Acta Oceanol. Sin., 2020, Vol. 39, No. 12, P. 65–75
https://doi.org/10.1007/s13131-020-1689-7
http://www.hyxb.org.cn
E-mail: hyxbe@263.net

The complete mitochondrial genome of the hybrid grouper *Epinephelus moara* (♀)×*Epinephelus tukula* (♂), and phylogenetic analysis in subfamily Epinephelinae

Zhentong Li[1, 3], Yongsheng Tian[2, 3*], Meiling Cheng[3, 4], Linna Wang[2, 3], Jingjing Zhang[1, 3], Yuping Wu[3, 4], Zunfang Pang[5], Wenhui Ma[5], Jieming Zhai[5]

[1] College of Fisheries and Life Science, Shanghai Ocean University, Shanghai 201306, China

[2] Laboratory for Marine Fisheries Science and Food Production Processes, Pilot National Laboratory for Marine Science and Technology (Qingdao), Qingdao 266071, China

[3] Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China

[4] College of Fisheries and Life Science, Dalian Ocean University, Dalian 116023, China

[5] Laizhou Mingbo Aquatic Co. Ltd., Laizhou 261400, China

Received 11 July 2019; accepted 27 April 2020

Abstract

The mitochondrial genome (mitogenome) of hybrid grouper *Epinephelus moara* (♀)×*Epinephelus tukula* (♂), a new hybrid progeny, can provide valuable information for analyzing phylogeny and molecular evolution. In this study, the mitogenome was analyzed using PCR amplification and sequenced, then the phylogenetic relationship of *E. moara* (♀)×*E. tukula* (♂) and 35 other species were constructed using Maximum Likelihood and Neighbor-Joining methods with the nucleotide sequences of 13 conserved protein-coding genes (PCGs). The complete mitogenome of *E. moara* (♀)×*E. tukula* (♂) was 16 695 bp in length, which contained 13 PCGs, 2 rRNA genes, 22 tRNA genes, a replication origin and a control region. The composition and order of these genes were consistent with most other vertebrates. Of the 13 PCGs, 12 PCGs were encoded on the heavy strand, and ND6 was encoded on the light strand. The mitogenome of the *E. moara* (♀)×*E. tukula* (♂) had a higher AT nucleotide content, a positive AT-skew and a negative GC-skew. All protein initiation codons were ATG, except for COX and ND4 (GTG), ATP6 (CTG), and ND3 (ATA). ND2, COXII, ND3, ND4 and Cytb had T as the terminating codon, COXIII's termination codon was TA, and the remaining PCGs of that were TAA. All tRNA genes, except for the lacking DHU-arm of $tRNA^{Ser\ (AGN)}$, were predicted to form a typical cloverleaf secondary structure. In addition, sequence similarity analysis (99% identity) and phylogenetic analysis (100% bootstrap value) indicated that the mitochondrial genome was maternally inherited. This study provides mitogenome data for studying genetic, phylogenetic relationships and breeding of grouper.

Key words: *Epinephelus moara* (♀)×*Epinephelus tukula* (♂), *Epinephelus moara*, *Epinephelus tukula*, mitochondrial genome, phylogeny

Citation: Li Zhentong, Tian Yongsheng, Cheng Meiling, Wang Linna, Zhang Jingjing, Wu Yuping, Pang Zunfang, Ma Wenhui, Zhai Jieming. 2020. The complete mitochondrial genome of the hybrid grouper *Epinephelus moara* (♀)×*Epinephelus tukula* (♂), and phylogenetic analysis in subfamily Epinephelinae. Acta Oceanologica Sinica, 39(12): 65–75, doi: 10.1007/s13131-020-1689-7

1 Introduction

The mitochondrial is a semi-autonomous organelle that can replicate autonomously under the control of nuclear genes ranging from 15 to 20 kilobases in length (Boore et al., 1999), and contains 37 genes: 13 protein-coding genes (PCGs), 2 RNA (12S RNA and 16S RNA), 22 tRNAs, and 1 control region that contains the initial sites for mtDNA replication and RNA transcription. Compared to nuclear DNA, mitogenome are small and relatively simple, and have the characteristics of coding content conservation, maternal inheritance, rapid evolution, and low levels of intermolecular genetic recombination (Boore, 1999). Therefore, mitogenome has been widely used to molecular research, such as the identification of species, the analysis of molecular evolution, the study of population genetic structure, and the analysis of phylogenetics (Brown et al., 1979; Moritz et al., 1987; Ballard and Whitlock, 2004; Liu et al., 2013). The complete mitogenome of hundreds of vertebrates species have been determined from mammals (Anderson et al., 1981; Bibb et al., 1981; Peng et al., 2007), chicken (Liu et al., 2016) and fish (Chang et al., 1994). As for Epinephelinae fishes, the first complete grouper mitogenome that was sequenced was Leopard coral grouper (*Plectropomus leopardus*) (Zhu and Yue, 2008), and mitogenomes from more than 30 species have been published since then (Zhuang et al., 2010; Qu et al., 2012; Oh et al., 2012; Zhu et al., 2016).

Mitogenome sequencing can accurately reflect the variation of individual bases. It is the most sensitive and reliable genetic

Foundation item: The Key Research and Development Program of Shandong Province under contact No. 2019GHY112063; the Breeding Project of Shandong Province under contract No. 2019LZGC020; the Central Public-interest Scientific Institution Basal Research Fund Chinese Academy of Fishery Sciences under contract Nos 2020XT0601, 2020TD19 and 2020TD25; the Yellow Sea Fisheries Research Institute Research Fees under contract Nos 20603022019002 and 20603022020015.
*Corresponding author, E-mail: tianys@ysfri.ac.cn

analysis method and one of the most widely used markers in molecular phylogeography (Avise, 2009). At present, research about the mitogenome of Epinephelinae species helps us understand the evolutionary relationship of them. The detail of their evolutionry relationships based on the mitogenome phylogenetic trees was consistent with that conventional morphology-based classification to some extent (Zhu and Yue, 2008; Gao et al., 2017). However, the mitogenome sequence of most Epinephelinae species was not available, which will limit our understanding of actual evolutionary relationships. Hybrids may provide more information leading to a more scientific classification (Gao et al., 2017). In the process of biological evolution, hybridization has an important influence on species formation (Wang, 2017). More and more examples of homoploid hybrid speciation were found, such as *Gila seminudea* (DeMarais et al., 1992) and *Cottus* sp. (Stemshorn et al., 2011). There are many species in subfamily Epinephelinae and most them are found in tropical and subtropical. By studying the mitochondria of hybrids to reveal the inherent genetic mechanism of species formation, which is of great significance to study the molecular phylogeography of subfamily Epinephelinae (Avise, 2009; Gao et al., 2017).

