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RESEARCH ARTICLE

Molecular phylogenetic relationship of *Epinephelus* based on sequences of mtDNA *Cty b*

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Abstract The mtDNA *Cyt b* gene was sequenced partially for *Variola louti* of Serranidae, Epinephelinae and seven endemic species of groupers—*Epinephelus awoara*, *E. brunneus*, *E. coioides*, *E. longispinis*, *E. sexfasciatus*, *E. spilotoceps* and *E. tauvina* in China. The seven endemic species and other seven foreign species of groupers—*E. aeneus*, *E. caninus*, *E. drummondhayi*, *E. haifensis*, *E. labriformis*, *E. marginatus* and *E. multinotatus* from the GenBank were combined and analysed as ingroup, while *Variola louti* was used as outgroup. We compared the 420 bp sequences of *Cyt b* among the 15 species and constructed two types of molecular phylogenetic trees with maximum parsimony method (MP) and neighbor-joining method (NJ) respectively. The results were as follows: (1) As to the base composition of mtDNA *Cyt b* sequence (402 bp) of 14 species of *Epinephelus*, the content of (A + T) was 53.6%, higher than that of (G + C) (46.4%). The transition/transversion ratio was 4.78 with no mutation saturation. (2) The cluster relationships between *E. awoara* and *E. sexfasciatus*, *E. coioides* and *E. tauvina*, *E. longispinis* and *E. spilotoceps* were consistent with phenotypes in taxonomy. (3) In the phylogenetic tree, the species in the Atlantic Ocean were associated closely with those in the Pacific Ocean, which suggested that the *Cyt b* sequences of *Epinephelus* were highly conserved. This may be attributed to the coordinate evolution. (4) In wel1-bred mating or heredity management, mating *Epinephelus* of the same branch should be avoided. It is likely to be an effective way to mate the species of the Atlantic Ocean with those of the Pacific Ocean to improve the inheritance species.

Keywords Cytochrome *b*, *Epinephelus*, molecular phylogenetic relationships

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1 Introduction

The genus *Epinephelus* comprises approximately 100 species of perciform fishes worldwide commonly known as groupers. In China, the genus *Epinephelus* includes 36 species and distributes primarily in the South China Sea (Cheng and Zheng, 1987). There were discrepancies in the classification of *Epinephelus* in the academia. Scholars have disagreements to the reports on the classification of *Epinephelus* species (Shen, 1986; Cheng and Zheng, 1987; Heemstra and Randall, 1993; Huang, 1994; Wu et al., 1999; Chen and Chen, 2002). The morphological characteristics such as color, body and pattern (typically used to identify individual species in the field) have obvious limits, which lead to confusion in many species within *Epinephelus*. For example, people often confuse *E. awoara*, *E. coioides*, *E. sexfasciatus* and *E. tauvina* with each other, all of which were commonly named ''blue grouper''. But presently, little is known about molecular studies to reveal the difference within *Epinephelus* (Gilles et al., 2000; Maggio et al., 2005).

The cytochrome *b* gene of mtDNA, a useful molecular marker for biologic evolution researches, has been widely used for the research of fishery phylogenetic relationships between genus and species, which has solved some classification and phylogenetic problems (Peng et al., 2002; Tang et al., 2003; He et al., 2004; Xiang et al., 2004). Owing to the reasons above, we determined the *Cyt b* sequences of 7 species of *Epinephelus* including *Epinephelus awoara*, *E. brunneus*, *E. coioides*, *E. longispinis*, *E. sexfasciatus*, *E. spilotoceps* and *E. tauvina*, which live in South China Sea, and *Variola louti* which belongs to the genus *Variola* of the subfamily Epinephelinae. The seven species and other seven foreign species of groupers whose mtDNA *Cyt b* sequences were downloaded from the GenBank, including *E. aeneus*, *E. caninus*, *E. drummondhayi*, *E. haifensis*, *E. labriformis*, *E. marginatus* and *E. multinotatus*, were combined and designed as ingroup, and *Variola louti* was used as

outgroup. In this study, to understand the phylogenetic relationships in the genus *Epinephelus*, the partial *Cyt b* sequences were analysed, compared and made into molecular phylogenetic trees. It was expected to get more molecular biologic information for well-bred selection and hybridization in practice.

