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GENETIC DIVERSITY OF THE ORANGE-SPOTTED GROUPER (*Epinephelus coioides*) IN TERENGGANU MALAYSIA BASED ON MITOCHONDRIAL CYTOCHROME B SEQUENCE DATA

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ABSTRACT

Orange-spotted groupers (*Epinephelus coioides*) are marine fish which can be found in the state of Terengganu (in Malaysia) and are economically important. Overfishing and fish farmers' dependency on wild orange-spotted grouper seed fish have caused a decline in their numbers. Hence, the aim of this study was to investigate the genetic diversity of wild orange-spotted grouper populations in Terengganu via partial cytochrome *b* (cyt-*b*) gene analysis. A total of 60 fish, which were reared from wild seed fish, were collected from farms in Besut and Setiu, Terengganu. Their DNA was extracted using the Qiagen Blood and Tissue kit. PCR amplification was conducted using cyt-*b* primers, and a sequence of 460 bp in length was obtained for each sample. Next, phylogenetic analysis was performed to study the relationships among the individuals. The haplotype and nucleotide diversities of the populations were investigated to measure genetic diversity. The haplotype diversity (Hd) of *E. coioides* was relatively low for both Besut (Hd = 0.4161) and Setiu (Hd = 0.8782) populations. The nucleotide diversity (π) for both Besut and Setiu populations was also low. Moreover, 15 haplotypes were identified among the 60 individuals, with 33 individuals sharing a single haplotype (Hap01). These findings similar with a previous study that reported that seed fish dependent can led to a decline in grouper numbers and in turn cause genetic deprivation in the population.

Key words: *Epinephelus coioides*, orange-spotted grouper, partial cyt-*b*, genetic variation, phylogenetic analysis

INTRODUCTION

Orange-spotted groupers (*Epinephelus coioides*) belong to the subfamily Epinephelinae (family Serranidae). They are known to live in areas with coral reefs, but can sometimes be found in brackish waters. Juveniles, in particular, are often found in brackish estuaries. This species covers a large area; from eastern Africa southwards to Durban (South Africa) or beyond, and eastwards to the western Pacific (which ranges from the Ryukyu Islands at its West to Australia or even Palau and Fiji at its East) (Heemstra & Randall, 1993).

Orange-spotted groupers have important economical value in marine fishing and aquaculture industries (Wang *et al.,* 2011). However, according to the International Union for Conservation of Nature (IUCN) Red List 2015, orange-spotted groupers are considered to be "near-threatened" as their numbers have been declining greatly in the past few years due to overfishing and habitat destruction (Wang *et al.,* 2011). The dependency of fish farmers on wild orange-spotted grouper seed fish also contributed to this decline. According to Sadovy de Mitcheson (2000), capturing and growing of wild seed fish accounted for 66 – 80 percent of grouper culture activities, and the number of seed fish caught each year exceeds a few hundred million globally.

Wild seeds are the major source of the supply of grouper seeds for aquaculture in Malaysia (Robert *et al.,* 2002). Most fish farms in Terengganu reared orange-spotted grouper seeds that were caught in wild using traditional fishing traps (temerang in

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Malay) placed approximately 30 metres from the coast line. However, Tupper and Sheriff (2008) reported that capture-based aquaculture is not the best solution to ensure a steady and sustainable supply of groupers due to low availability of seed, destructive and wasteful seed collection techniques, as well as removal of large numbers of young groupers, the last factor of which gives adverse effects to the adult populations. Currently, the genetic diversity studies can provide good insight on the population of fish (Oleksiak, 2010). Therefore, the genetic studies are needed to assess the population condition of *E. coioides*.

In order to study the genetic diversity and population conditions of *E. coioides* obtained from Terengganu, a mitochondrial DNA (mtDNA) molecular study was performed. According to Nguyen (2008), molecular data has been proven to be very useful in defining genetic variations. Studies on genetic variations can lead to the formulation of essential theoretical and practical guidelines for improving breeding programs as genetically-differentiated populations can potentially be used to improve the genetic quality of the fish (Yue *et al.,* 2009).