Hybridization can combine the advantageous traits of different species of fish, and provide so-called heterosis of hybrid progeny, which improves the viability and tolerance to the environment, as well as resolving issues around germplasm during culturing. Hybridization as an effective breeding method has been widely used in fish breeding, including grouper (James et al., 1999; Glamuzina et al., 2001). Grouper is an extremely diverse group of marine fish, providing abundant genetic materials for hybridization (Heemstra and Randall, 1993), such as *Epinephelus moara* (♀) × *Epinephelus lanceolatus* (♂) (Gao et al., 2017), *E. fuscoguttatus* (♀) × *E. tulcula* (♂) (Tian et al., 2019). *Epinephelus moara* and *E. tukula*, which both belong to Serranidae in the order Perciformes, are widely loved by consumers due to its delicious flavor. Both *E. moara* and *E. tukula* are mainly distributed in western Pacific region (Guo et al., 2004; Tian et al., 2019). *Epinephelus moara* has high market value, tolerance to high temperature and salt concentrations and high adaptability to aquaculture, while *E. tukula* possesses the merits of strong disease resistance and rapid growth. Therefore, the good traits of both groupers can be collected by hybridization. The grouper *E. moara* (♀)×*E. tukula* (♂) (EMET) were obtained by artificial insemination with *E. moara* as the female parent and *E. tukula* as the male parent (Fig. 1). As hybrid progeny, EMET have superior performance, such as high disease resistance, higher survival rate, and lower deformity rate. However, there have been no studies on *E. moara* (♀)×*E. tukula* (♂) mitochondrial sequences.

In this study, we present the complete sequence of the hybrid EMET mitogenome. The overall composition and organization of the EMET mitogenome was similar to the typical mitochondria structure in vertebrates. Further more, we verified that the mitogenome was maternally inherited through phylogenetic analysis. The EMET mitogenome provides a useful genomic resource for breeding hybrids in Epinephelinae.

Fig. 1. Six month old *E. moara* (♀)×*E. tukula* (♂) female individual.

2 Materials and methods

2.1 *Sample collection and DNA extraction*

The groupers *E. moara* and *E. tukula* cultured at Laizhou Mingbo Aquatic Co., Ltd. (Shandong, China) were collected from the wild. The hybrid were obtained by artificial insemination with *E. moara* as the female parent and *E. tukula* as the male parent. Three hybrid (EMET 1, 2, 3), six month-old, and mean total length (23.0±0.7) cm, were sampled and the fin were rapidly conserved in absolute ethanol and kept at –20°C for DNA extraction. Total genomic DNA was extracted from EMET fins using the OMEGA tissue DNA extraction kit (OMEGA, USA) according to the manufacturer's instructions. All experiments were followed the National Institutes of Health's Guide for the care and use of laboratory animals.

2.2 *PCR amplification and sequencing*

The complete mitogenome of EMET 2 was amplified by PCR using 19 primer pairs (Table 1), which were designed using Primer Premier 5.0 based on the complete mitogenome of *E. moara* (GenBank: JQ518290). Primers were synthesized by Ruibiotech Co. Ltd. (Hefei, China). PCR reactions were conducted using a total volume of 30 μL consisting of 22.1 μL deionized H_2O, 3 μL 10× PCR buffer (15 mmol/L Mg^{2+}), 2.4 μL dNTPs (2.5 mmol/L), 1 μL DNA template (50 ng/μL), 0.3 μL ExTaq DNA polymerase (5 U/μL, Takara), 0.6 μL forward primer (10 μmol/L) and 0.6 μL reverse primer (10 μmol/L). The PCR reaction conditions were as follows: initial denaturation 95°C for 10 min, followed by 36 cycles of 95°C for 30 s, 58–59°C for 35 s, 72°C for 90 s, followed by final extension 72°C for 8 min and terminated at 4°C. The PCR products (5 μL) were separated by a 0.8% agarose gel electrophoresis and visualized under UV light (BIO-RAD, Italy). Sequencing of the samples were conducted by Ruibiotech Co. Ltd. (Hefei, China).

2.3 *DNA sequence annotation and analysis*

The circular mitogenome of EMET was assembled using the overlapping contiguous fragments with SepMan (DNASTAR software package). The protein-coding gene and rRNAs sequences were predicted using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The tRNAs and potential RNA secondary structures were identified by using tRNAscan-SE 2.0 (Lowe and Chan, 2016). The structure of $tRNA^{Ser\ (AGY)}$ was predicted using sequence comparisons with other published grouper mitogenome. We used the mfold web server to predict the structure of O_L (the stem and loop structure) (Zuker, 2003). The EMET mitogenome map was drawn with OGDRAW (http://ogdraw.mpimp-golm.mpg.de/). Mitogenomes from 36 Epinephelinae species were used for comparative analysis. The base composition and codon usage patterns were predicted by MEGA 7.0, and trends of the composition of PCGs and rRNAs were measured by the formulas (Perna and Kocher, 1995):

$$\text{AT skew} = (\text{A} - \text{T})/(\text{A} + \text{T}), \tag{1}$$

$$\text{GC skew} = (\text{G} - \text{C})/(\text{G} + \text{C}). \tag{2}$$

2.4 *Phylogenetic analysis*

Phylogenetic relationships of EMET and other Epinephelinae species were analyzed using data from 35 fish species, including 3 hybrids grouper. Thirteen mitogenomic PCG sequence data were analyzed with Maximum Likelihood (ML) and Neighbor-Joining (NJ) methods (Table 2). The nucleotide sequences of 13 PCGs were aligned with MEGA 7.0 by default settings. The best partitioning scheme and nucleotide substitution models were GTR+I+G selected by PartitionFinder 1.1.1 (Lanfear et al., 2012), and node support was calculated with 1 000 bootstrap replicates.

Table 1. Primer pairs used for mitogenome amplification that was designed based on the mitogenome of *E. moara* using Primer Premier 5.0

Primer label	Forward primer sequence (5′–3′)	Primer label	Reverse primer sequence (5′–3′)	Melting temperature/°C
MT1-F	GGCTTGGTCCTGACTTTCCT	MT1-R	TATCGCTCCGTTGTTCCTTT	58
MT2-F	CCCGACCAGTCAAAAACAAC	MT2-R	AAGAGGAGACAGTCAAGCCC	58
MT3-F	ACACCCAAACAGACAACCGT	MT3-R	GGCTGACCTCGTAGGAAATAG	58
MT4-F	CCCCCATTTTATTCCTCCTC	MT4-R	GTGCTGGCAAATAAGAGGGTAG	58
MT5-F	ATGCCTGAAGTAAAGGACCAC	MT5-R	GCTTTGAAGGCTCTTGGTCT	59
MT6-F	CCTCCCATCCCTACAACTAACAC	MT6-R	AAAGAATCGGGTCTCCTCCTC	58
MT7-F	TCTTTCCTGCTCCTTCTTGC	MT7-R	CCCTTTCTCGTTTAGTGTGGTC	58
MT8-F	CGATACTCAGACTACCCAGACG	MT8-R	CCACTACGATTGGCATAAAGC	58
MT9-F	CGAATGGTCGTTCCCTTAGA	MT9-R	GCTACGGTTGGTATGAGTGGT	58
MT10-F	GAAACCAACCAAATCACGCA	MT10-R	TGAGCCATAAACGCCATCTG	58
MT11-F	TCGCATCAGGAGTAACAGTAACC	MT11-R	GGAGAGGGCAATAAATAGGGAG	58
MT12-F	CCATAACCACCTAATGACCCC	MT12-R	GGCTGTGTGTTCGTTCGTAGT	59
MT13-F	CATCTTCGCATTATGAGGGG	MT13-R	TCGGTTTATGTTAGGGTCGG	58
MT14-F	CTTTGTTAGCCTCCTCCCTC	MT14-R	GTAAGATGGTGTATGCCGCC	58
MT15-F	CTTTGACCTCACCTACCCACTC	MT15-R	GCTGGCAATGGATTGACCTA	59
MT16-F	TGAGCCCTTACCTTGACCCT	MT16-R	GCCATTGGTCCTGGTTAGAGT	59
MT17-F	AAAGGGGACGGATTAGAAGC	MT17-R	CGTTGTTTGGAGGTGTGAAG	58
MT18-F	CACCTCCTGTTCCTTCACGA	MT18-R	TCGGCTTGCTGGGTAATGA	59
MT19-F	GAGGGACAATAACTGTGAGGG	MT19-R	CTTCTTACTTTCGGGAGCGT	58