2 Materials and methods

2.1 Materials

The seven specimens of *Epinephelus awoara*, *E. brunneus*, *E. coioides*, *E. longispinis*, *E. sexfasciatus*, *E. spilotoceps and E. tauvina* were collected from South China Sea and northern East China Sea. The mtDNA *Cyt b* partial sequences of other seven foreign species of groupers, including *E. aeneus* (GenBank accession No. AJ420206), *E. caninus* (GenBank accession No. ECA420204), *E. drummondhayi* (GenBank accession No. AY313997), *E. haifensis* (GenBank accession No. AJ420207), *E. labriformis* (GenBank accession No. AY426255), *E. marginatus* (GenBank accession No. AB179760) and *E. multinotatus* (GenBank accession No. AY426254) from the GenBank were combined and analysed as ingroup, and *Variola louti* of Epinephelinae was used as outgroup.

2.2 DNA extraction and PCR

Genomic DNA was isolated from 0.1 g of muscle tissue, which was kept in 95% ethanol. The tissue samples were washed by distilled water and STE buffer (0.1 mol/L NaCl, 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH8.0) until the ethanol in muscle tissue was eliminated, then were digested in prewarmed (55°C) buffer (900 μL STE, 90 μL 10%SDS, 10 μL 20 mg/mL proteinase K) until the tissue was dissolved completely. The genomic DNA was extracted by using a phenol–chloroform method and kept at −20°C.

Partial mitochondrial DNA *Cyt b* sequence were amplified by PCR with two primers, CGA ACG TTG ATA TGA AAA ACC ATC GTT G and AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A (Cantatore et al., 1994) . PCR was carried out in 50 μL reaction mixtures containing 39.5 μL sterile distilled water, 5 μL 10× EX PCR buffer (TaKaRa), 2 μL dNTP (2.5 mM), 1 μL of each primer (20 μM), 0.5 μL EX *Taq* polymerase (2U), and 1 μL of template (100 ng DNA). PCR cycling was performed in a Perkin-Elmer Model 9700 thermal cycler with 35 cycles: initial denaturation at 94°C for 4 min, denaturation (94°C for 45 s) , annealing (54°C for 45 s), and extension (72°C for 1 min), then a final primer extension step (72°C for 10 min). The PCR products were electrophoresed in a 1.0% agarose gel, stained with ethidium bromide and visualized by ultraviolet transillumination.

PCR products were purified with 3S Spin Agarose Gel DNA Purification Kit (Shenergy Biocolor, Shanghai), then ligated into the pUCm-T vectors and cloned using the pUCm-T TA Cloning Kit (TaKaRa) according to the manufacturer's instructions. The clones were transformed in competent cell DH5α to screen out the positive clones, which were amplified cultured. Then the cloned plasmid was extracted. Finally, all samples were sequenced in the double direction with an automated DNA-sequencer (Applied Biosystems 3730, Shanghai invitrogen).

2.3 Sequence analysis

After sequencing, the partial *Cyt b* sequences, where the sequences of two primers had been deleted before the start codon (ATG) at the 5′ end, aligned with the homologous sequences of 7 species of genus *Epinephelus* downloaded from the GenBank. This was performed with the CLUSTAL W program. Nucleotide composition frequencies, Kimura-2 parameter genetic distance, variable sites and transition/transversion ratios were analyzed with the MEGA 3.0 software (Kumar et al., 2004). Molecular phylogenetic trees were constructed by Most parsimony method (MP) and Neighbor joining method (NJ), which were performed using PAUP 4.0b 10. Heuristic most parsimony (MP) analyses were conducted with tree bisection-reconnection branch swapping. Support for internal branches was assessed using 1000 bootstrap replications, all characters were of equal weight. Neighbor joining (NJ) analyses were conducted with the following parameters: substitution model set was all substitution, distance measure was Kimura 2-parameter, support for internal branches was assessed using 1000 bootstrap replications. The values of bootstrap confidence level (BCL) of nodes that are higher than 45% are indicated above the branch.

