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Phylogeography and Genetic Diversity of Humpback Grouper *Cromileptes altivelis* based on Cytochrome C Oxidase I

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ABSTRACT

Humpback grouper is one of the most popular fish group in the international live trade in Asia-Pacific regions. The price for one kilogram live of humpback grouper, especially in Spermonde Archipelago South of Sulawesi, is range from 350.000-400.000 IDR, whereas in the retail level in Hong Kong the price was about 92 US$. This condition leads to the reduction of nature population due to overexploitation. Population decreasing due to overexploitation may cause loss of genetic diversity within population and lead to reduce of potential adaptive, population resistance, and productivity. Therefore, it is important to do some efforts to avoid adverse effect of overexploitation on humpback grouper population in Indonesia. One of the valuable efforts is providing genetic information such as phylogeography and genetic diversity of humpback grouper *Cromileptes altivelis*. Analysis was based on 618 base pairs fragment of cytochrome c oxidase I gene from 36 individuals (sequences) of *Cromileptes altivelis* collected at four different sites (e.g. Pulau Seribu, Jepara, Situbondo and Spermonde Archipelago). The results showed that humpback grouper population has a high haplotype and nucleotide diversity. However, high genetic diversity and polymorphisms could not reveal population fragmentation (Φ_{stt} = 0.000). It is suggested that high gene flow rather than population sub structuring was occurred. High level genetic diversity and polymorphisms are vital related to adaptive potential to environmental alteration.

Keywords: genetic diversity, humpback grouper, phylogeography

INTRODUCTION

Grouper are the most popular fish in the international live fish industries in Asia-Pacific areas. Therefore, it is not surprising that those areas are the biggest producer on International live fish trade, including Indonesia (SEAFDEC 2001). There are several well known grouper species by Indonesian fisherman, one of which is humpback grouper. Humpback grouper (*Cromileptes altivelis*) has highest economic value. Muchtadi (2007), has been reported that the price for 1 kg live of humpback grouper at the fisherman level might reach 200.000 IDR. However during the field trip in the Spermonde Archipelago South of Sulawesi in May 2009, the price for 1 kg live was reached 350.000–400.000 IDR (personal observation), whereas the price for 1 kg live humpback grouper in the retail level in Hong Kong was 92 US$ (McGilvary & Chan 2003).

The high price of humpback grouper leads to uncontrolled and unsafe exploitation.These had caused population decreasing due to over exploitation. Population decreasing might cause a loss of genetic diversity within a given population and potentially reduce adaptive capability, population resistance and productivity (Hauser *et al.* 2002). Overexploitation and the use of unsafe collecting method are suggested as reason for the reduction of natural population of humpback grouper in almost around Indonesia. It is, therefore, important to make some efforts to avoid adverse effect of over exploitation on humpback grouper population in Indonesia for their sustainable use.

Mariculture is one of the alternative techniques to minimize a pressure on natural population of humpback grouper. There are some efforts to develop mariculture of humpback grouper. At present, there are four govermental breeding centres for humpback grouper. The rest of those are only grew the collected wild fry into market size. However, these efforts are depend on the availability of natural fry.

In order to support mariculture efforts by providing stock information with better genetic quality, a study on phylogeography and genetic diversity is needed. Phylogeographic analysis will permit detailed studies of landscape evolution, including the dispersal of taxa through a region, speciation, addaptive radiation, and extinction. In other words, phylogeographic analysis is an investigation of the fundamental linking between population processes and regional patterns of diversity and biogeography.

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The mtDNA-based phylogeography has been the improved description of geographical distribution, phylogenetic relationships, and genetic distances among evolutionary lineages of animals (Bermingham & Moritz 1998).

Genetic study on given populations are intended to evaluate genetic diversity of those populations. Compare to morphological analysis, genetic data are relatively stable and are not or less affected by the environment. Surjadi (2002), has note that genetic variation study is an important aspect on preservation and the sustainable use of germ plasma. Moreover, Indriani *et al.* (2002), notes that higher genetic diversity provide a higher opportunity to obtain organisms with the expected performance. Furthermore, genetic diversity is needed by the organisms to keep their capability on reproduction and adaptation to their environmental (Fe´ral & Jean Pierre 2002), including their resistance to several diseases.

