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Molecular characterization of four giant gourami strains from Java and Sumatra

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Abstract. *Nuryanto A, Amalia G, Khairani D, Pramono H, Bhagawati D. 2018. Molecular characterization of four giant gourami strains from Java and Sumatra. Biodiversitas 19: 578-584.* Giant gourami (*Osprhonemus goramy*) are widely distibuted across Indonesia, such Java, Sumatra, and Kalimatan which lead to the emerge of various gourami strains due to morphological differences. However, no scientific data about the relationship between morphological and genetic differences among strains. This research aimed to obtain information on molecular characteristics of four giant gourami strains from Java and Sumatra based on partial sequences of cytochrome c oxidase 1 gene. This information is vital to strengthen their taxonomic status. Caudal fin clips were sampled from each strain. Nucleotide sequencing was performed using bigdye terminator tehcnique. Pairwise *Fst* comparison was carried out using arlequin software, whereas sequences of divergence analysis was performed in DnaSP software. Homology of the sequences were checked with previous published data available in Boldsystem data base. Homology test resulted in 98.79 to 100% similarity to the previous published sequences. This means that all strains belonged to single species, i.e. *Osphronemus goramy*. This placement was supported by low-level of genetic divergences among strains. Although they have low-level genetic divergences, this value is suitable to separate each strain clearly as indicated by pairwise Fst comparison analysis and AMOVA, which showed differences among strains. However, phylogenetic tree shows that all stains formed a monopyletic group with boostratp value of 100. Phylogenetic analysis supports the placement of all strains into a single species that is *O. guramy*. Those morphological differences are also reflected in their genetic character, except for Tambago and Oranye strains.

Keywords: Fixation index, gourami, Java, molecular divergence, *Osphronemus goramy*, Sumatra

INTRODUCTION

Giant gourami is an Indonesia's indigenous fish species which is now widely distributed to other Southeast Asia countries and Chinese region (Pusat Penyuluhan Perikanan dan Kelautan 2011). In Indonesia, geographic distribution of this species spanning from Java, Sumatra, and Kalimantan Islands (Froese and Pauly 2007). This condition is predicted to cause geographic isolation among giant gourami populations from those islands.

Geographic isolation has caused morphological variation among populations leading to the emergence of several different geographic strains. In Java, there are at least five strains of giant gourami, e.g. Soang, Jepang, Paris, Bastar, and Porselen. In Sumatra, especially in West Sumatra, five giant strains are also popular among fish farmer, i.e. Palapah, Tambago, Jepun, Merah, and Krista (Azrita dan Syandri 2015). In addition, another giant gourami strain was also popular in Jambi. This strain called as Batanghari giant gourami (Nugroho et al. 2013).

Previous studies has proven that all giant gouramy strains show several morphological variations including phenetic (Nugroho et al. 1993; Nugroho et al. 2013; Azrita and Syandri 2015), colour (Nugroho 2011), and growth potential (Nugroho et al. 1993). The other studies also reported the variation of morphometrics and biochemical charateristic of giant gourami strains (Soewardi et al. 1995; Soewardi 1995; Kusmini et al. 2000; Suseno et al. 2000, Abulias et al. 2005; Nugroho dan Kusmini 2006; Bhagawati and Abulias 2008). However, those studies were only emphasized on giant gourami strains from Java. So far, only one study was performed on molecular and morphological characteristics on geographic strains of giant gourami from Java and Sumatra (Nugroho et al. 2013). However, that study was used RAPD as genetic marker and could not differentiate among strains. In fact, this information is vital to clarify taxonomic status of giant gourami strains from Java and Sumatra and has also important implication in breeding and culture development of this species. Therefore, a study on molecular characteristics of giant gourami strains from Java and Sumatra is urgently reuqired in order to define taxonomic status of each strain and support morphological data.