3 Results

3.1 Sequence characters

The length of *Cyt b* gene segments of 7 endemic species from Genus *Epinephelus* and *Variola louti* which was designed as outgroup were 402 bp, amplified by PCR (Fig. 1), purified, cloned and sequenced, and where the sequences of two primers were deleted before the start codon (ATG) at the 5′ end. The resulting data were combined with the homologous sequences of 7 species of Genus *Epinephelus* downloaded from the GenBank to form the analysis matrix. There were no insertion and deletion. Considering a total of 402 bp for the analysis, 148 bp (36.8%) were variable sites, and 122 bp were parsimony-informative sites, 254 bp were constant sites

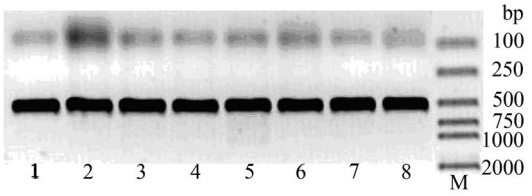

Fig. 1 The PCR products of mtDNA Cty *b* gene segment (1.5% agarose gel, 1 × TAE). 1–7: *E. coioides*, *E. awoara*, *E. sexfasciatus*, *E. brunneus*, *E. longispinis*, *E. spilotoceps*, *E. tauvina*.; 8: *Variola louti*; M: Marker (DL2000).

(Fig. 2). Considering the variable sites, 129 bp were at third codon positions. The frequencies of base composition in *Cyt b* gene segment of *Epinephelus* are shown in Fig. 3. The mean content of T, C, A and G was 29.2%, 30.0%, 24.4%, and 16.4%, respectively. It exhibited a strong bias anti-G. The content of A + T (53.6%) was higher than that of C + G (46.4%). The three codon positions differed greatly in their base composition. The frequencies of base composition were similar at the first codon positions, but at second positions T was 38.8% and at third positions C was 42.2%, G was 5.9%. This model was similar with which of other mammal (Kumar, 2003). The mean transition/transversion ratio was 4.78 (Kimura 2-parameter), which showed that transition was obviously more than transversion and these sites were no substitution saturation.

Except the outgroup, the mean percentage divergence in 14 species from Genus *Epinephelus* was 15.88%. The percentage divergence between *E. coioides* and *E. tauvina* was only 1.00%, which was the lowest. However, the percentage divergence between *E. sexfasciatus* and *E. drummondhayi* was 17.16%, which was the highest. The percentage sequence divergences ranged from 8.96% to 16.92% among other species of *Epinephelus* (Table 1).

3.2 Phylogenetic relationships

3.2.1 Neighbor joining (NJ)

Based on the partial *Cty b* sequences of *Epinephelus* fishes and using *Variola louti* as outgroup, a molecular phylogenetic tree was constructed by Neighbor-Joining method (Kimura 2-parameter). The values of bootstrap confidence level of nodes were indicated above the branch (Fig. 4). The figure showed that all species of the genus *Epinephelus* in this study produced one monophyletic tribe. The cluster relationship between *E. awoara* and *E. sexfasciatus*, *E. caninus* and *E. marginatus* was close, so the 4 species were clustered to sisters. *E. tauvina*, *E. coioides* and *E. brunneus* were clustered to sisters. Furthermore, *E. drummondhayi* and *E. haifensis*, *E. longispinis* and *E. spilotoceps* also were close. The relationship was highly supported by bootstrap (more than 70%).

