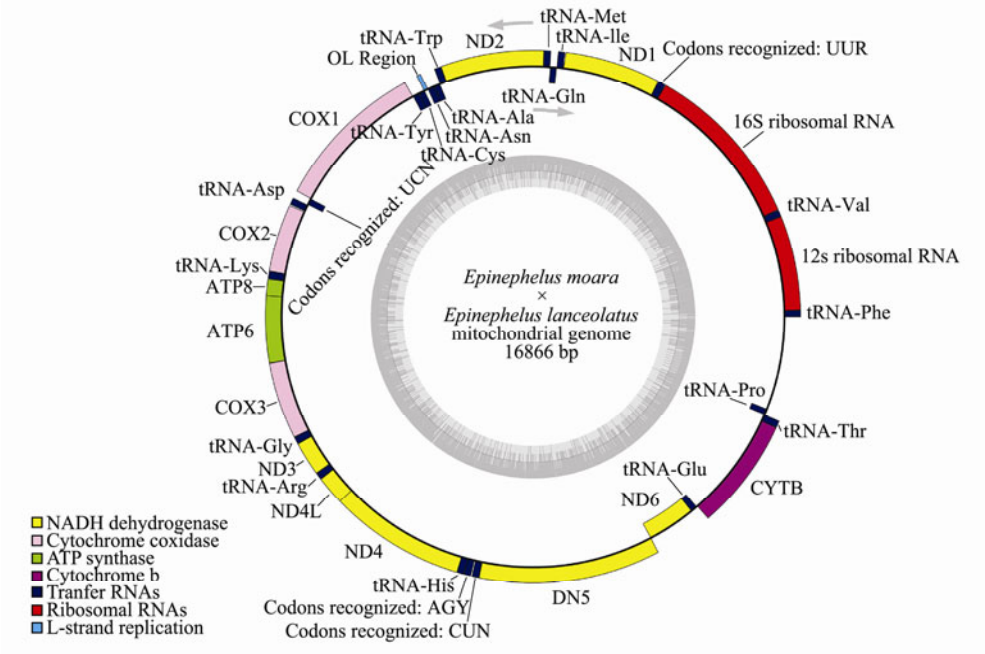

Fig.1 Gene map of the EMEL mitogenome. Genes encoded on the L-strand and H-strand are shown outside and inside the circular map, respectively. *ND1–6* represents genes of the NADH dehydrogenase subunits 1–6; *COI-III* represents cytochrome c oxidase subunits I–III; ATP6 and ATP8 represent ATPase subunits 6 and 8, respectively; Cytb represents cytochrome b. The inner ring indicates the GC content.

Table 3 The best partition scheme and nucleotide substitution models for ML analyses

	Best substitution model	Best partition scheme
ML	GTR+I+G	ND1, ATP6, COX3, ND3, ND4L, ND4, ND5, CYTB
	GTR+I+G	COX1, COX2
	GTR+G	ND2
	GTR+I+G	ATP8, ND6

Table 4 Nucleotide composition (A+T only) of key components and full-length mitogenomes of Epinephelus species