Table 2. List of species from the subfamily Epinephelinae for phylogenetic analysis

No.	Species name	Genus name	Accession ID
1	*Aethaloperca rogaa*	*Aethaloperca*	KC593376
2	*Anyperodon leucogrammicus*	*Anyperodon*	GQ131336
3	*Cephalopholis argus*	*Cephalopholis*	KC593377
4	*Cephalopholis sonnerati*	*Cephalopholis*	KC593378
5	*Cromileptes altivelis*	*Cromileptes*	KC845547
6	*Diploprion bifasciatum*	*Diploprion*	KP256530
7	*Epinephelus akaara*	*Epinephelus*	EU043377
8	*Epinephelus areolatus*	*Epinephelus*	KC466080
9	*Epinephelus awoara*	*Epinephelus*	JX109835
10	*Epinephelus bleekeri*	*Epinephelus*	KF556648
11	*Epinephelus bruneus*	*Epinephelus*	JQ518289
12	*Epinephelus coioides*×*Epinephelus akaara*		KX575834
13	*Epinephelus coioides*	*Epinephelus*	EU043376
14	*Epinephelus epistictus*	*Epinephelus*	KC816460
15	*Epinephelus fasciatomaculosus*	*Epinephelus*	KC480085
16	*Epinephelus fuscoguttatus*	*Epinephelus*	JX119192
17	*Epinephelus fuscoguttatus*×*Epinephelus lanceolatus*		KM605254
18	*Epinephelus lanceolatus*	*Epinephelus*	FJ472837
19	*Epinephelus malabaricus*	*Epinephelus*	KM873711
20	*Epinephelus moara*	*Epinephelus*	JQ518290
21	*Epinephelus moara*×*Epinephelus lanceolatus*		KU881800
22	*Epinephelus quoyanus*	*Epinephelus*	KC790539
23	*Epinephelus sexfasciatus*	*Epinephelus*	KC959953
24	*Epinephelus stictus*	*Epinephelus*	KC527593
25	*Epinephelus tukula*	*Epinephelus*	KJ414470
26	*Grammistes sexlineatus*	*Grammistes*	KJ489014
27	*Hypoplectrus gemma*	*Hypoplectrus*	NC013832
28	*Hyporthodus octofasciatus*	*Hyporthodus*	JX135579
29	*Macropodus opercularis*	*Macropodus*	NC025932
30	*Plectropomus areolatus*	*Plectropomus*	KC262636
31	*Plectropomus leopardus*	*Plectropomus*	DQ101270
32	*Pseudanthias dispar*	*Pseudanthias*	NC028286
33	*Triso dermopterus*	*Triso*	NC022140
34	*Variola albimarginata*	*Variola*	KC593370
35	*Variola louti*	*Variola*	KC593369
36	*Epinephelus moara*×*Epinephelus tukula*		MH748091

3 Results

3.1 *Genome structure and organization*

The complete mitogenome of EMET was 16 695 bp in length (GenBank accession No. MH748091), containing the typical 37 genes: 13 PCGs, 22 tRNAs, 2 ribosomal RNAs (12S rRNA and 16S rRNA), a control region (D-loop) in 997 bp length and a 36 bp initiation site for transcription and replication (Fig. 2). Of the 37 genes, 28 genes (12 PCGs, 14 tRNA genes, 2 rRNA genes) were encoded on the heavy (H)-strand, and the remaining 9 genes (1 PCGs, 8 tRNA) were encoded on the light (L)-strand (Table 3).

The lengths of the Epinephelinae mitogenome range from 16 418 bp in *E. coioides* to 17 227 bp in *E. bleekeri*, with average length of 16 688 bp (Table 4). The EMET mitogenome was longer than the average length in Epinephelinae. The nucleotide base composition of the EMET mitogenome was 28.6%, 28.9%, 16.1% and 26.4% for A, C, G and T, respectively. The calculated A+T content of the complete genome, PCGs, srRNA, lrRNA and the control region was 55.0%, 54.3%, 51.1%, 53.7%, and 66.9%, which were slightly lower compared to the other Epinephelinae species (Table 4). The EMET mitogenome had differences in base preference, and the value of the AT-skew and GC-skew were 0.04, –0.28, respectively. This illustrates that the EMET mitogenome had a bias to A and C.

3.2 *Protein-coding genes*

In the complete EMET mitogenome, 13 PCGs were encoded from 11 428 bp, accounting for 68.45% of all bases in the mitogenome. The order and location of PCGs were consistent with other vertebrate fish (Table 3). Twelve of the 13 PCGs were encoded on the H-strand, including ND1, ND2, COXI, COXII, ATP8, ATP6, COXIII, ND3, ND4L, ND4, ND5 and Cytb, and ND6 was encoded on the L-strand.

For translation initiation sites, 9 PCGs had ATG, while COXI and ND4 had GTG, ATP6 had CTG, and ND3 had ATA, and all of these patterns were shared by other vertebrates. Translation termination sites included TAA for 7 of the 13 PCGs, COXIII used TA, and the remaining PCGs (ND2, COXII, ND3, ND4, Cytb) used T as their termination codon.

In the complete EMET mitogenome, base content proportion order was as follows: C (28.87%)>A (28.58%)>T (26.46%)>G (16.09%). The utilization rate of G was the lowest, and the A+T was slighter higher than G+C. This pattern is commonly found in PCGs, except for ND4L. In addition, the base composition of the 13 PCGs were different depending on the gene, where the A+T content was highest in Cytb (64.51%), and second highest in COXII (56.73%). Interestingly, the PCGs had a high GC preference, where the GC-skew was 0.28 (Table 5).

The base composition of the coding chain leads to the preference of codon usage. By analyzing the usage of Relative Synonymous Codon Usages (RSCU) (Table 6), we found that C and T were the 2 most frequently used nucleotides, Leu, Ala, Thr, Ile were the 4 most frequently used amino acids, CUC (Leu), CUA (Leu), GCC (Ala) and AUU (Ile) were the 4 most frequently used codons, accounting for 17.07% of all codons in the mitogenome.