Various molecular markers has been used in population study and species identification. Among them, the cytochrome c oxidase 1 gene is a commonly applied marker for such studies in animals. This marker has been successfully used on population genetic studies of various animals, such as population genetic study on *Tridacna crocea* and *T. maxima* (Kochzius and Nuryanto 2008; Nuryanto and Kochzius 2009), and species identification studies in various animals, such as in Australian fish (Ward et al. 2005, 2008a,b, 2009), Antartic ocean's animals (Grant et al. 2010); marine crustacea (Radulovici et al. 2009), marine metazoa (Bucklin et al. 2011); marine Indian fishes (Lakra et al. 2011). Most recently, Nuryanto et al.

(2017) proved that the COI gene is a reliable marker for fish larvae identification. Those successful studies proved that the COI gen is a potential candidate gene due to its high mutation rate. It has been reported by Bucklin et al. (2011) that mutation rate of the COI gene is higher than other mitochondrial genes. Therefore, it is expected that this gene can be used to differentiate four giant gourami strains from Java and Sumatra.

Here we characterized Soang, Batanghari, Tambago, and Oranye giant gourami strains using partial sequences of cytochrome c oxidase 1 to obtain information on morphological and molecular divergences among giant gourami strains and to evaluate the taxonomic status of those four strains of giant gourami.

MATERIALS AND METHODS

Sampling sites and samples collection

Fish samples were collected purpossively from three locations in Java and Sumatra, namely: Ciamis (West Java), Payakumbuh (West Sumatra), and Jambi. Fin clips were cut off from caudal fin of each individual. The fin clip samples were preserved in 96% of ethanol.

DNA isolation

Total DNA was extracted using DNAeasy kits from Thermoscientific following the procedures from the company (www.thermofisher.com).

Marker amplification

Partial sequences of the COI gene was amplified using a pair of universal primers from (Ward et al. 2005) as follow: FISHF2: 5'-TCG ACT AAT CAT AAA GAT ATC GGC AC-3' dan FISHR2: 5'-ACT TCA GGG TGA CCG AAG AAT CAG AA-3'. Amplifications were conducted in 50 µl total volume of reagents. The PCR reactions containing of 1X buffer PCR, 2 mM of $MgCl_2$, 0.2 mM of each primer, dNTP mix for 0.2 mM, 1 U of Taq polymerase, 0.5-2.0 ng/µl of template DNA. Final volume was obtained after the addition of ultrapure water (Thermoscientific) until the reagents reached 50 µl. Thermal condition was set as follows, predenaturation on 95 °C for 4 minutes and followed by 35 cycles which was consisted of 30 seconds of denaturation on 95 °C, 2 minuteson 55 °C for denaturation, and 1 minute extension on 72 °C. Finale extension was performed on temperature of 72 °C for 5 minutes. Amplicons were visualized in 1% agarose gel electrophoreses.

Sequencing

Qualified PCR products were sent to 1st BASE (www.base.asia.com) for sequencing.

Sequences editing

The sequence of COI gene were edited using freely availaible Bioedit software (ver.7.0.4.1; Hall 1999) and double checked manually. All sequences were submitted to genbank and BOLD system to check their orthology and were aligned toghether using ClustalW (Thompson et al. 1994) in Bioedit software (ver.7.0.4.1; Hall 1999). All haplotype sequences has been deposited in genbank with the accession number of KY950358-KY950369.

Data analysis

Sequences divergences among strains were estimated based on Juke and Cantor subtitution model using DnaSP software ver. 4 (Rojas et al. 2003). The *Fst* value was estimated through analysis of molecular variance (AMOVA) which was run in Arlequin software (version 2.0; Schneider et al. 2000). Taxonomic tree was constructed based on K2P neighbor-joining algorithm using MEGA 5.0 software (Kumar et al. 2008). Branching topology was supported by 1000 non parametrics bootstraps replicates. Polarization of branching pattern was performed by added the sequences of Sabah strain (Nuryanto et al. 2012), *Trichogaster trichopterus* (accession number of JQ667586.1, JQ667584.1, and JQ667580.1.) as outgroup comparison.