	Whole		PCGs		rrnL		rrnS		CR	
	Length (bp)	AT (%)	Length (bp)	AT (%)	Length (bp)	AT (%)	Length (bp)	AT (%)	Length (bp)	AT (%)
E. moara × *E. lanceolatus*	16866	55.27	11428	54.30	1701	53.73	953	51.10	1167	68.38
E. kaara	16795	55.97	11429	55.04	1708	54.57	953	52.26	1093	70.45
E. areolatus	16893	55.62	11429	54.62	1706	54.92	953	52.26	1191	69.35
E. awoara	16802	55.76	11429	54.82	1706	54.87	953	52.15	1102	69.42
E. bleekeri	17227	54.97	11429	54.36	1704	54.34	955	51.41	900	67.11
E. bontoides	16903	55.95	11429	55.19	1705	54.37	954	51.36	1200	69.17
E. coioides	16418	55.24	11425	54.90	1705	54.43	954	51.89	720	65.28
E. coioides × *E. lanceolatus*	16418	55.29	11425	54.98	1706	54.22	953	52.05	720	65.69
E. corallicola	16647	55.26	11428	54.80	1704	52.88	952	51.68	950	68.21
E. epistictus	16920	55.38	11429	54.17	1709	54.59	953	51.63	1217	69.93
E.fasciatomaculosus	16682	55.85	11431	54.84	1707	55.30	953	52.89	980	71.02
E. fuscoguttatus	16648	56.10	11428	55.55	1705	53.78	952	52.31	948	69.20
E. fuscoguttatus × *E. lanceolatus*	16644	56.06	11428	55.55	1705	53.67	953	52.05	947	69.27
E. lanceolatus	16574	55.92	11428	55.36	1705	54.49	953	52.05	947	69.27
E. latifasciatus	16389	54.96	11427	54.57	1707	53.25	953	51.21	684	69.01
E. malabaricus	16423	55.25	11430	54.89	1706	54.57	954	51.89	720	65.28
E. merra	17017	54.86	11428	54.05	1707	54.48	955	51.52	988	68.32
E. moara	17017	54.86	11428	54.05	1707	54.48	955	51.52	988	68.32
E. quoyanus	16797	56.51	11429	56.02	1708	54.63	954	51.99	1093	68.98
E. septemfasciatus	16558	55.39	11429	54.83	1709	55.00	956	51.57	850	67.88
E. sexfasciatus	16786	66.61	11429	54.62	1708	54.45	950	52.84	1090	70.83
E. stictus	16524	55.50	11429	54.75	1705	53.37	952	52.42	824	70.27
E. trimaculatus	16761	56.09	11429	55.55	1706	54.10	953	51.31	1056	69.32
E. tukula	16503	54.74	11428	53.94	1705	54.08	952	50.00	805	70.06
A. rogaa	16538	56.71	11431	56.34	1711	54.57	953	52.26	830	68.55
A. leucogrammicus	16616	55.68	11428	54.90	1706	54.57	953	51.31	916	69.76
C. argus	16767	56.85	11430	57.14	1722	55.28	956	54.18	813	62.24
C. boenak	16771	56.94	11429	56.58	1711	55.11	955	52.67	1064	70.11
C. sexmaculata	16589	55.37	11429	54.83	1710	54.15	957	52.35	877	65.56
C. sonnerati	16587	55.83	11429	55.46	1713	54.23	957	52.98	878	65.49
C. altivelis	16497	55.31	11428	54.82	1706	53.93	952	50.63	799	67.83
H. octofasciatus	16545	55.94	11429	55.51	1708	54.57	953	51.73	840	70.00
P. areolatus	16770	56.43	11429	55.95	1709	56.00	952	53.57	1080	65.37
P. leopardus	16714	56.75	11410	56.34	1691	56.59	951	53.42	1065	66.48
T. dermopterus	16605	53.94	11429	52.41	1708	55.56	954	51.26	901	69.70
V. albimarginata	16758	56.06	11428	56.37	1659	54.04	959	51.20	979	61.08
V. louti	16770	56.20	11428	56.24	1696	53.60	961	52.65	975	62.87
G. sexlineatus	16502	52.53	11428	51.44	1711	52.54	950	52.84	818	64.91
D. bifasciatum	16805	53.96	11439	53.22	1705	55.37	954	51.68	843	63.23
Average	16693	55.84	11428	54.96	1705	54.43	954	52.00	945	67.77

is thought to be due to variations in tandem repeat elements of the control region, the length of intergenic regions and gene overlaps (Moritz and Brown, 1987; McKnight and Shafer, 1997; Rand, 1993). The complete mitogenome length of EMEL, 16866 bp, is larger than the average length (16693 bp) of other Epinephelinae mitogenomes (Table 4).

The complete mitogenome sequence of EMEL was submitted to GenBank (accession No. KU881800). Its nucleotide base composition is 28.66% A, 26.61% T, 16.05% G and 28.68% C. Based on this data, the calculated A+T content is 55.27 %, which is slightly below the average value (55.84%) of Epinephelinae species (Table 4). The calculated A+T content of PCGs (54.3%), rrnL (53.73%) and rrnS (51.1%) are also slightly lower than the average values (54.96%, 54.43% and 52%, respectively) for Epinephelinae species (Table 4). However, the A+T content of the EMEL mitogenome D-loop (68.38%) is slightly higher than the average value (67.77%) for other Epinephelinae species.