3.3 *tRNA and rRNA genes*

The 22 typical transfer RNA genes ranged from 67 bp in tR-

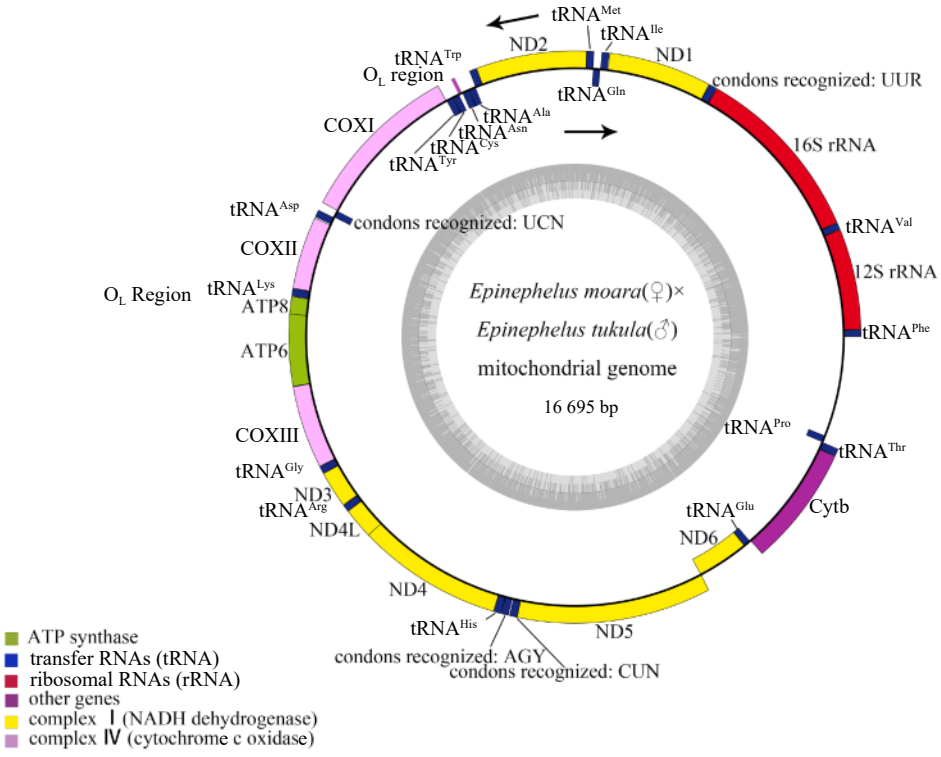

Fig. 2. Gene map of the *Epinephelus moara* (♀)×*Epinephelus tukula* (♂) mitogenome. Genes encoded on the H-strand and L-strand are shown outside and inside the circular map, respectively. ND1–ND6 represent genes of the *E. moara* (♀)×*E. tukula* (♂) dehydrogenase subunits 1–6; COXI–COXIII represent 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. Organization of the *Epinephelus moara* (♀)×*Epinephelus tukula* (♂) mitogenome

Name of gene	Position		Size-nucleotide/bp	Amino acid	Anticodon	Codons		Spaceoverlap	Strand
	From (bp)	To (bp)				Initiation	Termination		
$tRNA^{Phe}$	1	69	69		GAA			0	H
12S rRNA	70	1 022	953					0	H
$tRNA^{Val}$	1 023	1 092	70		TAC			0	H
16S rRNA	1 093	2 798	1 706					0	H
$tRNA^{Leu(UUR)}$	2 799	2 874	76		TAA			0	H
ND1	2 875	3 849	975	324		ATG	TAA	4	H
$tRNA^{Ile}$	3 854	3 923	70		GAT			–1	H
$tRNA^{Gln}$	3 923	3 993	71		TTG			0	L
$tRNA^{Met}$	3 994	4 063	70		CAT			0	H
ND2	4 064	5 108	1 045	348		ATG	T––	0	H
$tRNA^{Trp}$	5 109	5 179	71		TCA			1	H
$tRNA^{Ala}$	5 181	5 249	69		TGC			0	L
$tRNA^{Asn}$	5 250	5 322	73		GTT			0	L
O_L	5 323	5 358	36					0	–
$tRNA^{Cys}$	5 359	5 425	67		GCA			0	L
$tRNA^{Tyr}$	5 426	5 496	71		GTA			1	L
COXI	5 498	7 048	1 551	516		GTG	TAA	1	H
$tRNA^{Ser(UCN)}$	7 050	7 120	71		TGA			3	L
$tRNA^{Asp}$	7 124	7 196	73		GTC			8	H
COXII	7 205	7 895	691	230		ATG	T––	0	H
$tRNA^{Lys}$	7 896	7 969	74		TTT			1	H
ATP8	7 971	8 138	168	55		ATG	TAA	–10	H
ATP6	8 129	8 812	684	227		CTG	TAA	–1	H
COXIII	8 812	9 596	785	261		ATG	TA–	0	H
$tRNA^{Gly}$	9 597	9 668	72		TCC			0	H
ND3	9 669	10 017	349	116		ATA	T––	0	H
$tRNA^{Arg}$	10 018	10 086	69		TCG			0	H
ND4L	10 087	10 383	297	98		ATG	TAA	–7	H
ND4	10 377	11 757	1 381	460		GTG	T––	0	H
$tRNA^{His}$	11 758	11 827	70		GTG			0	H
$tRNA^{Ser(AGY)}$	11 828	11 899	70		TGA			7	H
$tRNA^{Leu(CUN)}$	11 907	11 979	73		TAG			0	H
ND5	11 980	13 818	1 839	612		ATG	TAA	–4	H
ND6	13 815	14 336	522	173		ATG	TAA	0	L
$tRNA^{Glu}$	14 337	14 406	70		TTC			7	L
Cytb	14 414	15 554	1 141	380		ATG	T––	0	H
$tRNA^{Thr}$	15 555	15 628	74		TGT			0	H
$tRNA^{Pro}$	15 629	15 698	70		TGG			0	L
D–loop	15 699	16 695	997						–

NA^{Cys} to 76 bp in $tRNA^{Leu\ (UUR)}$, and were interspersed across the mitogenome. Of the 22 tRNA genes, 21 were predicted to fold into the typical cloverleaf secondary structures. However, $tRNA^{Ser\ (AGY)}$ lacked the DHU loop and was predicted using comparative genomics (Fig. 3). Among tRNAs, eight were encoded on the L-strand, and the rest of were encoded on the H-strand. The small subunit of 12S rRNA gene was 953 bp in length, and was located between $tRNA^{Phe}$ and $tRNA^{Val}$. The length and position of the 12S rRNA were consistent with the common vertebrate arrangement. The large subunit of the 16S rRNA gene was 1 706 bp in length and was located between $tRNA^{Val}$ and $tRNA^{Leu\ (UUR)}$, similar to other vertebrates. We found that the lengths of 12S rRNA ranged from 940 bp to 961 bp, and the lengths of 16S rRNA ranged from 1 673 bp to 1 840 bp in Epinephelinae species (Table 4).