3.2.2 Most parsimony (MP)

Unweighted MP analysis produced a single tree with the tree length = 469, consistency index (CI) = 0.4670, retention index (RI) = 0.3573. The MP tree presented a similar topological structure with the NJ tree, except the different positions of *E. multinotatus* and *E. aeneus* in two trees. The relationship with the values of bootstrap confidence level was higher than 45% in the MP tree and was identical with the NJ tree.

4 Discussion

The groupers belong to fish of protogynous hermaphrodite and undergo sex reversal from female to male, which can be different from other genera of family Serranidae. At the same time, the interspecific fishes of *Epinephelus* had difficulty in morphologically distinguishing, especially at the larval period, which was more difficult. Thus, synonymic phenomena often occur (Felsenstein, 1985). This paper used 7 species of groupers (*Epinephelus awoara*, *E. brunneus*, *E. coioides*, *E. longispinis*, *E. sexfasciatus*, *E. spilotoceps* and *E. tauvina*), which mainly distributed in broad water areas of the western Pacific Ocean, northern Indian Ocean, and China Sea, primarily South China Sea. Whereas, the other 7 groupers downloaded from the GenBank (*E. aeneus*, *E. caninus*, *E. drummondhayi*, *E. haifensis*, *E. labriformis*, *E. marginatus* and *E. muhinotatus*) mainly distributed in the eastern Pacific Ocean, western Africa inshore of the Atlantic Ocean and Mediterranean Sea, no record was in China. The samples in the experiment were purchased at fishery ports or rafts. Because the Chinese ocean environment has been grievously destroyed, the natural seedings were quite limited in restricted sea areas (mostly southern North Bay and Hainan). We could not directly obtain samples that were captured from diverse sea areas. To some extent, the determined results reflect the phylogenetic relationships of 14 species of groupers at the molecular level. The NJ and MP trees, which were tested according to *Cyt b* partial sequence, had almost the same topological structure (Fig 4, Fig 5). In the pictures, the similar morphology species clustered respectively, demonstrating that the morphological identification and the results of analysis the *Cyt b* partial sequence were almost same: (1) *Epinephelus awoara* and *E. sexfasciatus* first clustered together. It was difficult to distinguish these two groupers from each other in morphology because the appearances in the larva and juvenile period are identical, adults all have sex distinct bars and similar distribution (Huang, 1994). Nevertheless, there were difference in the adult period: the *E. sexfasciatus*'s bars are almost at equal distance and the soft dorsal fin and caudal fin both have spots;

