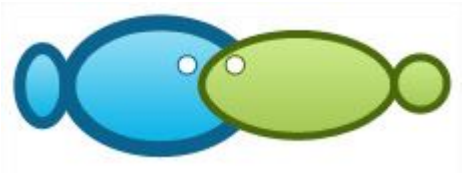

Genetic characteristics of senggaringan fish (*Mystus singaringan*) from Klawing river, Brantas River and Thailand as the basis of conservation and domestication

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Abstract. The aim of this study was to determine the genetic relationship and distance of *Mystus singaringan* from Klawing river in Central Java, from Brantas river in East Java and from Thailand. Five samples of *M. singaringan* were taken, and the *CO1* gene was isolated and amplified by PCR. The sequencing results were analyzed for kinship using the Maximum Likelihood method based on Tamura-Nei model, and genetic distance assessed using Kimura two-parameter method. The results revealed that the *COI* genes of *M. singaringan* from Klawing river had 99% sequence identity with *M. singaringan* from Brantas river and from Thailand. The base identifier of the *M. singaringan* sequences in Indonesia from Klawing river was guanine, while the Brantas river was adenine. The genetic distance of *M. singaringan* from Klawing river and from Brantas river (the origin of Klawing river) was 0.07 and 0.09 respectively, while the genetic distance from *M. singaringan* of Thailand was 0.14. These genetic distances were very low, and within the local population of the Klawing river, no genetic distance or polymorphism was seen. The results of this study provide an adequate basis for the conservation and the domestication of senggaringan fish.
Key Words: mitochondrial *CO1*, genetic variation, genetic kinship, fish conservation, *M. singaringan*.

Introduction. There are 60 freshwater fish species of Bagridae reported in Indonesia and only found in Java, Sumatra, and Kalimantan (Hubert et al 2015). Some species in Bagridae Family are also found in Thailand (Supiwong et al 2011; Supiwong et al 2013) and India (Punhal et al 2018). One native fish of Bagridae family with high commercial value is *Mystus singaringan* that is found around Klawing River, Purbalingga Regency, Central Java Province. The local name of this species is Senggaringan fish and the classification as *M. singaringan* has been confirmed (Pramono et al 2017) using a molecular approach of DNA barcoding with mitochondrial DNA of cytochrome-c-oxidase subunit 1 (mtDNA COI) from *M. nigriceps* (Heltonika 2009; Hendri 2010; Suryaningsih et al 2012). The exploitation of *M. singaringan* for commercial purposes and consumption is relatively high. This activity will certainly influence the decline of *M. singaringan* population; therefore, domestication of the species is urgently needed (Teletchea & Fontaine 2014).

The success and the development of domestication strategies require an understanding of several biological aspects, particularly regarding the genetic characteristics, structure, and diversity of target species (Han et al 2011). Genetic information is key for the development of appropriate aquatic resource management (So et al 2006; Kaleshkumar et al 2015; Yodsiri et al 2017) and population stability, which is related to the adaptability, survivability, and reproductive abilities of the fish (Frankham 1996; Hughes et al 2008). Genetic diversity reflects the ability of the fish population to adapt to changes in the habitat and to indicate the growth performance (Barasa et al 2014). Low genetic diversity can damage the fitness and the reproduction of a species (Han et al 2011), and this parameter can be used as one of the selection criteria for

restocking programs (Nuryanto et al 2007), conservation strategies (Jang et al 2017), and successful breeding (Barasa et al 2014). The genetic potential of *M. singaringan* in Klawing river has not been evaluated yet. Intraspecific genetic information is crucial to confirm the future adaptive potential and the resilience of both wild and domesticated fish.