3.4 *Intergenic spacers and overlapping regions of the mitogenome*

The complete mitogenome contains 9 intergenic spacers that vary from 1 bp to 8 bp, which were 33 bp in total. The largest intergenic spacer was 8 bp (AACCGTCA), and was inserted between $tRNA^{Asp}$ and COXII. The second largest was 7 bp, including 2 fragments (AAACATA, CCTATCA), and were inserted between $tRNA^{Ser\ (AGY)}$ and $tRNA^{Leu\ (CUN)}$, and between $tRNA^{Glu}$ and Cytb, respectively. The remaining spacers were shorter than 4 bp. In addition, a total of 23 bp short overlaps, ranging from 1 bp to 10 bp, were observed in 5 gene junctions. The biggest overlapping sequence was 10 bp (CTGAGTGTAA), which was located between ATP8 and ATP6. The second was 7 bp, and was located between ND4L and ND4.

3.5 *Replication origin of L-strand and control region*

In the EMET mitogenome, there were 2 fragment sequences or non-coding regions, including the replication origin of L-strand and the control region. The L-strand replication sequence was 36 bp, and was between $tRNA^{Asn}$ and $tRNA^{Cys}$. It was located

Table 4. Nucleotide composition of key components in the mitogenome of *Epinephelus*

	Whole				PCGs		rrnS		rrnL		DL	
	Length /bp	AT /%	AT-skew	GC-skew	Length /bp	AT /%	Length /bp	AT /%	Length /bp	AT /%	Length /bp	AT /%
Epinephelus moara×*Epinephelus tukula*	16 695	55.0	0.04	–0.28	11 428	54.3	953	51.1	1 706	53.7	997	66.9
Aethaloperca rogaa	16 538	56.7	0.04	–0.27	11 431	56.3	953	52.7	1 711	55.4	830	68.6
Anyperodon leucogrammicus	16 616	55.7	0.03	–0.29	11 428	54.9	953	51.3	1 706	54.6	916	69.8
Cephalopholis argus	16 767	56.9	0.03	–0.25	11 430	56.7	956	54.2	1 722	55.3	813	62.2
Cephalopholis sonnerati	16 587	55.8	0.06	–0.28	11 429	55.5	957	53.0	1 713	54.2	878	65.5
Cromileptes altivelis	16 497	55.3	0.05	–0.23	11 428	54.8	952	50.6	1 706	53.9	799	67.8
Diploprion bifasciatum	16 805	54.0	0.05	–0.26	11 439	53.3	954	51.7	1 705	55.4	843	63.2
Epinephelus moara	16 696	55.1	0.04	–0.29	11 428	54.3	953	51.1	1 706	53.7	999	67.3
Epinephelus moara×*Epinephelus lanceolatus*	16 866	55.3	0.04	–0.28	11 428	54.3	953	51.1	1 707	53.7	1 167	68.4
Epinephelus akaara	16 795	56.0	0.03	–0.27	11 429	55.1	953	52.3	1 708	54.6	1 093	70.5
Epinephelus areolatus	16 893	55.6	0.03	–0.26	11 429	54.6	953	51.7	1 706	54.9	1 191	69.4
Epinephelus awoara	16 802	55.8	0.02	–0.25	11 429	54.8	953	52.2	1 706	54.9	1 102	69.4
Epinephelus bleekeri	17 227	55.0	0.04	–0.28	11 429	54.3	955	51.4	1 704	54.4	900	67.1
Epinephelus bruneus	16 692	55.1	0.03	–0.29	11 428	54.4	953	50.7	1 704	53.7	995	66.9
Epinephelus fuscoguttatus	16 648	56.1	0.04	–0.29	11 428	55.6	952	52.3	1 705	53.8	948	69.2
Epinephelus fuscoguttatus×*Epinephelus lanceolatus*	16 644	56.1	0.04	–0.29	11 428	55.6	953	52.1	1 705	53.7	947	69.3
Epinephelus coioides	16 418	55.2	0.04	–0.29	11 425	54.9	954	51.9	1 705	54.4	720	65.3
Epinephelus coioides×*Epinephelus akaara*	16 458	55.3	0.03	–0.29	11 425	55.0	953	52.1	1 706	54.2	760	66.6
Epinephelus lanceolatus	16 642	56.1	0.06	–0.31	11 428	55.3	953	52.2	1 705	54.6	940	70.4
Epinephelus malabaricus	16 423	55.2	0.04	–0.29	11 430	54.9	954	51.9	1 706	54.6	720	65.3
Epinephelus fasciatomaculosus	16 682	55.9	0.03	–0.26	11 431	54.8	953	52.9	1 707	55.3	980	71.0
Epinephelus epistictus	16 920	55.4	0.04	–0.28	11 429	54.2	953	51.6	1 709	54.6	1 217	69.9
Epinephelus quoyanus	16 797	56.5	0.03	–0.27	11 429	56.1	954	52.0	1 708	54.6	1 093	69.0
Epinephelus sexfasciatus	16 786	55.6	0.02	–0.26	11 429	54.6	950	52.9	1 708	54.5	1 090	70.8
Epinephelus stictus	16 524	55.5	0.03	–0.27	11 429	54.8	952	52.4	1 705	55.0	824	70.3
Epinephelus tukula	16 503	54.7	0.04	–0.28	11 428	53.9	952	50.0	1 705	54.1	804	70.1
Hypoplectrus gemma	16 911	57.5	–0.04	–0.21	11 448	56.8	947	53.4	1 693	57.4	868	69.2
Hyporthodus octofasciatus	16 545	56.0	0.02	–0.26	11 429	55.5	953	51.7	1 708	54.6	840	70.0
Grammistes sexlineatus	16 506	52.4	0.10	–0.31	11 427	51.3	954	51.4	1 695	52.9	820	64.6
Macropodus opercularis	16 496	60.5	0.02	–0.25	11 436	61.1	948	56.2	1 673	58.3	830	66.9
Plectropomus areolatus	16 770	56.4	0.02	–0.25	11 429	55.9	952	53.6	1 709	56.0	1 080	65.4
Plectropomus leopardus	16 714	56.8	0.02	–0.25	11 413	56.3	951	53.4	1 691	56.0	1 065	66.5
Variola albimarginata	16 768	56.1	0.01	–0.26	11 428	56.4	959	51.2	1 695	54.0	979	61.1
Variola louti	16 770	56.2	0.01	–0.26	11 428	56.2	961	52.7	1 696	53.6	975	62.9
Triso dermopterus	16 605	53.9	0.05	–0.28	11 429	52.4	954	51.3	1 708	55.6	901	69.7
Pseudanthias dispar	16 954	55.0	–0.01	–0.22	11 427	54.2	940	53.0	1 840	55.8	995	63.6
Average	16 693	55.7	0.032	–0.27	11 429	55.1	953	52.2	1 708	54.7	941	67.5

in a typical cluster of 5 tRNA genes known as the WANCY region. This sequence was predicted to fold into a hairpin structure, consisting of a 28 bp stem and 8 bp loop (Fig. 4), forming a signal to initiate the replication of the L-strand.