RESULTS AND DISCUSSION

Morphological characteristics

Increasing number of giant gourami strains shows significant morphological differences. This condition is interesting to be studied, especially on the taxonomic status among each other. Our observation on four different geographic strains showed that Soang (Figure 1.A), Batanghari (Figure 1.B), Tambago (Figure 1.C), and Oranye strains (Figure 1.D) had different body form and coloration. Soang and Batanghari strains had similar scales color on upper part of the body, i.e. blackish brown. However, both strains had different color of their scales on the abdomen part. Abdomen scales of Soang strain had beige coloration, whereas those on Batanghari strain was blackish brown similar to the scales on the upper part of their body. Scales color on upper body part of Tambago strain were darker than those on Soang and Batanghari strains with lighter scales on abdomen part. The scales of Oranye strain were orange. Moreover, Batanghari strain could be distigushed from three other strains based on the length of ventral fins (filaments). The filaments of Batanghari strain only reached in the midle part of the caudal fin, whereas the other three strains had longer filaments and they filaments exceeded the caudal fin.

Soang and Batanghari strains could also be differentiated by their body and caudal peduncle height, fin rays in dorsal and anals fins, scales number on lateral lines, and operculum color. Our result was congruence with the result of Setijaningsih et al. (2007) who observed morphological differences among bastar, blue sapphire, and Paris strains. Those phenomena was also observed by Suwardi et al. (1995) on other gourami strains. Moreover Tanjung et al. (2011), noted that Soang gourami (angsa/galunggung) has a specific colour which is a bit lighter than normal, growth rate and viability are relatively higher than others, may reach a particular size which is bigger than other strains. Nugroho (2011) also observed a different scales color among strains. According those

previous studies, it seems that morphological variation in different gourami strains was a common phenomenon. Therefore, it was not surprising if in this study we observed some morphological differences among Soang, Batanghari, Tambagao, and Oranye strains of gourami.

If we follow the morphological species concept which stated that species status is defined solely based on morphological similarities and differences, those four giant gourami strains could be categoryzed as different species. This argument in agreement with Claridge et al. (1997) who stated that individuals or populations which having morphological divergences could be referred as different species. However, it was common that prominent morphological diferences occured among individulas from different populations, especially on geographically separated populations. In animal taxonomy, geographic populations that shows morphological divergences were called as subpopulations or sub species (Mayr and Ashlock 1991). However, in fiseries the morphological divergent among populations are usually named as strains. However, morphological divergences among giant gourami from Java and Sumatra were still unclear whether they belonged to different species or on subspecies level (or strain). To solve this problem, additional character is needed. In this study we used partial sequences of cytochrome c oxidase 1 gene.

Molecular characteristics

Multiple alignment of the COI gene from 38 individuals of four giant gourami strains showed that the length size of fragments was 452 base pair (bp). Of all sequences analysis from 38 individuals resulted in a total of 9 haplotypes with 10 (2.21%) polymorphic sites with the haplotype diversity and nucleotide diversity values were 0.760 ± 0.051 and 0.277% ± 0.199, respectively. From all polymorphism value obtained in this study, it indicated that the COI gene of giant gourami had low genetic polymorphism because the frequency of the most common sequence sites reached more than 95% (110%-2.21% = 97.79%). According to Hartl and Clark (1997), loci was referred as polymorphic loci when the most common allele had the polymorphic frequency less than 95%.

The obtained low polymorphisms might occur due to the COI gene of giant gourami strains had a low nuclotide diversity (0.277%). According to Kochzius and Nuryanto (2008), the nucloetide diveristy value less than 1% indicates that the marker has low nucleotide diversity. Low level of nucleotide diversity was also observed on the cytochome b gene in three giant gourami strains from Java (Nugroho et al. 2008). However, the result of this study was not congruence to that conducted by Nugroho et al. (2008) since we used different molecular markers. Nevertheless, our present result was in line with Nuryanto et al. (2012) study that used the same molecular marker. Both our present study and Nuryanto et al. (2012) study observed low level of the COI nucleotide diversity on giant gurami strains. This means that the COI gene on giant gourami had less divergence compare to those on other fish species (Castro et al. 2007) and other group animals (Nuryanto and Kochzius 2009; Kochzius and Nuryanto 2008).