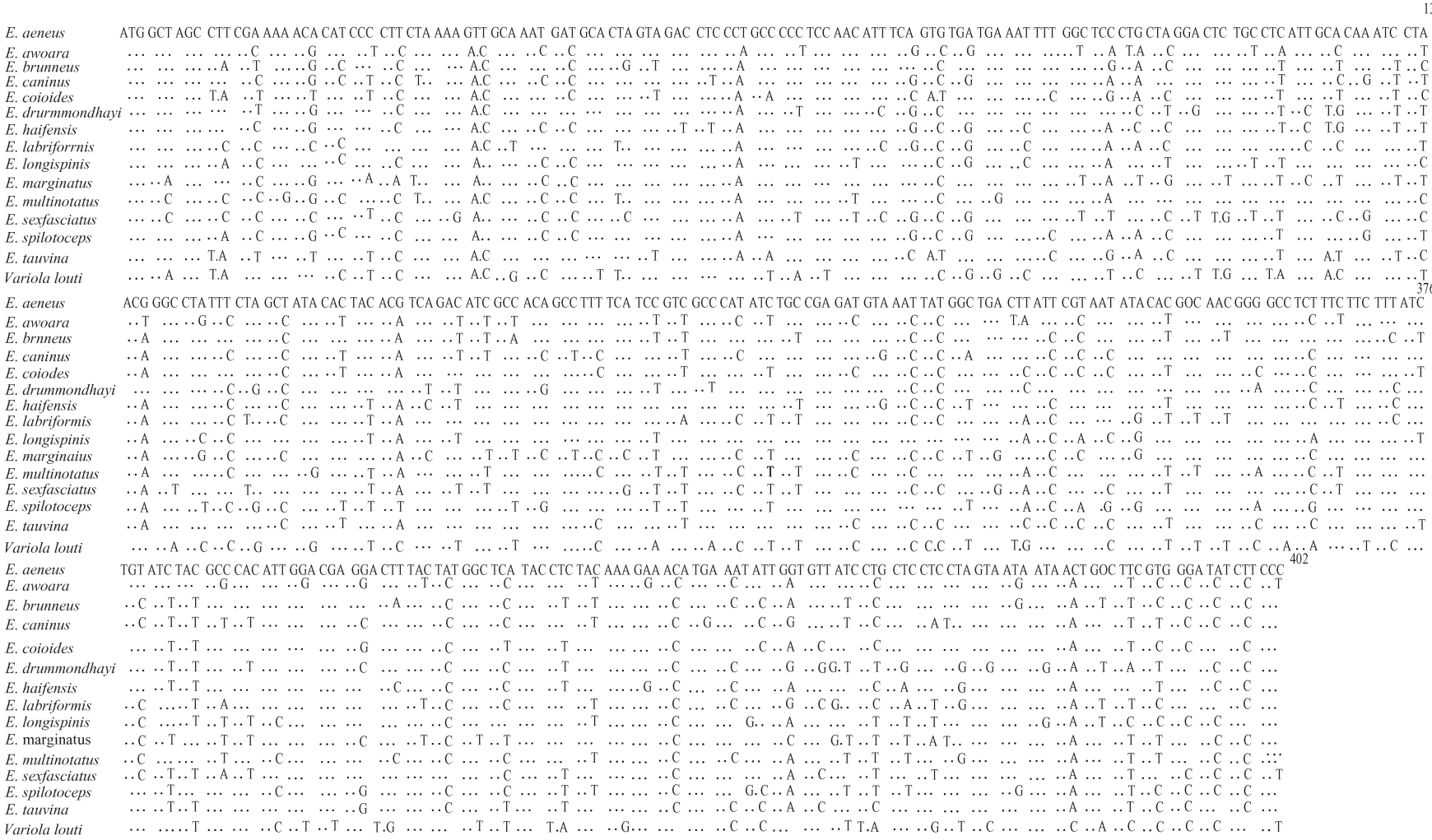

Fig. 2 Alignment of partial *Cyt b* gene sequences

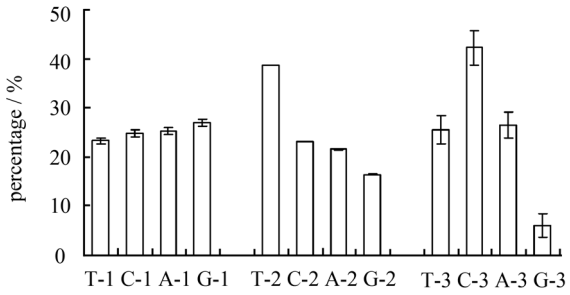

Fig. 3 Frequencies of base composition in *Epinephelus*

Epinephelus awoara have bars of unequal distances, and the distance between the third and fourth stripe is the widest, only the caudal fin has spots (Cheng, 1987). Some scholars thought that these two groupers were synonyms of the same fish (Chen, 2002). We found that although *Epinephelus awoara* and *E. sexfasciatus* first clustered together, but the sequence divergence reached 12.69% they should be considered as two species. (2) *E. coioides* and *E. tauvina* were similar in morphology, clustering together in the phylogenetic tree, larva and juvenile growth process are alike, sequence divergence was only 1%. Some reports mislabeled *E. coioides* to be *E. tauvina* (Heemstra and Randall, 1993), the biggest difference in anatomic structure is that pyloric cecum of *E. coioides* has 50–60 ramus, while that of *E. tauvina* only has 16–18 ramus (Heemstra and Randall, 1993), thus they should be two different species, and cannot be confused. (3) *E. longispinis* (once called as *E. fario* as synonym (Wu et al., 1999)) clustered with *E. spilotoceps*. They are both skewbald, the spots of *E. spilotoceps* are big and dense while that of *E. longispinis* is small and distant. This cluster was same to morphological characteristics.