Boore first described vertebrate mitogenome organization (Boore, 1999) and the data presented here suggest that this organization is conserved between EMEL and other vertebrates. Specifically, the mitogenome consists of 13 PCGs, 2 rRNA, 22 tRNA genes, 1 light-strand replication origin (O_L) and 1 control region (CR) (Fig.1). The

majority of the genes (28), are encoded on the heavy-strand (H-strand) while only 9 are encoded on the light-strand (L-strand) (Table 5). Briefly, 8 tRNA genes (*tRNA*^{Gln}, *tRNA*^{Ala}, *tRNA*^{Asn}, *tRNA*^{Cys}, *tRNA*^{Tyr}, *tRNA*$^{Ser(UCN)}$, *tRNA*^{Glu} and *tRNA*^{Pro}) and *ND6* are encoded on the L-strand (Table 5).

3.2 Protein-Coding Genes

In the EMEL mitogenome, a total of 11428 bp are predicted to encode 13 PCGs. The location of these 13 PCGs is identical to that of other bony fish (Table 5). In EMEL only 1 PCG (*ND6*) is encoded on the L-strand, while the remaining 12 PCGs (*ND1*, *ND2*, *COXI*, *COXII*, *ATP8*, *ATP6*, *COXIII*, *ND3*, *DN4L*, *ND4*, *ND5* and *Cytb*) are encoded on the H-strand. In addition, this study also analyzed codon usage in the EMEL mitogenomeand and found that of the 13 PCGs, 10 are predicted to use the canonical ATG start codon, while *COXI* and *ND4* use GTG, and *ATP6* uses CTG. Wolstenholme identified TNN (TAA, TA, or T) as the most frequently used stop codons in animal mitogenomes (Wolstenholme, 1992). Consistent with these findings several of the EMEL mitogenome PCGs utilize these stop codonds, as 6 PCGs are terminated with the complete stop codon TAA, 1 PCG with the incomplete stop codon TA (*COXIII*) and 5 PCGs with the incomplete stop codon T (*ND2*, *COXII*, *ND3*, *ND4* and *Cytb*). However *ND5* is terminated with the universal stop codon, TAG. It is presumed that, in vertebrates, these in

Table 5 Organization of the EMEL mitochondrial genome

Name of gene	Position		Size Nucleotide (bp)	Amino acid	Codons		Space (+) overlap (−)	Strand
	From	To			Initiation	Termination		
tRNA^{Phe} *(F)*	1	69	69				0	H
12S rRNA	70	1022	953				0	H
tRNA^{Val} *(V)*	1023	1092	70				0	H
16S rRNA	1093	2799	1707				0	H
tRNA$^{Leu(UUR)}$ *(L1)*	2800	2875	76				0	H
ND1	2876	3850	975	324	ATG	TAA	4	H
tRNA^{Ile} *(I)*	3855	3924	70				-1	H
tRNA^{Gln} *(Q)*	3924	3994	71				0	L
tRNA^{Met} *(M)*	3995	4064	70				0	H
ND2	4065	5109	1045	348	ATG	T--	0	H
tRNA^{Trp} *(W)*	5110	5180	71				1	H
tRNA^{Ala} *(A)*	5182	5250	69				0	L
tRNA^{Asn} *(N)*	5251	5323	73				0	L
O_L	5324	5359	36				0	
tRNA^{Cys} *(C)*	5360	5426	67				0	L
tRNA^{Tyr} *(Y)*	5427	5497	71				1	L
COX I	5499	7049	1551	516	GTG	TAA	1	H
tRNA$^{Ser(UCN)}$ *(S1)*	7051	7121	71				3	L
tRNA^{Asp} *(D)*	7125	7197	73				8	H
COX II	7206	7896	691	230	ATG	T--	0	H
tRNA^{Lys} *(K)*	7897	7970	74				1	H
ATP8	7972	8139	168	55	ATG	TAA	-10	H
ATP6	8130	8813	684	227	CTG	TAA	-1	H
COX III	8813	9597	785	261	ATG	TA-	0	H
tRNA^{Gly} *(G)*	9598	9669	72				0	H
ND3	9670	10018	349	116	ATA	T--	0	H
tRNA^{Arg} *(R)*	10019	10087	69				0	H
ND4L	10088	10384	297	98	ATG	TAA	-7	H
ND4	10378	11758	1381	460	GTG	T--	0	H
tRNA^{His} *(H)*	11759	11828	70				1	H
tRNA$^{Ser(AGY)}$ *(S2)*	11830	11899	70				8	H
tRNA$^{Leu(CUN)}$ *(L2)*	11908	11980	73				0	H
ND5	11981	13819	1839	612	ATG	TAG	-4	H
ND6	13816	14337	522	173	ATG	TAA	0	L
tRNA^{Glu} *(E)*	14338	14407	70				7	L
Cytb	14415	15555	1141	380	ATG	T--	0	H
tRNA^{Thr} *(T)*	15556	15629	74				0	H
tRNA^{Pro} *(P)*	15630	15699	70				0	L
CR	15700	16866	1167					-