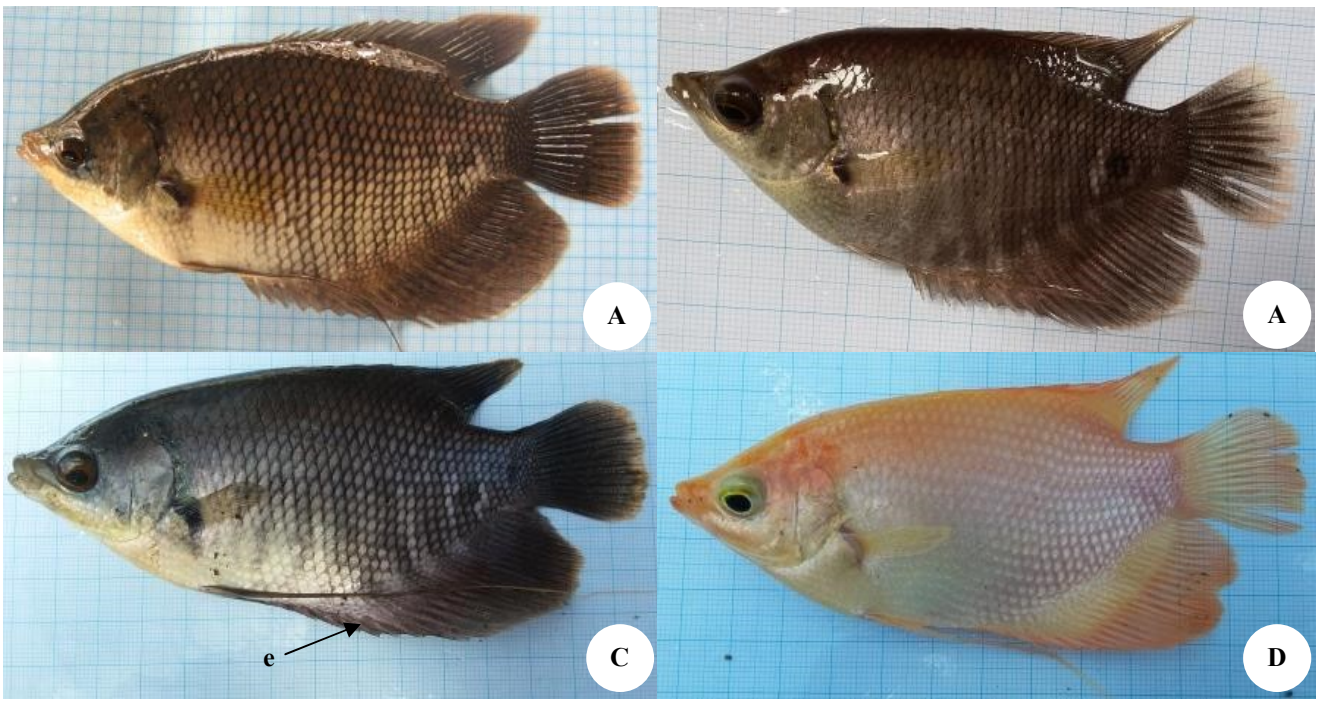

Figure 1. The observed samples of giant gourami strains. Note: A. Soang strain, B. Batanghari starin, C. Tambago strain (Payakumbuh), D. Orange strain (Payakumbuh), e. prominent copper color could be seen in life individuals

From population genetic diversity analysis, it showed that haplotype diversity values ranging from 0.298 ± 0.133 on Batanghari strain to 0.600 ± 0.215 on Oranye strain, while nucleotide diversity values ranged between 0.091% ± 0.097% on Batanghari strain and 0.446% ± 0.374% on Soang strain. The complete data including the number of sample, hapotype number, haplotype and nucleotide diveristy values for each strain was presented in Table 1.