The genetic characteristics and diversity of fish populations in public waters can be traced using mtDNA *CO1* as a genetic marker. Small segments of mtDNA that include the *CO1* genetic marker can be used to accurately sort and identify species that may be difficult to distinguish (Ward et al 2005; Muchlisin et al 2013), including various vertebrates and invertebrates, such as pufferfishes (Kaleshkumar et al 2015), bamboo shells/razor clams (Trisyani et al 2016), cuttlefish (Pratasik et al 2016), and sea urchins (Toha et al 2015). This approach of DNA barcoding can also be used for more in-depth studies, such as relationship studies (Erickson & Driskell 2012; Huang et al 2016), phytogeography (Yu et al 2014), population genetics (Craft et al 2010), analysis of genetic variation for population stability and resilience (Hughes et al 2008), and aquatic resource management (Kaleshkumar et al 2015).

The aims of this study were as follows: (1) identification and molecular characterization of *M. singaringan* from Klawing River; (2) determination of the relationship and genetic distances between *M. singaringan* from Klawing River Central Java; Brantas River in Mojokerto, East Java; and Thailand; (3) investigation of the level of genetic variation in *M. singaringan* in Klawing River.

Material and Method

Sample collection. Senggaringan fish were obtained from Klawing river in February 2017, using gill nets with the help of local fishermen. Sample collection was conducted in five stations; in the villages of Pasren (07°25, 284'S, 109°24, 003'E), Jetis (07°26, 145'S, 109°23, 208'E), Senon (07°27, 944', 109°21, 836'E), Bantaori (07°28, 586'S, 109°19, 306'E), and Congot Kedungbenda (07°29, 558'S, 109°20, 270'E) (Figure 1).

Station selection was based on existing land use. The minimum sample size was 40 individuals' catches per unit effort. For genetic studies, a single fish from each station was sampled. The remaining fish were used for adaptation studies in captivity (not published). A total of five fish were used to take caudal fin tissue samples and stored in 95% ethanol.

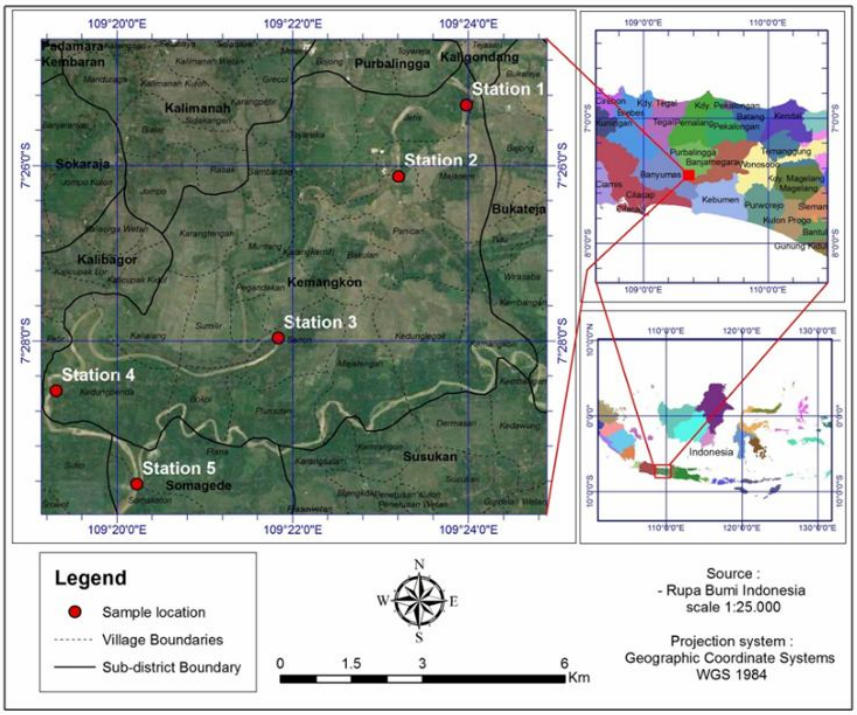

Figure 1. The research location map of Klawing river in Purbalingga Regency, Central Java.