Phylogeography and genetic diversity can be studied using genetic marker such as the fragment of the mitochondrial DNA cytochrome c oxidase I (COI) gene. The usage of this gene on the phylogeographic and genetic diversity of population studies have several advantages as follow e.g. high rate of mutation, has not had recombination process since it is inherited maternally (Hebert *et al*. 2003a), highly divergent among populations (Ward *et al*. 2009), and has broader phylogenetic sign (Hebert *et al*. 2003b). It was proved that COI gene is suitable for such genetic studies (Duran *et al*. 2004; Kochzius & Nuryanto 2008; Nuryanto & Kochzius 2009).

This research project is aimed to know genetic diversity and phylogeographic pattern of humpback grouper populations based on partial sequence of cytochrome c oxidase 1.

The result is expected to be used as a basis for applied research on humpback grouper mariculture such as a study on comparison of growth and survival rate of humpback grouper coming from variable genetic diversity stock. It is expected that the population from higher genetic diversity stock will show higher growth and survival rate, and also higher adaptive potential.

MATERIAL AND METHOD

Sample collection and DNA Isolation. Tissue samples were collected at four localities during the field trip in April-June 2009. Tissue samples were collected in Pulau Seribu (PS), Jepara (JP), Situbondo (ST), and Spermonde (SP) Archipelago South of Sulawesi. Fin clips were cut off from caudal fins with the help of forceps and scissors. Tissue samples were preserved on 96% of ethanol and later on ^{0}C until DNA analysis. Total genomic DNA was isolated using DNA tissue kits (Merchery Nagel GmBH, Germany) following the protocols from the company.

DNA amplification and sequencing. A fragment of Cytochrome c oxidase I (COI) gene was amplified using a pair of universal coral reef fish primers fwd_seq: TCAACCAACCACAAAG ACATTGGCAC, and rev_seq: TAGACTTCTGGG TGGCCAAAGAATCA (Ward *et al.* 2005). This primers were amplified a fragment of COI gene with approximately of 700 bp length.

The PCR reactions were carried out in a total volume of 50 µl contained 29,8 µl ultrapure water; 10X PCR buffer 5 µl, $MgCl_2$ (50 mM) 5 µl, 2 µl (0.01 mM) of each primer, 2 µl of dNTPs (0.05mM), 1 U Taq polymerase, and 4 µl of template DNA. The thermal cycles were as follow: one cycle at 95^{0}C for 5 minutes, followed by 35 cycles of 1 minute at 94^{0}C, 1 minute annealing temperature at 54^{0}C, and 1,5 minutes at 72^{0}C. The final extension was carried out at 72^{0}C for 10 minutes (Ward *et al.* 2005). PCR products were visualized on 1% Agarose gel. Clear and strong PCR products were selected for sequencing. Unpurified PCR products were sent to Macrogen Inc. Korea for sequencing.

Data Analysis. All sequences were initially aligned and edited manually using Bioedit software (ver. 7.0.4.1; Hall 1999). Multiple sequences alignment was done using ClustalW (Thompson *et al.* 1994), as implemented in Bioedt ver. 7.0.4.1 (Hall 1999). The orthology to previous published sequences available in NCBI sequence data base (*C. altivelis* DQ107097.1; DQ107905.1; DQ107892.1) was also verified.

Phylogeographic analysis was carried out by constructing phylogenetic tree for all sequences from all sampling sites, two sequences of humpback grouper from GenBank as in group and two sequences of *Epinephelus* from GenBank as *out groups*. Phylogenetic tree was constructed with the help of MEGA software 4.0 version (Kumar *et al*. 2008), using *neighbour joining method*. The support for tree branching was based on 1000 non-parametric bootstrap replicates. Significance of phylogeographic pattern was also tested with the Analysis of Molecular Variance (AMOVA, Excoffier *et al*. 1992), and pair wise Φ_{st}-values. Both statistical calculations were carried out using the software Arlequin (ver 2.0, Schneider *et al*. 2000).

Genetic diversity could be assessed by sequences polymorphisms, haplotype and nucleotide diversity. Haplotype diversity *h* (Nei 1987), and nucleotide diversity π (Nei & Jin 1989), were calculated with the programme Arlequin (ver. 2.0; Schneider *et al*. 2000).

RESULT AND DISCUSSION

Amplification of COI fragment using selected primers resulted of approximately of 700 bp length of PCR products. A length of 682 base pair of sequences was obtained from bigdye sequencing. However, a multiple sequences allignment result a length of 618 base pair of COI gene fragment from 36 individuals of *C. altivelis* collected at Jepara, Pulau Seribu, Spermonde, and Situbondo. Sequences verification proved that the resulted sequences have a sum of 98% similarity with the sequences from the same species available in GenBank sequences data base.