It can be seen from Table 1 that most of the strains had the medium level of genetic diversity except for Batanghari strain which shows the low level of haplotype diversity. According to Nei (1987), the value of genetic diversity in range of 0.5-0.7 was classified as medium category, while the value of 0.8-1 was high category, and 0.1-0.4 was low category. Low level of haplotypic diversity in Batanghari strain could be due to this strain is newly bred and still under higher breeding pressure compared to the established strains (Soang, Tambago, and Oranye strains). This selection has been done in order to obtain high quality offspring with higher growth rate and resistance to diseases. Therefore, it is reasonable that Batanghari strain had low haplotype diversity. The low to medium level of genetic diversity was a common situation in cultivated fish populations. Our result was similar to previous studies either in fish (Alarcon et al. 2004; Yoon and Park 2002) or in plant (Mandel et al. 2011), which also found that cultured populations are genetically less diverse than those wild populations. However, study from Yang et al. (2008) found that genetic diversity on cultivated mud carp populations did not decrease. The difference between our present study and Yang et al. (2008) study could be due to both studies used a different genetic marker. Here, we used COI gene while Yang et al. (2008) used microsatellite marker. Different genetic markers had different evolution rates leading to a different genetic diversity values and trends among studies, which used different genetic markers.

Genetic divergences among strains

Of all molecular identification among observed strains, genetic divergence value among sequences was 1.410, while genetic divergences among individuals within strain range from 0.409 in Batanghari strain to 2.000 in Soang strain. Molecular divergences among strains ranged between 1.289 (Batanghari: Oranye) and 3.167 (Soang: Oranye). Low level of molecular divergences could be also observed in branch length of the phylogenetic tree (Figure 2).

It could be pointed out from Table 2 that the COI gene on four giant gourami strains has low nucleotide divergence. A low sequence divergence was also reported by Nuryanto et al. (2012) on four giant gourami strains from Java (Soang, blue safir, jepang, and mutiara). This data was strengthen by the fact that all the strains were still threated as a single species, namely *Osphronemus goramy* Lacepede, 1801. This decision was made based on Peg et al. (2006) who obseved that intra-specific sequences divergences ranged from 1% to 3%. Even, when we refer to Nuryanto et al. (2007), sequences divergence among species might reach higher than 4%. This species delimitation was also supported by BLAST result which showed that sequences similarities of four samples ranged from 99% to 100% to COI gene sequences of *O. guramy* available in genbank. Similar result was also resulted when the samples were subjected to barcode of life data identification system (BOLD system) where the strains showed the sequence similarity of 98.79%-100% to the sequences of *O. guramy* available in BOLD system. Nuryanto et al. (2017) also found a low intraspecific genetic divergence on fish larvae collected in East Plawangan, Segara Anakan, Cilacap.

Table 1. Number of sample (N), haplotype number (nhp), haplotype diversity (*h*), and nucleotide diversity (π) of each giant gourami strain from Java and Sumatra

Strain	N	nhp	*h*	π (%)
Soang	4	2	0.500 ± 0.265	0.446 ± 0.374
Batanghari	19	4	0.298 ± 0.133	0.091 ± 0.097
Oranye	6	3	0.600 ± 0.215	0.222 ± 0.199
Tambago	9	3	0.667 ± 0.105	0.396 ± 0.285
Total	38	12		

Table 2. Juke and Cantor genetic divergences intra-and inter-strains of giant gourami (%) based on 456 basepairs nucleotide length

Strain	Soang	Batanghari	Oranye	Tambago	Sabah
Soang	**2.000**				
Batanghari	2.211	**0.409**			
Oranye	3.167	1.289	**1.000**		
Tambago	3.056	1.485	1.389	**1.778**	
Sabah	30.000	28.711	29.500	29.167	**1.000**

Note: Bold values indicate within strain sequences divergence

Table 3. Pairwise correlation analysis among gourami strains or populations

Strain	Soang	Batanghari	Orange	Tambago
Soang	-			
Batanghari	0.655^{***}	-		
Oranye	0.442^{*}	$0,595^{***}$	-	
Tambago	0.394^{**}	$0,551^{***}$	$-0,009^{ns}$	-

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

Table 4. Analysis of molecular variances among gourami strains

Source of variance	d.f.	Sum of square	Variance components	Percentage of variation
Among strain	3	6.452	0.231 Va***	50.85
Within strain	34	7.601	0.224 Vb	49.15
Total	37	14.053	0.455	
F_{ST}: 0.508^{***}				

