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Molecular Characteristics of Batanghari, Tambago, Orange, and Mandiangin Giant Gourami Strains

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Abstract

Morphological variations among geographic and can be identified as different species. However, a lot of studies proved that morphological variations are also common in conspecific individuals. Therefore, precise identification using additional characters is vital, such as using a molecular marker. Here, we characterized Batanghari, Tambago, Orange, and Mandiangin gourami strains using the cytochrome b gene to evaluate their taxonomic status. Partial sequences of cytochrome b gene were sequenced for 40 individuals. Taxonomic status was checked for giant gourami sequences available in GenBank. Kimura 2-Parameter genetic distances were calculated in MEGA6 software. Haplotype and nucleotide diversity within population and Φ_{st}-value among populations were estimated in Arlequin software. Phylogenetic relationship was reconstructed using the neighbor-joining method in MEGA6 software based on Kimura 2-parameter model with 1000 pseudobootstraps. Taxonomic identification results in 99% sequences homology to *Osphronemus goramy* sequences (accession number KU984978.1 and AY763768.1), means that all strains belong to single species. Low genetic distances, medium haplotype and low-level nucleotide diversity were observed among strains. Pairwise Φ_{st}-comparison indicates no genetic differences among Sumatera strain, whereas strong genetic structures observed between Sumatera and Mandiangin strains. The phylogenetic tree showed that Mandiangin formed separate subclades from other strains with bootstraps value of 100%. This finding has important implication for breeding sciences and efforts.

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INTRODUCTION

Giant gourami (*Osprhronemus gouramy* Lac.) is an indigenous freshwater fish species in Indonesia. Geographic distribution of this species in Indonesia includes Java, Sumatera, and Kalimantan (Froese and Paulin, 2017). Geographic distribution might lead to geographic isolation among geographic populations which might cause speciation (Losos and Glor, 2003). In case of geographic distribution of giant gourami, it leads to the emergence of geographic strains, such as Soang, Jepang, Paris, Bastar, and Porcelain (Setijaningsih *et al.*, 2007), Batanghari in Jambi (Nugroho, 2011), Tambago and Orange West Sumatera (Nuryanto *et al.*, 2017a).

All the strains showed variable morphological characteristics (Azrita and Siandri, 2015). Morphospecies status of individuals with different morphology is occasionally become difficult to be defined (Heinrichs *et al.*, 2004). However, large amount of studies proved that morphological variations are observed within species in a wide range of animal phyla (Kelley *et al.*, 2011; Hepp *et al.*, 2012). According to Tzeng *et al*. (2000), morphological variations among geographically separated populations involve their genetic components which are also variable. Flot *et al.*, (2008) had proved a positive correlation between genetic and morphological variations.

Previous studies showed molecular variation among strains (Sari *et al.*, 2014; Nugroho *et al.*, 2013; Nugroho, 2011; Nugroho & Kusmini, 2006). However, all those studies were used RAPD and Isozyme markers that less precise tools for species identification and mostly done in gourami from Java. So far, no published information on molecular characteristics of giant gourami strains from Sumatera and Kalimantan especially based on partial sequences of cytochrome b gene. Therefore, it is important to study on a molecular feature of morphologically differences giant gourami strains from Sumatera and Kalimantan based on partial sequence of cytochrome b (cyt b) gene to clarify their taxonomic status whether morphological differences among strains refer to either different species or only pointing out as geographic isolates or subspecies.

Taxonomic status and genetic differences among geographic strains can be evaluated based on their molecular characteristics. Several markers are commonly used in molecular characterization, one of which is partial sequences of cytochrome b gene. This gene has a strong phylogenetic sign and high variation among populations due to its high evolution rates in fish (Tang *et al.*, 2006). It has been proved that cytochrome b is a useful marker for fish species identification (Griffiths *et al.*, 2010; Perez *et al.*, 2007; Pepe *et al.*, 2005), fish genetic diversity (Perdices *et al.*, 2004), phylogenetic analysis (Tsigenopoulos *et al.*, 2002; Casey *et al.*, 2004; Doadrio dan Dominguez, 2004), and phylogeographic studies (Kotlik dan Berrebi, 2001; Santos *et al.*, 2003). It is expected that the use of the Cyt b gene in giant gourami strains characterization can be used to analyze their genetic diversity and differences and subsequently be used to strengthen the taxonomic status of giant gourami strains from Sumatera and Kalimantan.