The control region was 997 bp long, and was between $tRNA^{Pro}$ and $tRNA^{Phe}$. The AT content of the control region was 66.9%, which was little lower than the average (67.5%), falling between 61.1% in *Variola albimarginata* and 71.0% in *E. fasciatomaculosus* (Table 4). The grouper control region consists of several terminaiton-associated sequences (TAS) comprising the extended termination-associated sequences (ETAS) region. In addition, the control region of the *E. moara* (♀)×*E. lanceolatus* (♂) mitogenome has 170 bases more than that of *E. moara* (♀)×*E. tukula* (♂), and the 170 base sequence contains 10 repeatitive elements and the repeat unit is 17 bases (TATTACATATATGCTGA).

3.6 *Maternal inheritance*

Comparisons of the mitogenome between the hybrid EMET and its parents *E. moara* (♀) and *E. tukula* (♂) were conducted. EMET's mitogenome was 16 695 bp in length, whereas *E. moara* and *E. tukula* were 16 696 bp and 16 503 bp, respectively (Table 4). The mitogenome of EMET shared 99% sequence identify with *E. moara*, and 91% sequence identify with *E. tukula*. The PCGs of the mitogenome of the hybrid, *E. moara* and *E. tukula* shared the same length (11 428 bp). The main difference of mitogenome in length among the hybrid, *E. moara* and *E. tukula* were the length of control region. Compared with *E. moara*, EMET had lost 1 base, and had a 28 base substitution. All base substitution were purines replaced by the other purine (A/G), or pyrimidine replaced by the other pyrimidine (C/T). In addition, of the 28 base substitutions, 19, 6 and 3 were found in PCGs, control regions and tRNA.

3.7 *Phylogenetic analysis*

To examine the evolutionary relationship of the hydrid EMET to other Epinephelinae species, a phylogenetic analysis was con-

Table 5. Nucleotide composition and skews of the *Epinephelus moara* (♀)×*Epinephelus tukula* (♂) mitochondrial protein-coding and ribosomal RNA genes

Gene	Proportion of nucleotides/%				(A+T)/%	AT-skew	GC-skew	(A+C)/%	(G+T)/%
	A	C	G	T					
12S rRNA	29.38	26.97	21.93	21.72	51.10	0.15	–0.10	56.35	43.65
16S rRNA	32.65	25.26	21.04	21.04	53.69	0.22	–0.09	57.91	42.08
ND1	26.05	33.23	14.15	26.56	52.61	–0.01	–0.40	59.28	40.71
ND2	29.38	33.97	12.06	24.59	53.97	0.09	–0.48	63.35	36.65
COXI	25.34	27.92	17.34	29.40	54.74	–0.07	–0.23	53.26	46.74
COXII	30.10	27.79	15.48	26.63	56.73	0.06	–0.28	57.89	42.11
ATP8	27.38	33.93	12.50	26.19	53.57	0.02	–0.46	61.31	38.69
ATP6	27.92	30.99	12.57	28.51	56.43	–0.01	–0.42	58.91	41.08
COXIII	25.73	29.30	16.43	28.54	54.27	–0.05	–0.28	55.03	44.97
ND3	22.06	32.66	14.90	30.37	52.43	–0.16	–0.37	54.72	45.27
ND4L	21.89	36.70	15.48	25.93	47.82	–0.08	–0.41	58.59	41.41
ND4	26.94	32.37	14.12	26.57	53.51	0.01	–0.39	59.31	40.69
ND5	28.06	31.32	13.27	27.35	55.41	0.01	–0.40	59.38	40.62
ND6	15.90	14.94	31.42	37.74	53.64	–0.41	0.36	30.84	69.16
Cytb	24.89	30.67	14.81	39.62	64.51	–0.23	–0.35	55.56	54.43
Total	28.58	28.87	16.09	26.46	55.04	0.04	–0.28	57.45	42.55

Table 6. Codon usage of PCGs in the *Epinephelus moara* (♀)×*Epinephelus tukula* (♂) mitogenome

AA	Codon	Count	Proportion/%	RSCU	AA	Codon	Count	Proportion/%	RSCU
Ala(A)	GCU	62	1.63	0.71	Pro(P)	CCU	53	1.39	0.96
	GCC	154	4.05	1.77		CCC	105	2.76	1.90
	GCA	116	3.05	1.33		CCA	56	1.47	1.01
	GCG	16	0.42	0.18		CCG	7	0.18	0.13
Cys(C)	UGU	12	0.32	0.77	Gln(Q)	CAA	83	2.18	1.75
	UGC	19	0.50	1.23		CAG	12	0.32	0.25
Asp(D)	GAU	20	0.53	0.51	Arg(R)	CGU	8	0.21	0.41
	GAC	58	1.53	1.49		CGC	20	0.53	1.03
Glu(E)	GAA	79	2.08	1.65		CGA	45	1.18	2.31
	GAG	17	0.45	0.35		CGG	5	0.13	0.26
Phe(F)	UUU	105	2.76	0.87	Ser1(S1)	AGU	9	0.24	0.22
	UUC	136	3.58	1.13		AGC	45	1.18	1.12
Gly(G)	GGU	42	1.11	0.70	Ser2(S2)	UCU	51	1.34	1.26
	GGC	82	2.16	1.37		UCC	70	1.84	1.74
	GGA	80	2.11	1.33		UCA	60	1.58	1.49
	GGG	36	0.95	0.6		UCG	7	0.18	0.17
His(H)	CAU	24	0.63	0.44	Thr(T)	ACU	47	1.24	0.62
	CAC	85	2.24	1.56		ACC	118	3.11	1.56
Ile(L)	AUU	140	3.68	1.02		ACA	129	3.39	1.70
	AUC	134	3.53	0.98		ACG	9	0.24	0.12
Lys(K)	AAA	70	1.84	1.82	Val(V)	GUU	61	1.61	1.15
	AAG	7	0.18	0.18		GUC	50	1.32	0.94
Leu1(L1)	CUU	134	3.53	1.22		GUA	82	2.16	1.54
	CUC	152	4.00	1.38		GUG	20	0.53	0.38
	CUA	203	5.34	1.85	Trp(W)	UGA	106	2.79	1.78
	CUG	39	1.03	0.36		UGG	13	0.34	0.22
Leu1(L2)	UUA	113	2.97	1.03	Try(Y)	UAU	31	0.82	0.56
	UUG	18	0.47	0.16		UAC	80	2.11	1.44
Met(M)	AUA	106	2.79	1.42	Stop	AGA	0	0	0
	AUG	43	1.13	0.58		AGG	0	0	0
Asn(N)	AAU	38	1.00	0.66		UAA	0	0	0
	AAC	78	2.05	1.34		UAG	0	0	0

Note: RSCU represents synonymous codon usage.