Note: Va and F_{ST}: p-value = 0.000 ± 0.000; * = $0.05 \geq p \geq 0.01$, ** = $0.01 > p \geq 0.001$, *** = $p < 0.001$; NS = not significant

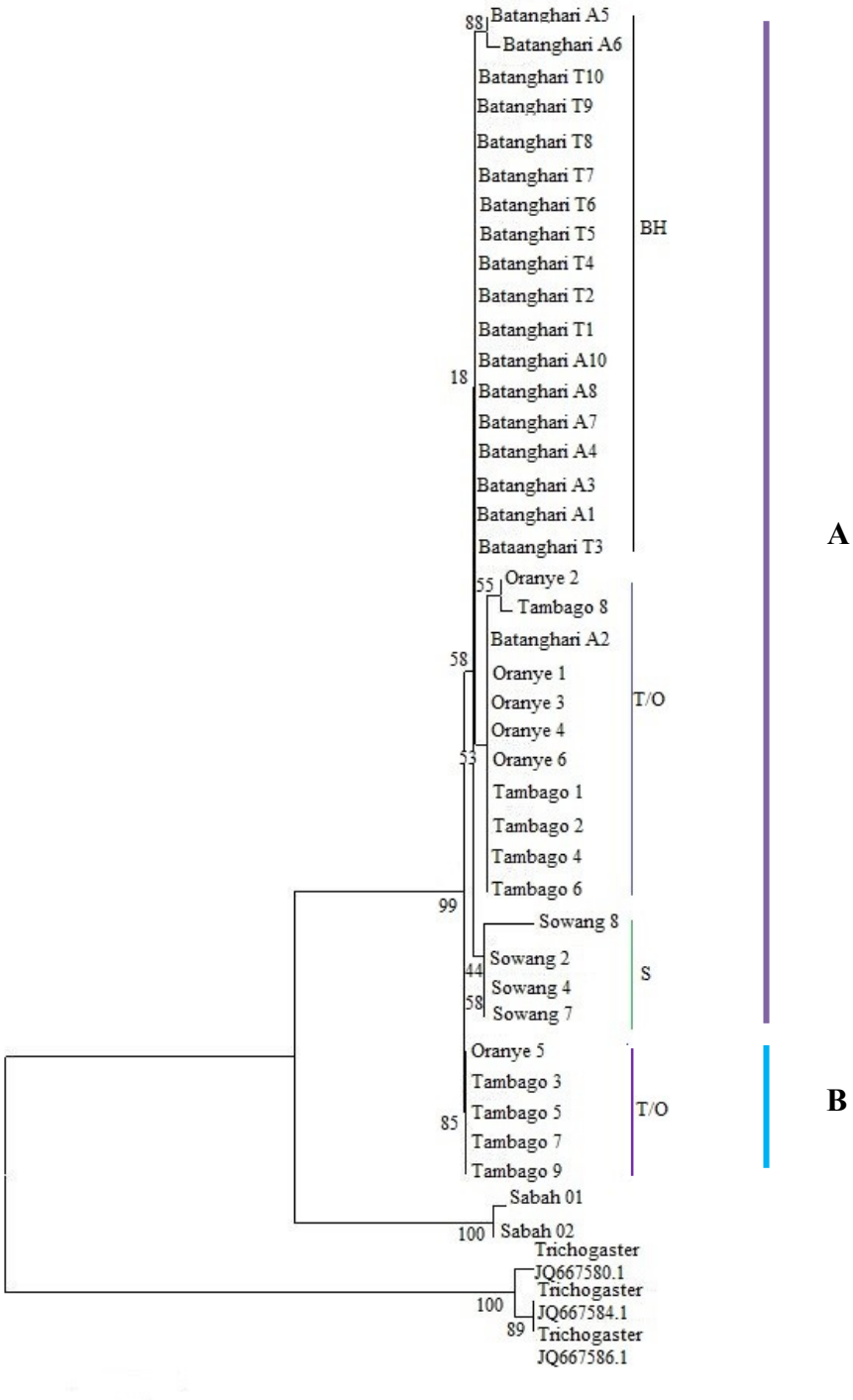

Figure 2. Neighbor-joining tree showing the separation giant gourami strains. Note: The values under or upper the lines indicates bootstraps values, BH = Batanghari, T/O = Tambago/Oranye, S = Soang

The result of sequence divergence analysis supported the morphological data, which placed the giant gourami populations from Java and Sumatra into different strains, except for Tambago and Oranye strains. In addition, the result of fixation index (Fst) data among populations also showed a significant fixation index among populations except for Tambango and Oranye strains. Detailed fixation index was presented in Table 3.