Experimentally, mtDNA is chosen for intra-specific genetic variation monitoring because multiple copies are present in each cell. Mito-chondrial gene contents are strongly conserved across generations, apart from undergoing very little duplication, having exceptionally short intergenic regions, and posessing no introns (Gissi *et al.,* 2010). At the same time, mtDNA is highly variable in natural populations because it has an elevated mutation rate – a property which can provide evidence regarding the history of tbe population over short time frames.

For example, mtDNA has been used to study the said relationship between Eastern Atlantic groupers of Epinephelus and Mycteroperca genera (Maggio *et al.,* 2006); *E. hexagonatus* and *E. fuscoguttatus* (Baharum & Nurdalila, 2011); as well as *E. septemfasciatus* and 48 other species of the same genus (Guan *et al.,* 2014). It also being used in phylogenetic studies on Malaysian grouper by Rahim *et al*. (2016) and population structure studies of the Indonesian giant tiger shrimp *Penaeus monodon* by Abdul-Aziz *et al.* (2015).

The objectives of this study were to study the genetic diversity of *E. coioides* populations in Terengganu waters and assess their genetic relationship. The information obtained will help in better understanding of the genetic diversity of marine species such as *E. coioides*, especially the species of Southeast Asian waters. It will also provide useful information for fishery management and aquaculture.

MATERIALS AND METHODS

Sample collection

A total of 60 wild orange-spotted grouper juveniles (TLs 7.6-10.2 cm; BWs 300-500 grams) were collected from Besut and Setiu, Terengganu, Malaysia with 30 orange-spotted grouper juveniles in respective place. In this study, an annotated and illustrated FAO species catalogue by Heemstra and Randall (1993) and Fishes of Terengganu East Coast of Malay Peninsula, Malaysia by Matsunuma *et al.* (2011) were used to help identify the fish samples of which the identification was further supported with molecular method.

Specimen preparations

To prepare the specimens, dorsal fin clipped (1 cm^2) from the juvenile orange-spotted grouper and immediately preserved in 95% ethanol at ambient temperature upon sampling and then kept at -80°C until DNA extraction was performed.

DNA extraction

Genomic DNA was extracted using a Blood and Tissue Kit from Qiagen following standard method with some modifications for isolating the DNA from fin samples.

PCR amplification of extracted DNA

Amplification of partial cytochrome *b* gene was done using the universal primers following Gilles *et al.* (2000), which were Cytb28f (5'- CGA ACG TTG ATA TGA AAA ACC ATC GTT G-3') and Cytb34r (5'- AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3'). A 20 µL reaction volume contained 0.4 µL of 5U Taq polymerase, 2 µL of 10× Buffer A, 0.6 µL of 50 µM $MgCl_2$ (Vivantis, Malaysia), 0.4 µL of the 10 µM dNTPs, 1 µL of 20 µM primer and the extracted DNA. The amplification was done in a Thermal Cycler (Eppendorf AG, Germany), with the cycling profile beginning with an initial denaturation at 94°C for 2 min. Amplification was performed as follows for 35 thermal cycles: denaturation for 30 s at 94°C, annealing for 30 s at 48°C, and extension for 30 s at 72°C, followed by a 10 min final extension at 72°C. The amplified product (5 µL) was visualized in 1.2% agarose gel. The purified PCR products were sent to First Base Laboratories Sdn. Bhd. for bi-directional sequencing using similar primers as were used for PCR amplification.