Molecular identification. Genomic DNA extraction was carried out using the Quick DNA Tissue/Insect Miniprep Kit (Zymo Research) method. The *CO1* gene was amplified using the universal primer pair L*CO1*490: 5'-ggtcaacaaatcataaagatattgg-3' and HCO2198: 5'-taaacttcagggtgaccaaaaaatca-3' (Folmer et al 1994). The polymerase chain reaction (PCR) reaction mixture was 12.5 µL PCR Buffer (2x), 0.5 µL forward primer (10 pmol μL^{-1}), 0.5 µL reverse primer (10 pmol μL^{-1}), KOD FX Neo polymerase (1.0 U μL^{-1}), 1 µL DNA template, and 5 µL ddH_2O for a total reaction volume of 25 µL. Amplification was performed using the KOD FX Neo buffer system (Catalog No KFX-201; Toyobo, Osaka, Japan) in an Agilent SureCycler 8800. The PCR program was 35 cycles of: pre-denaturation incubation at 95°C for 3 minutes, denaturation at 98°C for 10 seconds, annealing at 50°C for 30 seconds, and extension at 68°C for 1 minute; then the mixture was held at 40°C. The results of PCR were visualized by electrophoresis on 1% agarose gel. Bi-directional sequencing was performed by First Base CO (Malaysia) using Big Dye© terminator v3.1 cycle sequencing kit (Applied Biosystems).

Data analysis. Editing of the sequencing results and determination of nucleotide composition were carried out using Mega7 software (Tamura et al 2011), while the DNA sequences were aligned with ClustalW version 1.4 (Thompson et al 1994). The pattern and level of substitution were estimated using Kimura's two-parameter model. Species identification was performed online with GenBank data on NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with various access codes using the Basic Local Alignment Search Tool (BLAST) method (Altschul et al 1997) and Barcode of Life Data (BOLD) system (http://www.boldsystems.org/index.php/IDS_OpenIdEngine). Genetic distances were analyzed using Kimura two-parameter method, and phylogenetic trees identified using the maximum likelihood method based on Tamura-Nei model (Tamura & Nei 1993).

Results and Discussion

Molecular identification and characterization of M. singaringan. The results of genetic analysis using mtDNA *CO1* genetic markers revealed fragments of 704, 716, 716, 711, and 724 bp in length amongst others (Table 1). In a previous study, Pramono et al (2017) reported fragments reaching 697 bp. The sequences obtained in this study (Figure 2) confirm that the amplified product is a functional fragment of the *CO1* gene, which Folmer et al (1994) have reported to be around 700 bp in length. The sequencing results showed no codon stops or coherent amino acid partial codes. This agrees with the work of Chakraborty & Ghosh (2014) identifying 175 species of freshwater fish in India, and with the data published by Nuryanto et al (2007) on *Pinctada trocea* and *P. maxima* pearl oysters in Indonesian waters.

Table 1

Identification of similarity level of the fish DNA sequence

No	*ID sample, pb*	*Identification result (% similarity)*		*Species*
		BLAST	*Barcode System*	
1	MS1, 704	99	99.84	*Mystus singaringan*
2	MS2, 716	99	99.84	*Mystus singaringan*
3	MS3, 716	99	99.84	*Mystus singaringan*
4	MS4, 711	99	99.84	*Mystus singaringan*
5	MS5, 724	99	99.84	*Mystus singaringan*

The length of the conserved *CO1* gene base in this study was 616 bp (Figure 2). Hebert et al (2003) reported that to distinguish species, the length of the *CO1* gene used was approximately 650 bp. The length of the *CO1* gene varies between fish species. In *Eumicrotemus taranetzi,* the gene is 466 bp (Lee et al 2015), while in *Chilomycterus reticulus, Arothron hispidus* and *Lagocephalus guentheri* the gene length is 631, 636, and

615 bp, respectively (Kaleshkumar et al 2015), while 456 and 484 bp have been reported for *Tridacna crocea* and *T. maxima* (Nuryanto et al 2007), respectively.