Genetic diversity. Genetic diversity of population could be measured by DNA polymorphism and nucleotide diversity (Nei & Jin 1989). This value indicates a proportion of nucleotide differences among all sequences in the samples. For mitochondrial DNA, genetic diversity is also measured by haplotype diversity (h) (Nei 1987). Haplotype diversity is the proportion of different sequences from total sequences collected within population under study (Hartl & Clark 1997). Among a total of 36 (sequences) individuals, 36 haplotype were obtained with 61 polymorphic sites (9.87%) and 70 mutations. Therefore, the most common haplotype has a frequency of 90.13%. This proves that the marker (COI gene) showed high polymorphisms since the frequency of most common allele less than 99% (Hartl & Clark 1997). High polymorphism on COI fragment was also observed on *T. crocea* (Nuryanto *et al*. 2007; Konchzius & Nuryanto 2008; Nuryanto & Kochzius 2009), and also on blue sea stars (*Linckia laevigata*) and its gastropods parasite *Thyca crystallina* (Kochzius *et al*. 2009).

Genetic diversity analysis within each population showed high levels of haplotype diversity ($h = 1.00 \pm 0.05$). This means that all the sequences in each sampling site are different among one to another. High levels of nucleotide diversity were observed within all populations. Nucleotide diversity was ranges from 1.25% in the population from Situbondo to 2.22% in Jepara (Table 1). It is prove that all population showed high genetic diversity. High genetic diversity on COI gene fragment in this study proved that this gene has high divergences within species. A high genetic divergence of COI gene on *Lates carcarifer*, *Dasyatis kuhlii* and *D. laylandi* were observed (Ward *et al*. 2008a). The level of genetic diversity obtained in this study was comparable to other studies on several coral reef organisms such as in bivalve (Shefer *et al*. 2004; DeBoer *et al.* 2008; Kochzius & Nuryanto 2008; Nuryanto & Kochzius 2009), and shrimp (Barber *et al*. 2006).

Phylogeography. Phylogeographic pattern among all sampling sites based on 36 haplotype of humpback grouper *C. altivelis* are presented on phylogenetic tree (Figure 1). The figure showed that *C. altivelis* group is separated from *Epinephelus* group. This result proved that the used of COI gene is reliable enough for interspecies discrimination. According to deWard *et al*. (2011), COI fragment showed high interspecific divergence. This reliability is powerful enough for interspecies discrimination. The high

Table 1 Sample sites, number of sequences (n), number of halpotypes (Nhp), haplotype diversity (*h*) and nucleotide diversity (π) in *Cromileptes altivelis* from Pulau Seribu, Jepara, Situbondo, and Spermonde Archipelago

No	Sample site	n	Nhp	h	π (%)
1.	Jepara	9	9	1.00 ± 0.05	2.22 ± 1.25
2.	Pulau Seribu	9	9	1.00 ± 0.05	1.65 ± 0.94
3.	Spermonde	9	9	1.00 ± 0.05	1.86 ± 1.06
4.	Situbondo	9	9	1.00 ± 0.05	1.25 ± 0.73