Sequence alignment and phylogenetic analysis

The obtained sequences were edited using a sequence scanner to remove any unwanted sequences, noise and gaps. All sequences were identified using the Basic Local Alignment Search

Tool (BLAST) provided by the National Center of Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). The edited sequences were aligned and compared with the sequence of *Epinephelus coioides* available in the GenBank database using CLUSTAL W (Thompson *et al.,* 1994). Sequences were analysed for pairwise distance and phylogenetic tree was constructed by using MEGA7 software. Maximum-likelihood estimation with 1000 bootstraps was used to determine the genetic relationships among individuals. According to Librado and Rozas (2009), DnaSP (Version 5.1, Universitat de Barcelona) was used to estimate mitochondrial genetic variation, including haplotypic and nucleotide variation. The level of genetic variation within phylogenetic clades was determined with DnaSP.

RESULTS

All 60 wild fishes sampled from Besut and Setiu were successfully sequenced for their partial cytochrome b genes, which had a final sequence length of 460 bp after alignment. All 60 sequences had 99 – 100% similarity with the mtDNA Cyt-b sequence of *E. coioides* in the Genebank Database (Accession No.: DQ448043.1), confirming that all the samples are *E. coioides*.

In total, 15 haplotypes were identified among the 60 fishes in the sample (Table 1). 33 fishes shared a single haplotype (Hap01), which therefore had the highest relative frequency (0.550). Seven haplotypes (Hap09 to Hap15) showed unique nucleotides, with each present in only a single fish (relative frequency: 0.017), all of which were captured from Setiu.

Phylogenetic analysis showed that the 60 fishes were separated into two clades (Figure 1). In the main clade, Hap12, Hap13 and Hap14 were separated and distinct from the other haplotypes in the same clade with a strong bootstrap support. Pair-wise genetic distance values (Tamura-Nei parameter) of the cyt *b* genes of the samples, which ranged from 0.000 to 0.077, are shown in Table 3. The small genetic distance between the samples proves that all of them are nearly identical to one another and have low genetic diversity.

The composite sequences of the haplotypes and nucleotides discovered in the Besut and Setiu populations are shown in Table 2. All 30 wild *E. coioides* from Besut had relatively low haplotype (Hd = 0.4161) and nucleotide (π = 0.02178) diversities (Table 2). The neutrality test, measured via Tajima's D, also gave a low value of -2.80575 ($p > 0.05$) for *E. coioides* from Besut. Likewise, the 30 wild *E. coioides* from Setiu also showed low haplotypic (Hd = 0.8782) and nucleotidic (π = 0.01452) diversities (Table 2).

Table 1. The number of haplotypes, their locality, accession number and relative frequency of partial cytochrome *b* gene for specimens collected used in this study

Samples from Terengganu						Haplotype	Genbank Accession Number	Frequency
Besut			Setiu					
B1	B2	B3	S1	S3	S4	Hap01	AY738240.1 / DQ448044.1	0.550
B4	B5	B6	S5	S6	S7			
B10	B11	B12	S10	S16	S18			
B13	B14	B16	S25					
B17	B18	B19						
B22	B23	B24						
B25	B26	B27						
B29	B30							
B7	B15		S2	S8	S22	Hap02	DQ448044.1	0.083
B8			S9			Hap03	DQ448044.1	0.033
B9			S11	S20		Hap04	DQ448044.1	0.050
B20			S12	S23		Hap05	DQ448044.1	0.050
B21			S13			Hap06	AY738240.1	0.033
B28			S14			Hap07	AY738240.1 / DQ448044.1	0.033
			S15	S24	S26	Hap08	DQ448044.1	0.050
			S17			Hap09	AY738240.1	0.017
			S19			Hap10	DQ448044.1	0.017
			S21			Hap11	DQ448044.1	0.017
			S27			Hap12	DQ448044.1	0.017
			S28			Hap13	AY738240.1	0.017
			S29			Hap14	AY738240.1	0.017
			S30			Hap15	AY738240.1	0.017

The Hap01 is represented to 33 samples with the highest frequency, whereas the other seven haplotype (Hap09 – Hap15) represent single individuals and low frequency.