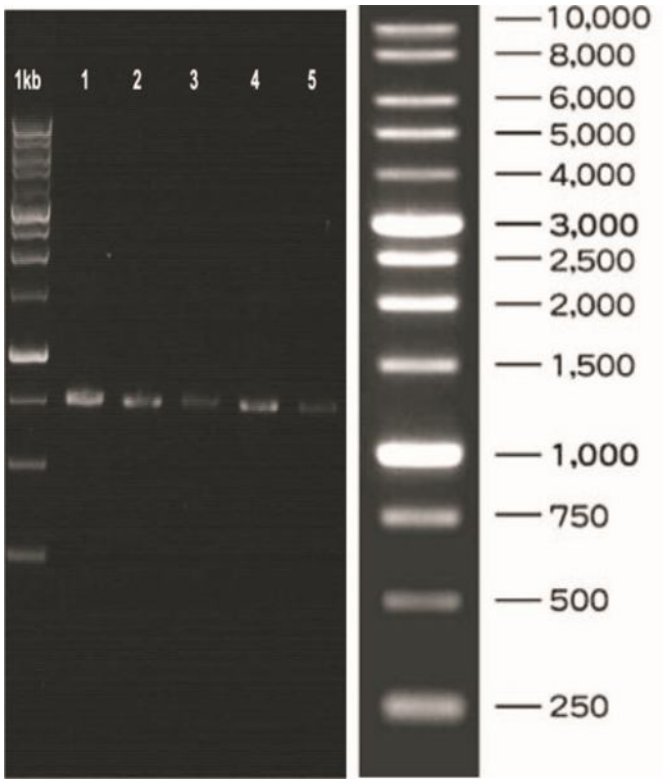

Figure 2. The *CO1* gene amplification result of *M. singaringan* from Klawing River.

Nucleotide sequencing and BLAST analysis revealed the sequence identity of samples with *M. singaringan* data to be 99%. Using the BOLD system, the sequence identity with *M. singaringan* was 99.84% (Table 1). These confirmed the taxonomic identity of the fish to be *M. singaringan*. Taxonomically, *M. singaringan* belong to the Phylum of Chordata, Class of Actinopterygii, Order of Siluriformes, Family of Bagridae and Genus *Mystus*. This study supported the results of Pramono et al (2017) in identifying the species, as well as Hubert et al (2015), who reported that fish from the Bagridae Family are only found in Sundaland and absent in Wallacea and Sahul.

The *CO1* gene has been used by several researchers for species identification, taxonomic confirmation, variation, and genetic relationships in both fish and biota in freshwater (Muchlisin et al 2013; Hubert et al 2015; Dahrudin et al 2016), brackish water, and sea water (Lee et al 2015; Kaleshkumar et al 2015; Toha et al 2015). The superiority of the *CO1* gene for species identification lies in its high mutation rate, lack of recombination (Hebert et al 2003), and large variation among populations (Bucklin et al 2003). Furthermore, Hubert et al (2015) proved that this gene can be used to identify freshwater fish in Indonesia, identifying 1,218 species of 84 families, including 1,172 native species from 79 families. In addition, there were also 630 species of endemic fish. Dahrudin et al (2016) validated the species name of several endemic fish, including *Barbodes platysoma*, *Mystus abbreviatus, Ompok javanesis*, *Puntius aphya* and *P. bramoides*.

Kinship and genetic distance. Kinship analysis of *M. singaringan* samples from Klawing river revealed a clear separation from *M. nigriceps* (access code KU692658.1) in phylogenetic trees. However, one main cluster of *M. singaringan* was observed from Brantas river (access codes KU692659.1, KU692660.1, KU692661.1, and KU692662.1) and from Thailand (access code JQ289146.1) (Figure 3). This cluster indicates past biogeographic connections, suggesting that this species in Sundaland originated from Peninsular Malaysia, Chao Phraya River, and Mekong (Hutama et al 2016).