complete stop codons are altered to a complete TAA stop codon through post-transcriptional polyadenylation (Ojala *et al*., 1981).

3.3 tRNA Genes and rRNA Genes

The methods used here predicted 22 tRNA genes in the EMEL mitogenome ranging from 67 to 74 bp (Table 5). Among the 22 tRNA genes, 21 were detected using the tRNAscan-SE Search Server v.1.21 (Lowe and Eddy, 1997). The remaining tRNA gene (*tRNA*$^{Ser\ (AGY)}$) were then predicted using comparative genomics as described in the section of materials and methods. In addition, the 2 rRNA genes (16S rRNA and 12S rRNA) were then detected at the conserved position (between *tRNA*^{Phe} and *tRNA*$^{Leu(UUR)}$, separated by *tRNA*^{Val}) with a total length of 2660 bp.

3.4 Non-Coding Regions

Two non-coding regions (O_L and CR) were both detected in the EMEL mitogenome. The data presented here indicate that O_L is located in a typical cluster of 5 tRNA genes known as the WANCY region, which is similar to that in other vertebrate mitogenomes. Based on folding models, this region is predicted to fold into a stable stem loop structure consisting of a 26 bp GC stem and an 8 bp loop (Fig.2).

The CR is predicted to have a total length of 1167 bp, located between $tRNA^{Pro}$ and $tRNA^{Phe}$, which is consistent with its organization in other vertebrates (Sbisà *et al.*, 1997). As stated previously, the A+T content is 68.38%, which is slightly higher than the average value (67.77%) of other Epinephelinae species. The observation of a repeat array at the 5' end, a signature feature of fish mitogenome CRs, supports this as the putative CR in the EMEL mitogenome (Fig.3). The repeat array consists of 31 copies of a 12 bp repeat unit (5'-ATATTACATATATG CTG-3'). The variable lengths of CRs among different Epinephelinae species (Table 4) are mainly due to the different number of basic unit repeats, suggesting that the CR may be the most variable region in the mitogenome (Wan *et al.*, 2003).

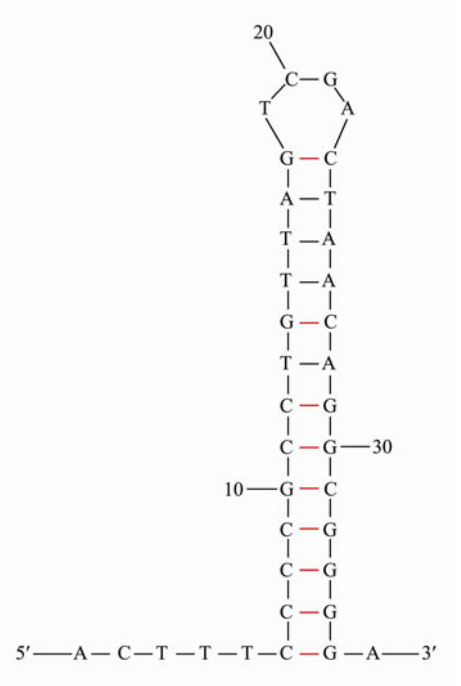

Fig.2 Stem and loop structure of the O_L in the EMEL mitogenome.