ducted using the nucleotide sequence from the conserved 13 PCGs of 35 Epinephelinae species, including 3 hybrid grouper individuals (Table 2). A similar topology were observed through both ML and NJ methods (Fig. 5), and the approximate values of

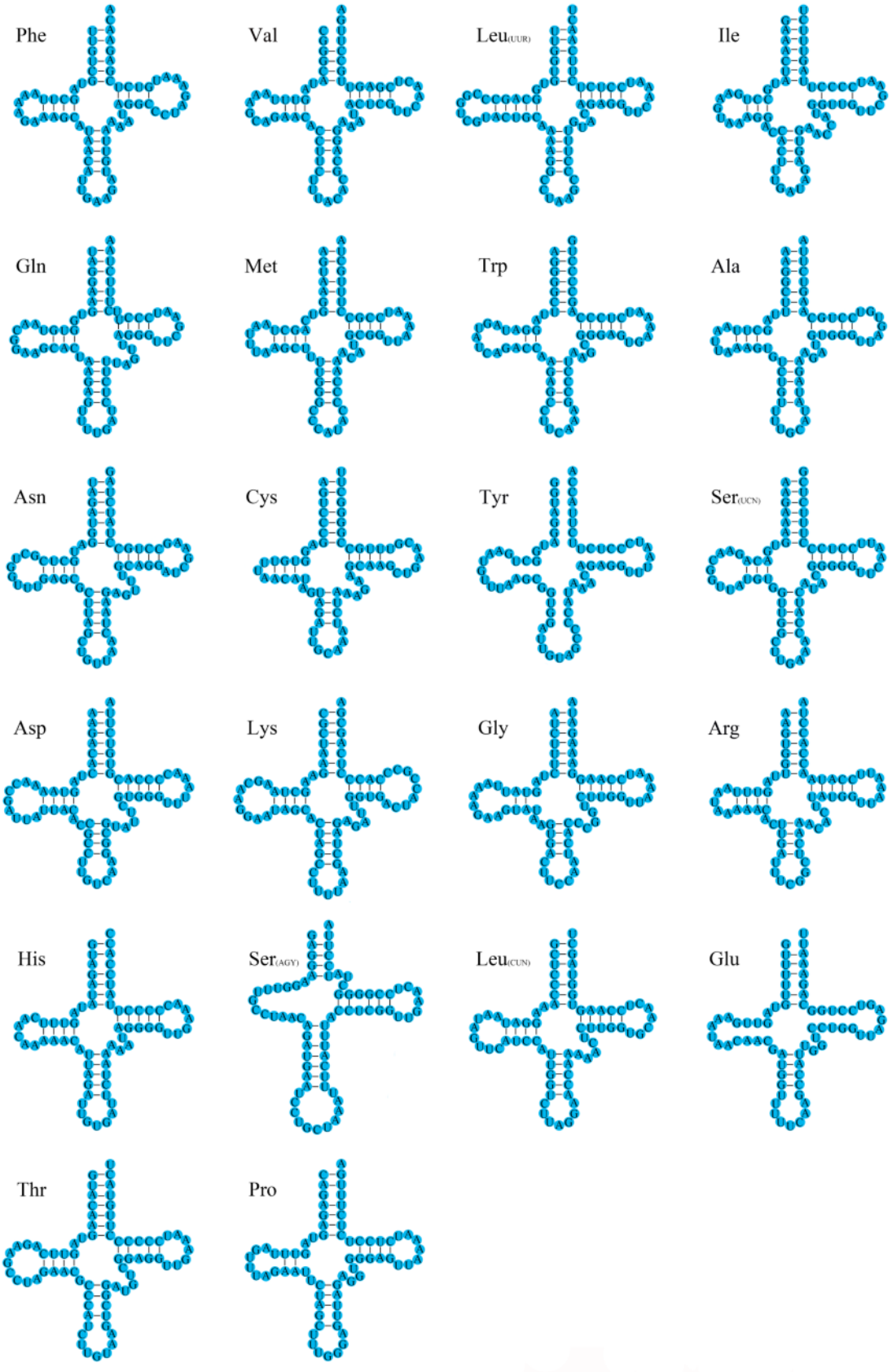

Fig. 3. Predicted secondary structures of tRNA in the *Epinephelus moara* (♀)×*Epinephelus tukula* (♂) mitogenome.

the nodes were similar. The 36 species from 14 genera were clustered into 6 different groups with high bootstrap resampling support. Interestingly, Group 1 consisted of 24 species, 20 belonging to the genus *Epinephlus* and 4 belonging to the genus

Cromileptes, *Anyperodon*, *Hyporthodus* and *Triso*, and this pattern was not consistent with conventional morphology-based classification of Epinephelinae species. Group 2 contained 2 species from the genus *Cephalopholis* and 1 from the genus *Aethaloperca*. Group 3 consisted of 2 species from the genus *Variola*. Group 4 consisted of 2 species from the genus *Plectropomus*. Group 5 contained 1 species from the genus *Diploprion* and 1 from *Grammistes*. Group 6 contained 2 different species from the genus *Hypoplectrus* and *Macropodus*. EMET was sister to *E. moara*×*E. lanceolatus*, and then most closely related to *E. moara* (100% bootstrap value), and these results support that the mitogenome was inherited maternally.

4 Discussion

The complete mitogenome of 1 hybrid grouper species, *E. moara* (♀)×*E. tukula* (♂), was determined in this study. The complete mitogenome of EMET was 16 695 bp in length, containing the typical 37 genes: 13 PCGs, 22 tRNAs, 2 ribosomal RNAs (12S rRNA and 16S rRNA), D-loop and an initiation site for transcription and replication. The gene content and mitogenome structure were conserved and in accordance with other vertebrates (Zhu and Yue, 2008; Zhuang et al., 2013).

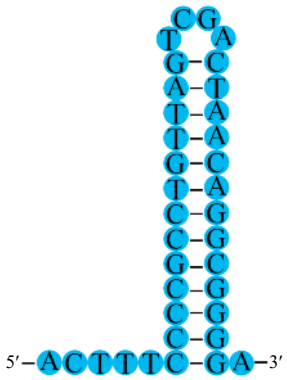

Fig. 4. Stem and loop structure of the OL in the *Epinephelus moara* (♀)×*Epinephelus tukula* (♂) mitogenome.

By comparing the mitochondrial genomes of 36 fish species, we found that there are some difference among them in length of complete mitogenome. The length of control region in Epinephelinae species ranged from 720 bp in *E. coioides* and *E. malabaricus* to 1 217 bp in *E. epistictus*, intergenic regions and gene overlaps distributed in mitogenome also plays a role in the length difference of the mitogenome. Variations in the length of the control region, the length of intergenic regions and gene overlaps were accountable to this phenomenon (Moritz et al., 1987). The control region also had several conserved structures, such as a repeat array, which is considered to be the putative control region (Moritz et al., 1987). The length of the control region depends on the length and repetition of the basic repeating units of this region. Some studies have concluded that control regions are associated with the sequences that form the stem and loop structure (Stanton et al., 1994). Interestingly, both samples of the EMET and *E. moara*×*E. lanceolatus* shared the same female parent, *E. moara*, there were differences in the control region of EMET, *E. moara*×*E. lanceolatus* and *E. moara*. One explanation about the variation in control region is DNA mismatch due to DNA secondary structure formation during mitochondria DNA replication (Brown et al., 1992; Cheng et al., 2015). The other explanation is slipped strand mispairing, illegitimate elongation and replication based on termination-associated sequence taken place at control region (Ludwig et al., 2000). The further study is needed to fully understand the mechanisms that give rise to this variability.