This study is expected to provide information which is valuable in developing a breeding strategy within and among strains to obtained high-quality strains and preserve unique characters of each strain.

METHODS

Tissue samples of caudal fin were collected in May 2016 for Tambago, Orange, (Payakumbuh, West Sumatera) and Batanghari (Jambi) strains, while for Mandiangin strain were collected in April 2017 from Mandiangin (South Kalimantan). Tissue samples were then preserved in the absolute ethanol 96% and kept in the refrigerator until DNA processing.

Total genomic DNA was extracted using Chelex methods (Walsh *et al.*, 1991) with optimization in incubation times. The fragment of cytochrome b gene was multiplied utilizing a pair of primer as follows; forwards L14725L:5'CGA AAC TAA TGA CTT GAA AAA CCA CCG TTG3' and reverse HMVZ16:5'AAA TAG GAA RTA TCA YTC TGG TTT RAT3' (Santos *et al.*, 2003). A total volume of 50 µl of PCR reactions was used during amplification. These mixtures consisted of 33.7 µl of ultrapure water (Thermo scientific), 1X of dream taq buffer (Thermofisher Sciencefitic), 0.4 mM of dNTPs, primer 0.4 picomols of each primer, 1 U DNA Taq polymerase and 2 µl DNA template. PCR process was observed in the thermal cycle Peqstar (PeqLab Company). The cycles were performed in the following temperature regime; predenaturation in 95°C for 3 minutes and continued with 40 cycles with 30 seconds denaturation in 95°C, annealing in 45°C + 0,1°C for 45 seconds, and chain elongation in 72 °C for 1 minute. Final elongation was done in 72°C for 9 minutes. The PCR products were stained with ethidium bromide and migrated in 1% agarose gel. Afterward, the PCR products were visualized over UV-light transilluminator.

Partial sequences of the cyt b gene were obtained by submitting qualified PCR products to 1st BASE Asia (www.base.asia.com) for sequencing.

Multiple sequences alignment was done in ClustalW (Thompson *et al.*, 1994) in Bioedit 7.0.5. (Hall, 2005). Species-level identification was conducted by submitting the sequences to GenBank using basic local alignment search tool (BLAST). Nucleotide sequences were translated into amino acid sequences using ORF Finder online version (https://www.ncbi.nlm.nih.gov/orffinder/) to ensure that the resulted sequence is a functional gene. Haplotype (Nei, 1987) and nucleotide (Nei & Jin, 1989) diversity were statistically calculated in *Arlequin* software version 2.0 (Schneider *et al.,* 2000). Overall haplotype and nucleotide diversity were estimated statistically using DnaSP version 4.0 (Rozas *et al.*, 2003). Pairwise Φ_{st}-comparisons were conducted in Arlequin version 2.0 (Schneider *et al.*, 2000). Kimura 2-Parameter (K2P) genetic distances were calculated using MEGA6 (Tamura *et al.*, 2013). Evolutionary relationships among strain were estimated through phylogenetic tree reconstruction using MEGA 6.0 (Tamura *et al.*, 2013). The phylogenetic tree was reconstructed using a *neighbor-joining* algorithm with 1000 *non-parametric bootstraps*. The polarity of branching pattern was obtained through outgroup comparison with *Trichopodus pectoralis* (accession number AY763758.1) and *T. trichopterus* (AY763759.1) as outgroups taxa.

RESULT AND DISCUSSION

Partial sequences of the cytochrome b gene were successfully sequenced from 40 individuals of four giant gourami strains. The resulted sequences range from three individuals of Orange strains to 20 individuals of Mandiangin strain. Homology test showed that all strains have sequences similarity of 99% to previously published sequences of *Osphronemus goramy* available in GenBank (KU984978.1 99% dan AY763768.1). This homology test means that all strains belong to *Osphronemus goramy* Lacepede, 1801. This placement is fulfilled the requirement of similarity among individuals as notes by Pereira *et al*. (2013) that two or more individuals can be referred to as a single species when they have sequences similarities between 97% and 100%. Species delineation of all strains to a single species was supported by the low level of K-2P genetic distances among strains (from 0.0% to 1.1%). Our finding is similar to what was reported by Pegg *et al*. (2006) in other fish groups which observed genetic distance among individuals from single species ranges from 1% to 3%. This similarity could be due to that we have used a similar genetic marker that was mitochondrial cytochrome genes.