E. multinotatus was on the root of the NJ tree, while *E. aeneus* was on the root of the MP tree. This was caused by statistical analysis. These two kinds of fish did not distribute in China. The whole result of phylogenetic analysis was consistent: all the species of *Epinephelus* assembled together, forming a monophyletic group, and the outgroup Variola louti was outside. On the upper of the tree, *E. caninus* and *E. marginatus* from the Atlantic, having horizontal stripes, clustered together and then formed a bigger cluster with the cluster of *E. awoara* and *E. sexfasciatus* from the Pacific, having bars too. On the other hand, *E. spilotoceps* and *E. longispinis,* which are from the Indian Ocean and the Pacific Ocean, clustered with species from the Atlantic and sit in the lower of the tree. Some categories in the Atlantic Ocean and some in the Indian Ocean and the Pacific Ocean clustered together, which indicated that the groupers' geographical distribution in the ocean had always been continuous. Today, desultory distribution in certain seas may be caused by human activities or something else which destroyed the tropic and subtropical coral reef environment and made the environment fragmented. Nevertheless, the conservation of the *Cyt b* sequence has been reserved to this day. It also may be caused by coevolution. In addition, the propagation in practice demonstrated that the inter-species distance of *Epinephelus* is very small, especially among *E. awoara*, *E. sexfasciatus*, *E. coioides*, and *E. tauvina*, which have similar morphological characteristics. Moreover, the interspecies hybridization of these fishes was frequent, existing broad gene flow (Felsenstein, 1985). The introgression of geographic inter-groups of some species was more distinct (Gilles et al., 2000). These posed challenges to the idioplasm protection. The question whether there was gene flow of the species distributing in the Pacific Ocean and the Atlantic Ocean after the forming of the two oceans need further study because groupers are reef inhabiting fish.

Table 1 Percentage divergence (below diagonal) and number of transitions/transversions (above diagonal) for partial *cyt b* sequences in *Epinephelus* and the outgroup (*Variola louti*)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1		41/8	42/10	51/9	43/8	41/9	44/10	48/18	37/9	49/13	40/9	48/7	43/15	50/5	54/33
2	12.19		30/6	41/9	45/12	39/9	30/6	40/18	37/9	42/13	35/9	39/11	47/15	45/7	56/33
3	12.94	8.96		51/11	47/12	40/11	4/0	37/20	39/11	46/15	43/11	50/11	46/17	54/9	56/35
4	14.93	12.44	15.42		41/13	39/2	53/11	35/17	42/12	51/14	45/12	44/12	43/18	42/6	54/32
5	12.69	14.18	14.68	13.43		52/13	51/12	50/18	46/13	54/15	44/13	48/9	46/19	46/9	57/33
6	12.44	11.94	12.69	10.2	16.17		44/11	42/19	38/12	46/14	42/12	48/12	40/18	44/6	63/30
7	13.43	8.96	1.00	15.92	15.67	13.68		37/20	41/11	43/15	42/11	50/11	50/17	56/9	54/35
8	16.42	14.43	14.18	12.94	16.92	15.17	14.18		49/17	40/17	40/17	39/21	46/21	47/13	55/31
9	11.44	11.44	12.44	13.43	14.68	12.44	12.94	16.42		45/12	36/10	47/12	35/8	47/6	62/34
10	15.42	13.68	15.17	16.17	17.16	14.93	14.43	14.18	14.18		39/4	47/14	47/14	57/10	55/38
11	12.19	10.95	13.43	14.18	14.18	13.43	13.18	14.18	11.44	10.7		36/10	46/12	44/8	51/36
12	13.68	12.44	15.17	13.93	14.18	14.93	15.17	14.93	14.68	15.17	11.44		48/18	43/8	51/36
13	14.43	15.42	15.67	15.17	16.17	14.43	16.67	16.67	10.7	15.17	14.43	16.42		44/12	67/34
14	13.68	12.94	15.67	11.94	13.68	12.44	16.17	14.93	13.18	16.67	12.94	12.69	13.93		52/32
15	21.64	22.14	22.64	21.39	22.39	23.13	22.14	21.39	23.88	23.13	21.64	21.64	25.12	20.9	