Repeat unit

```
   1 ATATTACATA TATGCTGATA TTACATATAT GCTGATATTA CATATATGCT GATATTACAT
  61 ATATGCTGAT ATTACATATA TGCTGATATT ACATATATGC TGATATTACA TATATGCTGA
 121 TATTACATAT ATGCTGATAT TACATATATG CTGATATTAC ATATATGCTG ATATTACATA
 181 TATGCTGATA TTACATATAT GCTGATATTA CATATATGCT GATATTACAT ATATGCTGAT
 241 ATTACATATA TGCTGATATT ACATATATGC TGATATTACA TATATGCTGA TATTACATAT
 301 ATGCTGATAT TACATATATG CTGATATTAC ATATATGCTG ATATTACATA TATGCTGATA
 361 TTACATATAT GCTGATATTA CATATATGCT GATATTACAT ATATGCTGAT ATTACATATA
 421 TGCTGATATT ACATATATGC TGATATTACA TATATGCTGA TATTACATAT ATGCTGATAT
 481 TACATATATG CTGATATTAC ATATATGCTG ATATTACATA TATGCTGATA TTACATATAT
 541 GCGTCTTGAT TCAACATTAT ATTTGATAAC AAATAAACTG CACAGTAAGA ACCTACCAAC
 601 AAGAGTTAGG TAATGCATAC GGTTATTGAT AATGAGGGAC AATAACTGTG AGGGTCTCAC
 661 TCAGTGAATT ATTCCTGGCA TTTGGTTCCT ACTTCAGGGC CATGACTTGA TTATATTCCT
 721 CACACTTTCA TTAACGCTGG CATAAGTTAA TAGTGTTAAC CATTAGATTC ATTACCCAGC
 781 AAGCCGAGCC TTCATTCCAG GGGGTGGGGG GTTCCCTTTT ATTTTTTTCC TTTCAACAGG
 841 CATTTCAGAG TGTAAGAAAA GGCTGAAAGT TTGAAGGTAG TACATCACTT TGCAGCTGAA
 901 CACAAGGTTA TGCAGGCTAA AAAGACATTC TTTTAGAAAT AATTACATAA CTGATTTCAA
 961 GAACATAAAT ATACTTATCC TACTCAGAGC ACCCCCCTAA TATTAGGATG CCCGGTCTGG
1021 TGGTTTTTAT CCGTTTAAAC CCCCCTACCC CCCTAAACCC CTGAGATTCC TAACACCCCT
1081 GTAAACCCCC CGGAAACAGG GCTAAACCTC AAGTAATAAG TTTTTAACCT AAAATGTGTT
1141 TATTACACTA ATGTAATTTT TAATTTG
```

Fig.3 Nucleotide sequence of the CR in EMEL mitogenome. The underlined sequence represents the repeat array.

3.5 Maternal Inheritance

Comparison of the mitogenome sequences between the hybrid EMEL and the parents was performed. The hybrid EMEL mitogenome shares high similarity with *E. moara* (the female parent, 99.8% identity), but lower similarity with *E. lanceolatus* (the male parent, 89.3% identity). The divergence between the hybrid EMEL and *E. moara* mitogenomes (0.2%) is mainly due to the loss copies of repeat elements in the CR during the heredity. This provides some evidence to show that the mitogenome is inherited from the female parent.

3.6 Phylogenetic Analysis

Phylogenetic analysis was performed using the ML method based on the nucleotide sequences of 13 conserved PCGs from 36 Epinephelinae species and three hybrids (Table 2). It is clear that EMEL forms a clade with *E. moara* with strong bootstrap support (bootstrap = 100). Additionally, the other 2 well-supported monophyletic clades were observed between the hybrid and female parent (*E. coioides*♀×*E. lanceolatus*♂, *E. fuscoguttatus* and *E. fuscoguttatus*♀× *E. lanceolatus*♂) (Fig.4), which demonstrates that the mitogenome is inherited maternally. Furthermore, *Anyperodon leucogrammicus* and *Cromileptes altivelis* form a clade with the species in the genus *Epinephelus*, which is slightly different from conventional morphology-based classification of Epinephelinae species. Additionally, another clade was formed with three *Cephalopholis* species and *Aethaloperca rogaa*, which is also different from conventional morphology-based classification of Epinephelinae species. The difference in the evolutionary relationships between the morphology-based and mitogenome-based phylogenetic trees may be due to the limited availability of mitogenomes from Epinephelinae species. Therefore, it is predicted that the phylogenetic relationships of species in Epinephelinae will become clearer when more Epinephelinae mitogenomes become available.