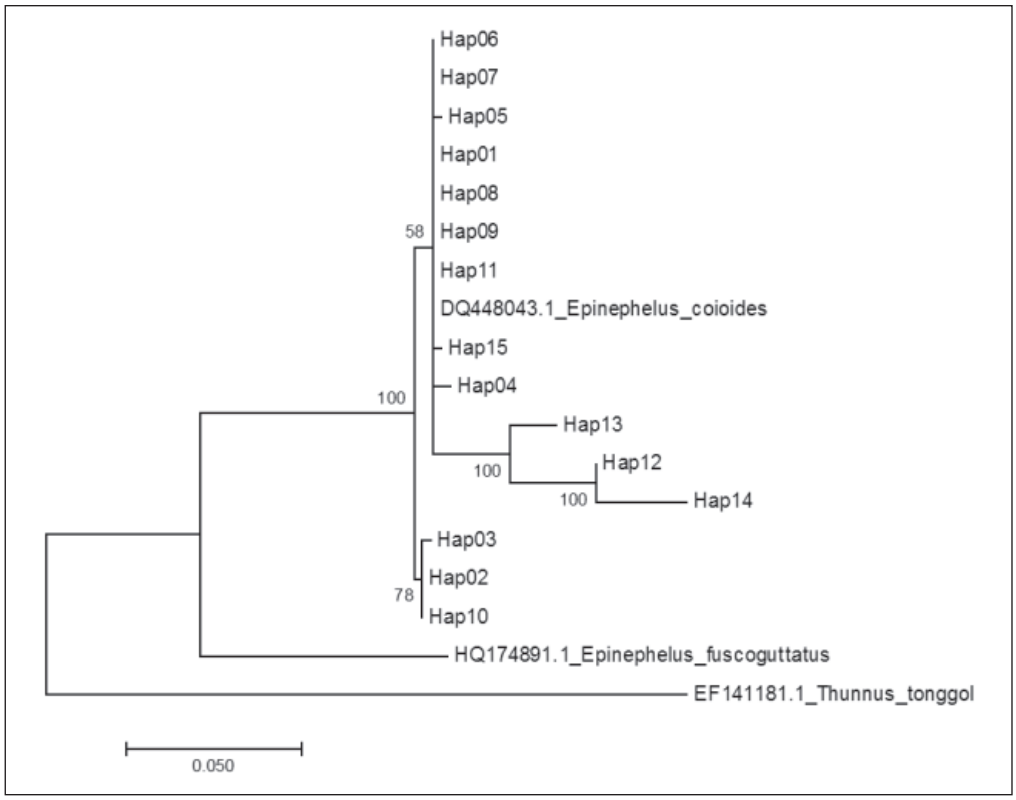

Fig. 1. Molecular phylogenetic analysis by the Maximum-likelihood method of *E. coioides* cyt-*b* haplotypes with *E. fuscoguttatus* and *Thunnus tonggol* as out-groups. The Hap01 is represented by 33 samples with the highest frequency, while Hap02 is shared among five individuals in Besut and Setiu, Terengganu. Meanwhile, the other seven haplotypes (Hap09 – Hap15) represented single and low frequency individuals in Setiu, Terengganu.

Table 2. Haplotype and nucleotide diversities for the *E. coioides* populations in Besut and Setiu, Terengganu, Malaysia

Locality	Haplotypic diversity (Hd)	Nucleotide diversity (π)	Tajima's D
Besut	0.4161	0.02178	-2.80575 (P>0.05)
Setiu	0.8782	0.01452	-1.78210 (P>0.05)

Haplotype diversity (Hd), nucleotide diversity (π) and Tajima's D test for neutrality for the population of *E. coioides* in Besut and Setiu, Terengganu. The haplotype diversity (Hd) shown is the mean of genetic diversity.