Similar result was obtained from AMOVA analysis, where a significant genetic difference was observed among populations (Table 4). This difference indicated that each population belongs to different strains which was in line with the present status of each population known by fish farmer in each region. However, no genetic difference was observed among Tambago and Oranye. This genetic similarity was rather surprising since both strains had

significantly different in their morphological performances (Figure 1), where Tambago had blackish brown scales in upper part of the body and bronze scales in ventral scales, while Oranye strain had orange scales in all part of the body. This genetic similarity among different individuals with varied morphological performances could be due to three reasons. First, it could be caused by interbreeding among them since they are cultivated toghether in the same pond and the same treatment. Secondly, it could be due to the convergence evolution among strains which result in genetic similarity due to they live in the same condition and the same selection pressures. Thirdly, it might be the scales color shown in sample strains was not genetic basis, although this hypothesis needs a further clarification.

Based on the Figure 2, it can be clearly seen that giant gourami strains formed a monophyletic clade compared to the out-groups samples with short branching pattern among subclades. This indicated that all strains belong to a single species. Accoding to Mishler and Brandon (1987) and Taylor et al. (2000) all individuals forming a monophyletic group are phylogenetically classified as single species. The short branching pattern proved that slight genetic divergences occured among strains (Table 2). However, that slight genetic differences were reliable enough to differentiate among strains, which could be observed from Fst and populations structure analysis (Tables 3 and 4). Therefore, it could be stated that phenotypically strains of giant gouramy has correlation with the genetic basis of those phenotype as shown from molecular divergences, Fst, populations structure, and NJ tree tests.

Figure 2 also indicates that giant gourami clade was divided into two subclades with strong support of bootstrap value (99). The first subclade consisted of Soang, Batanghari, Tambago and Oranye strains. The second clade was formed by one individual of Oranye strain (Oranye 5) and four individuals of Tamabgo strain (Tambago 3, 5,7, and 9). This means that giant gourami has more than one common ancestor although it originated from a single primitive ancestor.

Interestingly, individuals of Tambago and Oranye strains were separated into two different clades. This might be due to that Tambago and Oranye individuals on the subclade A is a result of breeding processes among soang, batanghari, Tambago, and Oranye broodstocks. This reason based on information from local informants from Payakumbuh where Payakumbuh Seed Centre was located. Moreover, in this location, the origin of sowang strain was imported from Java and Batanghari strain from Jambi, which were then breeded with local strains (Tambago and Oranye). On the other hand, Tambago and Oranye individuals from subclade B are suggested as original strain from Payakumbuh. Therefore, they were separated from other individuals of Tambago and Oranye strains and create separate subclade.

All observed populations had the range value from low to medium level of genetic diversity and had closed phylogenetic relationships. These were advantageous for giant gourami cultivation effort as a basis approach for breeding test among strains. Furthermore, the further breeding effort of giant gourami strain from different populations and different ancestral lines is important to rise the genetic variability. This breeding technique is expected to obtain giant gourami offspring with an expcted phenotypic characters, such as high diseases resistant and high growth rate.

It can be concluded that Soang, Batanghari, Tambago, and Oranye strains showed morphological and molecular differences among the sample gourami strains except for Tambago and Oranye strains. This means that partially, morphological divergences were possitively correlated with their molecular charactersitics although some cases had negative correlation. All strains showed low molecular divergences, yet those four giant gourami strains could be categoryzed as a single species, i.e. *Osphronemus goramy* Lacepede, 1801. Since they had significant molecular divergences, fixation index (Fst), significant population structure, and phylogenetic analysis, the grouping of giant gourami samples into four different strains is appropriated.

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