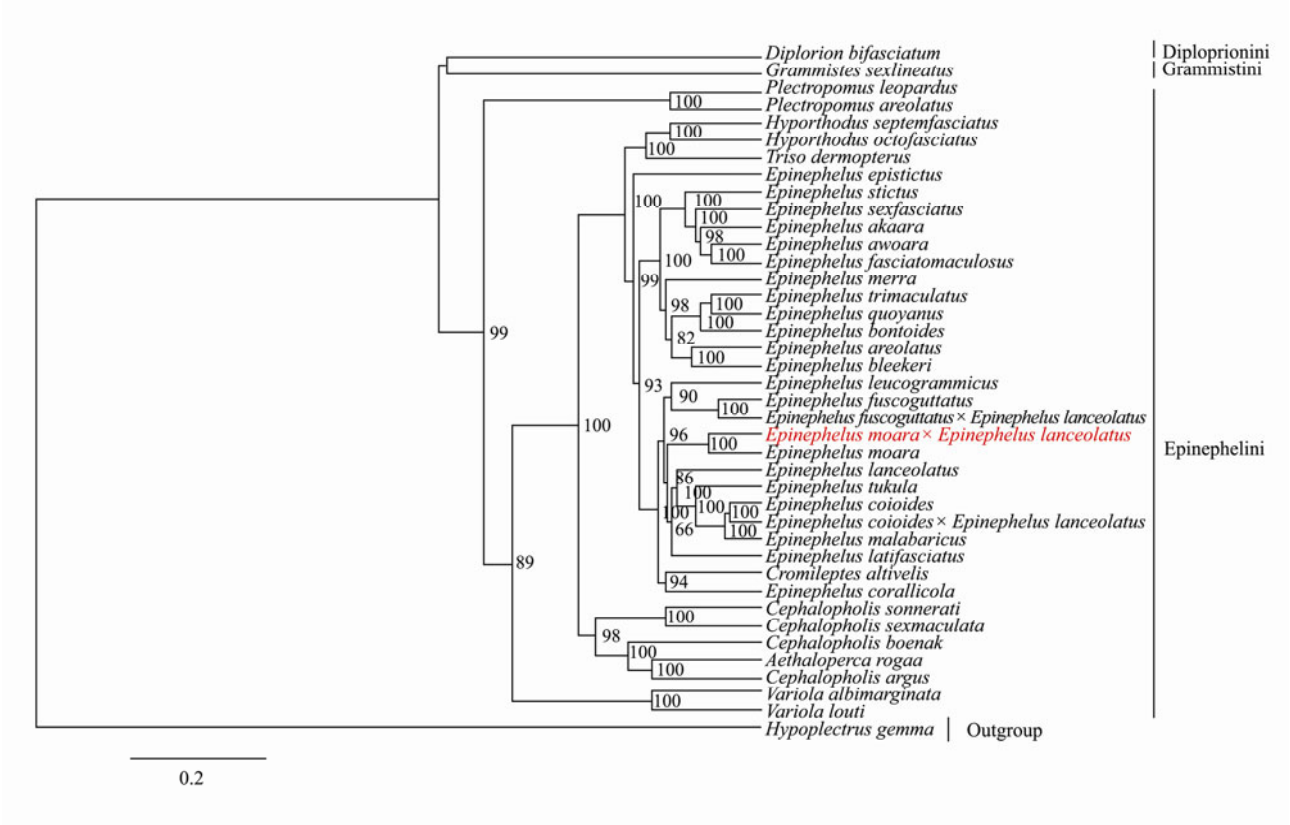

Fig.4 Phylogenetic tree of 36 Epinephelinae species and three hybrids. Phylogenetic analysis was based on nucleotide dataset of 13 PCGs using the ML method. Numbers at nodes are ML bootstrap values and only values above 50% are displayed.

4 Conclusions

The complete sequence of the EMEL mitogenome is 16866 bp in length, and contains 13 PCGs, 22 tRNA genes, 2 rRNA genes, an O_L, and a CR. Phylogenetic analysis based on nucleotide sequences of the 13 conserved PCGs was performed using the ML method, which demonstrated that the mitogenome is inherited maternally by the progenies. Furthermore, this relationship is slightly different from conventional morphology-based classification of Epinephelinae species. It is predicted that as more Epinephelinae mitogenomes become available, the phylogenetic relationship between species in this subfamily will become clearer.

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Conflict of Interest

All authors state that there is no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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