Table 3. Pair-wise genetic distance (Tamura-Nei parameter) of 60 *E. coioides* individuals in Besut and Setiu, Terengganu based on partial cytochrome *b* gene

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	Hap01															
2	Hap02	0.008														
3	Hap03	0.010	0.003													
4	Hap04	0.005	0.013	0.016												
5	Hap05	0.003	0.010	0.013	0.008											
6	Hap06	0.000	0.008	0.010	0.005	0.003										
7	Hap07	0.000	0.008	0.010	0.005	0.003	0.000									
8	Hap08	0.000	0.008	0.010	0.005	0.003	0.000	0.000								
9	Hap09	0.000	0.008	0.010	0.005	0.003	0.000	0.000	0.000							
10	Hap10	0.008	0.000	0.003	0.013	0.010	0.008	0.008	0.008	0.008						
11	Hap11	0.000	0.008	0.010	0.005	0.003	0.000	0.000	0.000	0.000	0.008					
12	Hap12	0.039	0.048	0.051	0.045	0.042	0.039	0.039	0.039	0.039	0.048	0.039				
13	Hap13	0.034	0.043	0.046	0.040	0.037	0.034	0.034	0.034	0.034	0.043	0.034	0.034			
14	Hap14	0.068	0.074	0.077	0.074	0.071	0.068	0.068	0.068	0.068	0.074	0.068	0.026	0.062		
15	Hap15	0.003	0.010	0.013	0.008	0.005	0.003	0.003	0.003	0.003	0.010	0.003	0.042	0.037	0.071	

Tajima's D gave a low value of -1.78210 ($p > 0.05$) as well.

The insignificant results of the Tajima's test in both localities indicate a population size expansion. The average FST value was -6.11671 ($p > 0.05$), suggesting a non-significant genetic variation among the two localities. The pair-wise FST data primarily demonstrates non-significant differentiation, which was the case for both Besut and Setiu specimens.

DISCUSSION

The aim of this study was to investigate the genetic diversity of wild orange-spotted groupers using the mtDNA cyt-*b* gene. According to Tseng *et al*. (2011), mitochondrial cyt-*b* genes have been used to analyse the genetic diversities and population genetic structures of many closely-related fishes. mtDNA has a number of specific biological properties, such as maternal inherintance, limited recombination, and high conservation, all of which make it an appropriate marker of molecular biodiversity. A study by Jackson *et al*. (2014) has been done on Nassau groupers to address their patterns of connectivity in mass-aggregating marine fish by analysing the patterns of genetic diversity using mtDNA.

Genetic diversity is important for the long-term survival of a species, because such variations can ensure the fitness of the species or population by providing the ability for them to adapt to changing environments. Fe'ral and Jean-Pierre (2002) mentioned that populations with lower genetic diversity have high homozygosity, which can adversely affect the survival traits and fitness of the individuals or populations. This will in turn lead to a reduction of the effective population size and affect the sex ratio. Inbreeding will result, which culminates in the loss of genetic diversity.

Though orange-spotted groupers are economically important in Southeast Asia, information on its genetic diversity remains limited. The results showed that wild orange-spotted groupers have low genetic diversity. Even though the genetic distances of the population ranged between 0.000 and 0.077, 46 out of 60 individuals demonstrated low genetic distances (0.000). According to Frankham (2005) and Willi *et al*. (2006), loss of genetic diversity can cause reductions in population size that give affect to the ability of populations to cope with heterogeneous environments and thus negatively influence species viability. Besides, Hap01 and Hap02 were also shared by a majority of the individuals (Hap01: 33 individuals; Hap02: 5 individuals). Apart from the 2 frequently-occurring haplotypes (Hap01 and Hap02), there were 7 unique haplotypes (Hap09 to Hap15), each of which was only present in one individual.