Multiple alignments of the cytochrome b genes from 40 individuals of four giant gourami strains resulted in 357 base pairs (bp) fragments. Seven polymorphic sites were observed among 357 bp fragment from 40 total sequences (1.9%). This value indicates that the used marker has a low level of polymorphisms. Low genetic polymorphisms were also observed in *Myripristis berndti* (Craig *et al.*, 2007) and in *Ephinepenlus itijara* (Craig *et al.*, 2009). This similar result indicates that low level of genetic polymorphisms can be observed in the broad scope of fish species.

Genetic diversity analysis result in haplotype diversity value ranges from 0.533 to 0.714 and nucleotide diversity ranged between 0.001 and 0.003. Complete data on haplotype and nucleotide diversity are presented in Table 1.

Table 1. Individual number, haplotype number, polymorphic loci, haplotype and nucleotide diversity

Strain	n	haplotype	Polymorphic loci	h	π
Batanghari	10	2	1	0.533	0.001
Mandiangin	20	6	7	0.684	0.003
Padang Tambago	7	3	2	0.714	0.002
Orange	3	2	1	0.667	0.002

It can be seen from Table 1 that with the range value of haplotype diversity between 0.533 and 0.714 indicate medium genetic diversity within strain. Medium haplotype diversity values of cyt b gene in our study are similar to what was reported by Craig *et al.* (2007) in twenty populations of coral reef fish *Myripristis berndti* (*h*= 0.286-0.829) from geographic widely separated populations, and Craig *et al*. (2009) in goliath grouper *Ephinepenlus itijara* from Panama, Bezile, Brazil, and Florida populations (*h*=0.324-0.684). According to our result in giant gourami and those previous studies, it is likely that moderate haplotype diversity is rather common in cyt b gene of fish taxa.

Our result is different to Akbar *et al*. (2014) study who had reported a high genetic diversity of cytochrome b gene on yellowfins tuna (*Thunnus albacares*; *h*=0.990). The difference between

our results to the study from Akbar *et al*. (2014) might be caused by two factors. Firstly, it could be due to that mutation rate of the cyt b gene on tuna species are different to that in giant gourami. Secondly, the difference between our study from the study of Akbar *et al*. (2014) might be caused by the source of populations. In this study, we used cultivated species, while Akbar *et al*. (2014) used natural populations. It is assumed that cultivated population receives different selection pressure than that natural population. Different selection pressures might cause different adaptive evolution and lead to various genetic diversity level.

The observed nucleotide diversity ranges from 0.001 to 0.003 indicates a low level of haplotype diversity. This result in accordance with Kochzius & Nuryanto (2008) that nucleotide diversity value less than 0.01 refers to low-level diversity. All genetic parameters indicate that the studied giant gourami strains had low genetic diversity in their cytochrome b gene. Low genetic diversity was also observed in cultivated fish species, such as *Carassius carassius* (Yoon and Park, 2002) and in sea bream, *Sparus aurata* (Alarcon *et al*., 2004).

Amova results in with Φ_{st} value of 0.281 and mean square value of 0.127. A statistical test of 95% significance level proved that strong genetic structure occurred among giant gourami strains (p<0.0001). However, when we look carefully to the result of pairwise Φ_{st} comparison analysis, only Mandiangin strain that contributes to the observed genetic structure, while Sumatera strains were not genetically different as indicated by the low level of pairwise Φ_{st} values (Table 2).

Pairwise Φ_{st} comparison values range from -0.264 between orange and Batanghari strains up to 0.379 between Mandiangin and Batanghari strains. The pairwise Φ_{st} values among strains are presented in Table 2.

Table 2 showed low levels of genetic differences among Sumatera strains indicated no genetic structure among Sumatera strains. This condition might be caused by high genetic exchanges among strains through seed transfer among areas in Sumatera. In case of Sumatera strains, our study has a similar result to a part of Craig *et al*. (2007) study in reef fish *Myripristis berndti* where no genetic differences were observed in rather narrow areas such as within Central Pacific (Φ_{st} = –0.007, p = 0.634), within Indian Ocean (Φ_{st} = –0.027, p = 0.528), and within the east Pacific (Φ_{st} = –0.061, p = 0.920). The present study and previous study from Craig *et al*. (2007) indicates low rates of molecular evolution in mitochondrial cytochrome b gene which lead to no genetic structuring among populations in small geographic scales. Therefore, it is not surprising if a low level of genetic differences were observed in cyt b gene among morphologically different giant gourami strains in Sumatera.