Codes 1–15 denote: *E. awoara*, *E. brunneus*, *E. coioides*, *E. longispinis*, *E. sexfasciatus*, *E. spilotoceps*, *E. tauvina*, *E. aeneus*, *E. caninus*, *E. drummondhayi*, *E. haifensis*, *E. labriformis*, *E. marginatus*, *E. multinotatus*, *Variola louti*

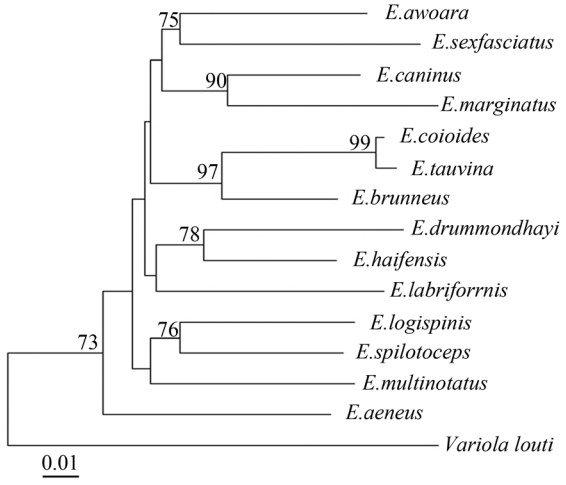

Fig. 4 Molecular phylogenetic tree on the partial *Cytb* sequences of *Epinephelus* constructed by Neighbor-Joining method of PAUP Version 4.0b10 with Bootstrap Test (1000 replications). The values of bootstrap confidence level (BCL) of nodes are indicated above the branch.

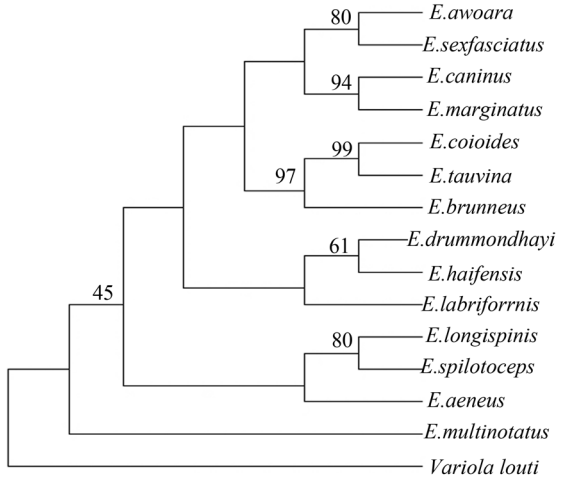

Fig. 5 Molecular phylogenetic tree on the partial *Cytb* sequence of *Epinephelus* constructed by Maximum Parsimony method of PAUP Version 4.0b10 with Bootstrap Test (1000 replications). The values of bootstrap confidence level (BCL) of nodes are indicated above the branch. Tree length = 469; Consistency index (CI) = 0.4670; Retention index (RI) = 0.3573

On the other hand, according to the partial sequence data of the *Cyt b* gene of groupers, people are guided to avoid raising the groupers of the same small clade together as far much as possible. This helps to prevent the heredity variety decline. However, the groupers from different clades can crossbreed to enrich the heredity variety. To recover the endangered groupers of China, such as *E. tauvina*, we should not only recover the natural environment of the coral reef, but should hybridize species that are in different clades of the evolution tree and distribute in Atlantic Ocean (e.g., *E. haifensis*). It may be a better way to improve heredity.

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