Based on the haplotypes present in the 60 fishes of *E. coioides* from two localities (Besut and Setiu), phylogenetic analysis was conducted to study their evolutionary relationships. The shallow divergence between the 12 haplotypes of the main clade might indicate that the wild *E. coioides* individuals with these were from the same *E. coioides* population. But as for Hap12, Hap13 and Hap14 that were separated from the other haplotypes in the main clade, it means more genetic evolutions occur in these haplotypes compared to others in the main clade. As for the other 3 haplotypes (Hap02, Hap03, and Hap10) that were separated from the main clade, it shows that the individuals were not from *E. coioides* populations of the main clade. However, the average FST value in this study was low at -6.11671 ($p > 0.05$), and as mentioned by Haslawati *et al*. (2011), a low pair-wise FST value did not mean that the shallow divergence between the specimens of the same species was significant. Therefore, individuals in these 3 haplotypes (Hap02, Hap03 and Hap10) can be grouped together with the individuals of the main clade even though they might have acquired some genetic differentiation.

E. coioides from the two localities (Besut and Setiu) were present in both clades of the phylogenetic tree. Since the orange-spotted grouper is not a migratory fish, drifting of grouper larvae by the ocean currents can cause the mixing of *E. coioides* individuals from Besut and Setiu populations. Although information for *E. coioides* is lacking, serranid fishes are generally high fecundity fish (Collins *et al.,* 2002; Whiteman *et al.,* 2005). They release large amounts of pelagic larvae dispersing through oceanic current (Thompson & Munro, 1978).

E. coioides usually spawn during the full moon night, during which the spring tide gives strong ocean currents that could result in the mixing of Besut and Setiu *E. coioides* individuals. Subjective information indicated *E. coioides* spawn during the months of the northeast monsoon (November to March with peak season from December to January). During northeast monsoon, the sea surface current from the South China Sea were forces northward towards Gulf of Thailand (Sharp, 1996), which could also further support the mixing between Besut and Setiu *E. coioides* individuals. It was also mentioned by Jackson *et al*. (2014) that ocean currents have an influence on the dispersal patterns of larvae which give rise to exchanges among populations.

The genetic diversity of *E. coioides* was also determined based on haplotype and nucleotide diversities. The results of this study showed low haplotype (Hd = 0.4161 to 0.8782) and nucleotide diversities (π = 0.01452 to 0.02178) in Besut and Setiu populations. Comparable results to this study with low haplotype and nucleotide diversity due to overfishing were observed in Dusky grouper, *E. marginatus* (Hd=0.743 to 0.934 ; π=0.004 to 0.014) (Maggio *et al.,* 2006) and Red grouper, *E. itajara* (Hd=0.53 to 0.86; π=0.001 to 0.005) (Silva-Oliveira *et al.,* 2008).

In case of *E. coioides*, the low Hd and π values indicate a low level of genetic diversity which is the result of overfishing of this species and excessive capturing of the juveniles for mariculture (Tupper & Sheriff, 2008). This practice has intensified in Southeast Asia over the last few decades as the larger fish became scarce and the interest in grouper mariculture to produce highly-valued live groupers for food grew.

According to Sadovy de Mitcheson and Colin (2012), an important biological aspect contributing to the declining number of groupers is their reproductive biology. Many groupers live long and take many years to mature sexually, hence making them vulnerable to fishing. Overfishing of the juveniles or adults of *E. coioides* will eventually decrease the variability in the nucleotide sequences of their cyt-*b* genes. This might cause an excessively low frequency of polymorphism (Tajima, 1989). Such a finding was also reported for the Dusky grouper (*E. marginatus*), the populations of which showed low haplotype and nucleotide diversities (Maggio *et al.,* 2006).

CONCLUSION

In general, the molecular phylogenetic result generated from this study are fast and reliable method to identifying the pairwise genetic distance and this method can be applied across highly divergent fish taxa. Nevertheless, the levels of diversity appear to decrease as the genetic distances between *E. coioides* samples in Terengganu becomes lower. Further study should be done to determine the validity of this finding in longer time elapse.

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