Another interesting finding that can be summarized from Table 2 was that high Φ_{st} values were observed between all strains from Sumatera and Mandiangin strain. Those Φ_{st} values indicate significant genetic structures or genetic differences were found between Sumatera strains and Mandiangin strain. This condition might be due to no genetic exchange between those two islands because seeds transportation mainly tends to occur on the island. This result was also similar to another part of Craig *et al*. (2007) and Craig *et al*. (2009) studies in two reef fish species where significant genetic differences were found among large geographically separated populations. Moreover, strong genetic structures were also observed among geographically separated cyprinids populations (Durand *et al*., 2002) and among *Orizyas latipes* populations Takehana *et al*. (2003). The finding of our study and all previous studies proved that cyt b gene is a suitable marker for genetic structure study in distantly separated populations but not for geographically closed populations.

Molecularly, the Mandiangin strain is differentiated by three transitions in their pyrimidine nucleotide. These shifts were observed in base position of 240 from T gourami Batanghari, Tambago, and Orange strain to C in Mandiangin strains. In the nucleotide position of 249 and 279, transitions occurred from C in Batanghari, Tambago and, orange strains become T in Mandiangin strain. Those changes made Mandiangin strain genetically separated from all other strains

Table 2. Pairwise Φ_{st}-values among strains

Strain	Batanghari	Mandiangin	Padang Tambago	Padang Oranye
Batanghari	-			
Mandiangin	0,379***	-		
Tambago	-0,086ns	0,304***	-	
Orange	-0,264ns	0,321**	-0,215ns	-

Keterangan: * = $0.05 \geq p \geq 0.01$, ** = $0.01 > p \geq 0.001$, *** = $p < 0.001$; ns = not significant

from Sumatera. However, since the different between Mandiangin and all other Sumatera's strains were still in the range of species variability. Therefore, they are conspecific strains.

Tree topology supports the results of the pairwise comparison, genetics distances, and amova (Figure 1). It can be inferred from Figure 1 that all giant gourami strains formed a monophyletic group compared to the outgroup species with maximum bootstraps value of 100. The tree topology proved that all strains are evolved from a single common ancestor. It means that all strains are belonging to single species. The argument was in accordance with the statement from Gill *et al*. (2005) that group of individuals which are originated from one speciation event or evolved from one ancestor can be delimited single group. In species level, this monophyletic group can be assigned to single species.

If we look into detail in Figure 1, the giant gourami strains were divided into two different subclades (A and B). The subclade A consisted of Batanghari, Tambago, and Orange strains. The subclade B only included Mandiangin strain. This subclade division between Sumatera and Kalimantan strains indicates that giant gourami from those islands showed that significant genetic structure occurred between Sumatera and Kalimantan strain. However, this division did not lead them belong to different species because all the strains were only separated by the short length of branching pattern in a phylogenetic tree with the branch length less than 0.02 (Figure3).

The K-2P genetics distances were range from 0.000 to 0.014. Complete K-2P genetic distances were presented in Table 3. These values showed low genetic distances among strains. Low genetic distances in cytochrome b gene among large gourami populations provide data that phenotypic differences were not always positively correlated to differences in molecular level. Therefore, based on low level of genetic distances among Batanghari, Tambago, Orange, and Mandiangin strain can be defined convincingly that all strains are belonging to single species, namely *Osphronemus goramy* Lacepede, 1801. This was because several studies note that different species can be delineated molecularly into species level if they have minimum genetic divergences of 0.02 (2%) (Barber *et al*., 2002). A more significant value was reported by Nuryanto *et al*. (2007) and Nuryanto *et al*. (2017b), which found genetic divergences among species was higher than 0.05 (5%).

In taxonomy, phenotypic differences among geographically separated individuals from single species are referred as to different ecophenotype. Several studies proved that many animal populations have various ecophenotypic individuals (Langerhans *et al*., 2003; Zieritz and Aldridge, 2009). Therefore, morphological variations in giant gourami strains from Sumatera and Kalimantan can be referred as to different ecophenotype.