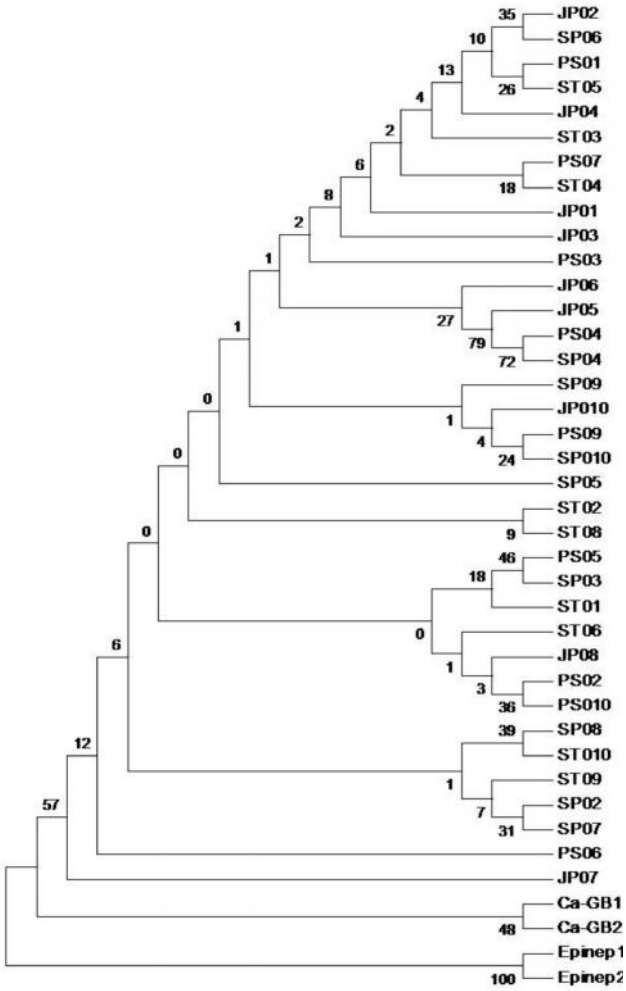

Figure 1 Neighbor Joining tree indicated random geographic distribution of haplotype of humpback grouper *Cromileptes altivelis*

diversgences on COI gene is due to of its high rate of mutations (Hebert *et al*. 2003a).

However, if we look into detail through Figure 1, all 36 haplotypes from four sampling sites are distributed randomly. In other words, the haplotypes were not made groups according to their origin (sampling sites). This fact suggests that COI gene fragment showed high divergences among individuals within populations but less divergence among populations. The result is comparable to Ward *et al*. (2008a), who showed low divergences of COI gen between populations on *Dasyatis kuhlii* and *D. laylandi.*

Random geographic distribution of humpback grouper haplotypes as shown in the Figure 1 indicated no fragmentations or substructuring was observed on humpback grouper populations under study. Unfragmented population was also observed on *Symbiodinium* from Indonesian Archipelago (Nuryanto *et al*. 2010). The phenomenon leads to an assumption that all samples have the same evolutionary origin, especially for the cultured samples from Pulau Seribu, Jepara, and Situbondo. However, when we looked at on the haplotype distribution of the three farmed populations we could not decided which population is the parental population. This assumption, however, need a further analysis.

Related to the samples from Spermonde Archipelago, which is separated about 2000 km away from the rest of populations but the haplotypes was also mixed with all others haplotypes from Java Island, it is suggested that the parental population of farmed populations in Java Island is Spermonde Archipelago population. This means that Spermonde Archipelago is one of the centres of origin of the cultured populations in Java Island. It has been reported that the populations from the center part of Indonesia (Wallacea areas inclufing Spermonde populations) are the evolutionary origin of the rest of Indonesias populations (Kochzius & Nuryanto 2008; Tim and Kochzius 2008, Nuryanto & Kochzius 2009). The argument is also appeared based on the fact that it was very difficult to obtain wild humpback grouper samples from Java Island (Pulau Seribu, Jepara, and Situbondo, or even from Segar Anakan and Pelabuhan Ratu). In other hand, it was quite easy to get tissue samples from Spermonde Archipelago. The mixes of all haplotypes from all sampling sites also indicated high gene flow was occurred among populations under study.

Table 2 AMOVA analysis among humpback grouper populations

Source of Variation	db	Φ_{ST}	Variation	P
Among populations	3	0.000^{ns}	0.000^{ns}	<0.001
Within population	32		0.500^{s}	
Total	35			

Remarks: ns= non significant, s= significant

Phylogeographic pattern of humpback grouper haplotypes was also verified by AMOVA and pairwise Φ_{st} value. The analysis showed a low of Φ_{st} value (Φ_{st} = 0.000; Table 2). A low of Φ_{st} value proved no differences among populations or no fragmentation among populations. According to Hartl and Clark (1997), Φ_{st} values between 0 and 0.05 indicating low genetic difference among populations. Moreover, high Φ_{st} values and low Nm indicating high genetic difference among population or low gene flow (Bossart & Prowell 1998).

No genetic differences were also indicates high gene flow among humpback grouper populations. High gene flow was also found on tasslefish (*Polynemus sheridani*, Chenoweth & Hughes 2003), and *Symbiodinium* (Nuryanto *et al.* 2010).

CONCLUSION

Based on cytochrome c oxidase 1 gene, humpback grouper population from Pulau Seribu, Jepara, Situbondo, and Spermonde Archipelago has a high level of polymorphisms, haplotype and nucleotide diversity. However, high level genetic diversity and polymorphisms could not reveal population fragmentation. It is suggested that high gene flow rather than population substructuring was occurred.

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