As is well-known, mitochondria are generally inherited from maternal. However, different degrees of paternal genetic phe-

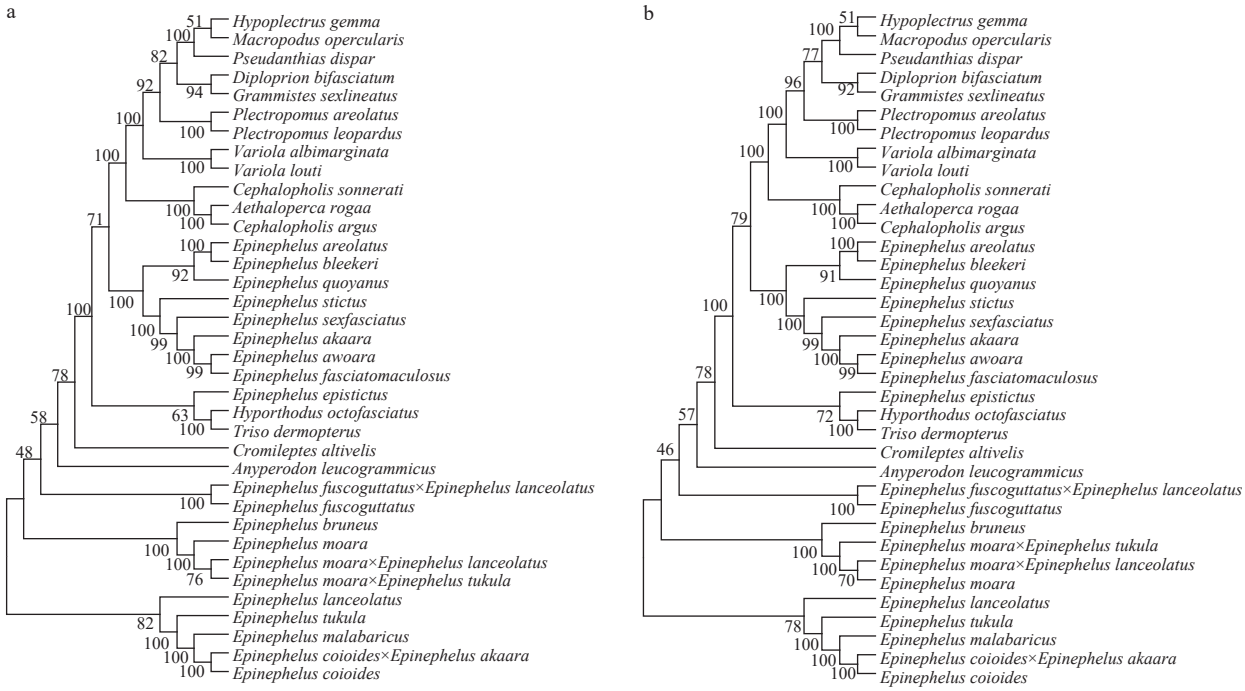

Fig. 5. Phylogenetic relationship of *E. moara* (♀)×*E. tukula* (♂) and other Epinephelinae species. The Maximum Likelihood tree (a) and Neighbor-Joining tree (b) was constructed with MEGA 7.0 using concatenated amino acids sequences of 13 protein-coding genes in the mitogenome. Numbers on each node are bootstrap values of 1 000 replicates.

nomena about mitochondria were found on a few creatures in many studies (Hoarau et al., 2002; Meusel and Moritz, 1993; Zhao et al., 2004), they provides some evidence for recombination in the mitogenome. In addition, mitogenome has both maternal genetic phenomena and special cases of recombination or introgression of paternal inheritance in hybridization of fish (Guo et al., 2004). Due to the existence of mtDNA paternal inheritance, the paternal genetic effects of mtDNA should be considered when conducting such study related to evolution, classification, disease and animal economic traits. In this study, there were 29 differences in the mitogenome of the hybrid and *E. moara*, and their distribution were scattered, which was the level of intraspecific variation. So it was not caused by the recombination of the paternal parent (Zhou et al., 2012). It was certain that the mitogenome of the hybrid follow strictly the laws of maternal inheritance.

With the application of mitochondrial and nuclear genes in phylogenetic, traditional views of systematics are experiencing challenges (Craig and Hastings, 2007). In our study, we performed ML and NJ analyses using the concatenated nucleotide sequences of the 13 PCGs, both phylogenetic analyses yielded congruent tree topologies with strong support on all nodes of concern. The trees constructed in this study are quite similar to the one reported by Gao et al. (2017). EMET clusters together with *E. moara*×*E. lanceolatus*, *E. moara*, and they together with other *Epinephelus*. The genus *Variola*, *Plectropomus*, *Cephalopholis* and *Epinephelus* are independent of each other, indicating that the molecular phylogeny is in good consistent with the traditionl systematics. There are, of course, examples of inconsistencies. Four species *Anyperodon leucogrammicus*, *Cromileptes altivelis*, *Hyporthodus octofasciatus*, and *Triso dermopterus* were a cluster with the species from the *Epinephelus*. These data may indicate that these 4 species should be classified into the genus *Epinephelus*, and similar evidence have been supported by other studies (Craig et al., 2001; Ding et al., 2006; Zhu and Yue, 2008). Although some monotypic species have their unique morphological features, the taxonomic revision to synonymize *Aethaloperca* within genus *Cephalopholis*, and *Grammistes* within genus *Diploprion* (Craig and Hastings, 2007; Gao et al., 2017), and this is corroborated by our phylogenetic analyses. We found that *Macropodus opercularis* has closed relationships with *Hypoplectrus gemma*, which suggests they diverged relatively later. In addition, mitogenome exhibits limitations in resolving complicated phylogenetic relationships in many fish lineages (Stepien and Kocher, 1997). So, more genetic data are necessary to fully elucidate the phylogenetic relationships among Epinephelinae fishes.

5 Conclusion

In this study, the complete mitogenome of the hybrid *E. moara* (♀)×*E. tukula* (♂) was 16 695 bp in length, which contained 13 PCGs, 2 rRNA genes, 22 tRNA genes, a replication origin and a control region, which was reported the first time. Phylogenetic analysis based on the sequence of 13 PCGs of 36 species by using ML and NJ methods, which indicated that the mitogenome was inherited maternally in the hybrid individuals, and there were some differences between molecular phylogeny and traditionl systematics. Thus, more mitogenome and further analyses are in urgent need to elaborate the phylogenetic relationship of Epinephelinae fishes.

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