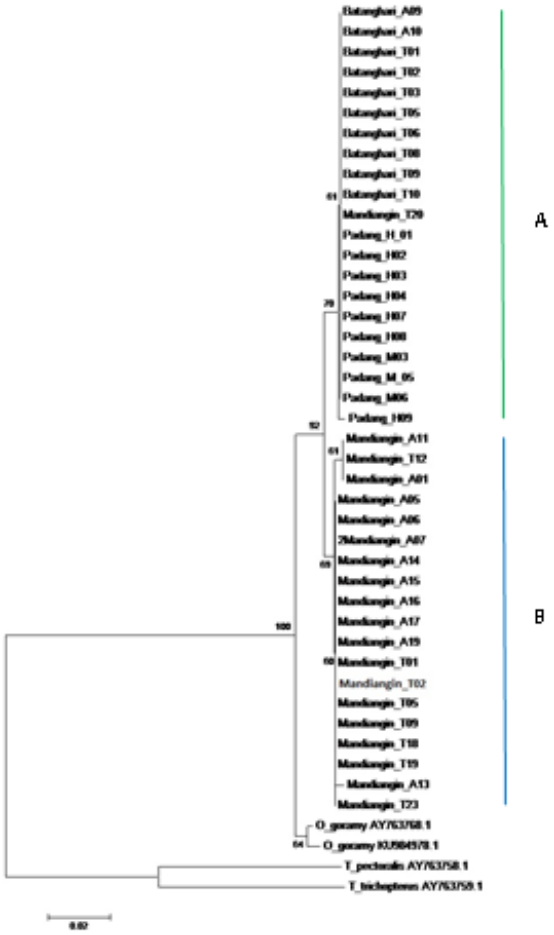

Figure 1. Phylogenetic tree indicates giant gourami strains divided into two subclades (A and B)
Remark: The number upper and below the lines are bootstraps values; Clade Sumatera (Batanghari, Tambago, Orange); Clade Kalimantan (Mandiangin)

Our result was different to Santos *et al.* (2003) study which found high genetic distances in cyt b gene of geographically separated *Macrodon ancylodon* populations and Craig *et al.* (2009) which also observed a high genetic difference among goliath grouper (*Epinephelus itajara*) populations. The differences between our result and the study from Santos *et al*. (2003) and Craig *et al*. (2009) might due to we used a different source of populations. In one hand, we used cultivated populations which might be under similar selecti-

Table 3. The K-2P genetic distances among giant gourami strains

Strain	Batanghari	Mandiangin	Tambago	Orange
Batanghari	0.000			
Mandiangin	0.009-0.011	0.000-0.011		
Tambago	0.000-0.003	0.000-0.014	0.000-0.003	
Oranye	0.000	0.000-0.011	0.000-0.003	0.000
Trichopodus	0.215-0.217	0.211-0.217	0.215-0.221	0.215-0.217
T.Pectoralis vs T.trichopterus = 0.118				

on pressure from breeder to develop high-quality strains. This directional selection will cause genetic changes and has an evolutionary impact on those populations and lead to homogenization of gene component of the giant gourami populations. According to Mysterud (2011) and Jorgensen *et al*. (2007), directional selection might lead to genetic alteration and has a significant impact on the evolutionary success of populations. In case of breeding selection might cause loss of genetic diversity within population, such as in giant gourami strains from Sumatera and Kalimantan. Previous studies proved low genetic diversity in cultivated fish species of *Carassius carassius* (Yoon & Park, 2002) and sea bream, *Sparus aurata* (Alarcon *et al*., 2004)

Our finding on taxonomic status and molecular characters of giant gourami strains from Sumatera and Kalimantan has necessary implication for the development of giant gourami cultivation, especially for developing high-quality strain. Information from this study is also vital for strains conservation to sustain the variability within each strain as germ plasma for sustainable of giant gourami resources. Our data also showed the importance of fundamental science to support applied science or even culture science and breeding science.

CONCLUSION

Gant gourami strains from Sumatera are genetically similar, whereas Mandiangin strain from Kalimantan showed significant differences compared to Sumatera strains. All strain formed monophyletic group compared to outgroups species and separated into two subclades in the phylogenetic tree. In general, all strains had low genetic differences. Based on all the analysis, Batanghari, Tambago, Orange, and Mandiangin strains belongs to *Osphronemus goramy* Lacepede, 1801.

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