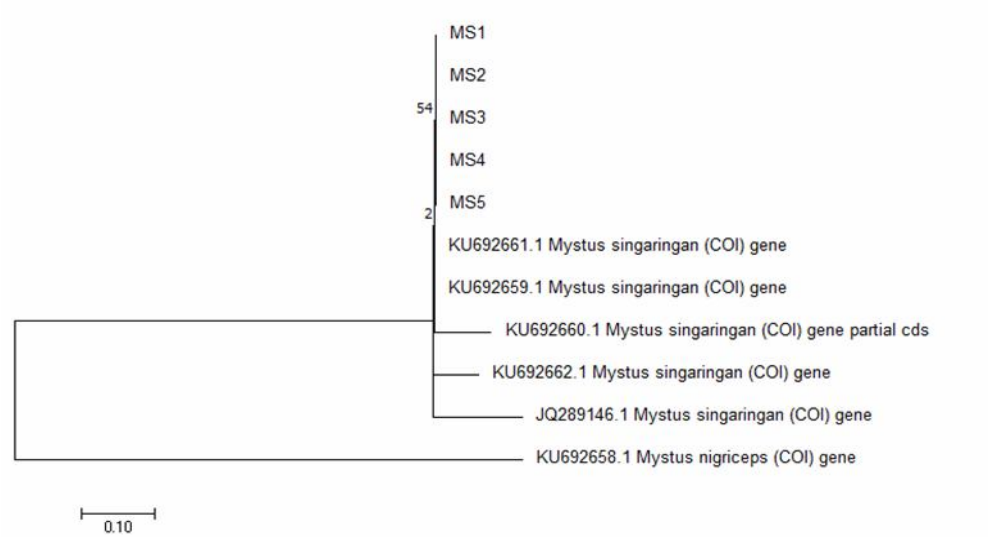

Figure 3. The P-distance phylogenetic tree of senggaringan fish (*Mystus singaringan*) according to the *CO1* gene. Note: KU692662.1 = Mojokerto (Dahruddin et al 2017); KU692661.1 = Mojokerto (Dahruddin et al 2017); KU692660.1 = Mojokerto (Dahruddin et al 2017); KU692659.1 = Mojokerto (Dahruddin et al 2017); JQ289146.1 = Thailand (Supiwong et al 2011); KU692658.1 = *Mystus nigriceps* Mojokerto (Dahruddin et al 2017); MS1-5 = fish sample from Klawing River Purbalingga.

The separation of the kinship levels confirms that *M. singaringan* and *M. nigriceps* have different nucleotide characteristics, and confirms that the fish sampled were not *M. nigriceps* that were known as Senggaringan fish to previous researchers, Heltonika (2009) and Hendri (2010).

Kinship of Siluriformes is generally divided into three lineages forming a single subfamily, and the kinship of *M. singaringan* is a lineage with *M. vittatus, M. horai, Batasio tengana, M. malabaricus, M. bocourti, M. bleekeri, M. gulio, M. multiradiatus, M. rhegma, M. cavasius, M. tengara, Sperata aor, S. seenghala, Bagrus bajad, B. filamentosus, B. macracanthus, Pseudomystus siamensis, Eutropiichthys vacha,* and *Batasio travancoria* (Punhal et al 2018).

Sequencing and sequence alignment results of Senggaringan fish from Indonesia showed differences in the nucleotide sequences. The nucleotide bases of *M. singaringan* in the present study contained guanine (G), while the nucleotide bases of fish from Brantas river contained adenine (A). These differences were used to differentiate *M. singaringan* from Brantas and Klawing rivers. The differences in nucleotide bases did not affect the *CO1* gene nor the species identification. In accordance with Yao et al (2010), the *CO1* gene method is a reliable method to identify different species even when considerable variation between species and low intra-specific variations are present.

Genetic distance describes the number of mutations or evolutionary events between two samples. The genetic distance in this study was analyzed using the Mega7 (Maximum Composite Likelihood) program to compare *M. singaringan* population of Klawing River with those of the Brantas River and Thailand. The genetic distance between *M. singaringan* from Klawing river and those from Brantas river was 0.07 and 0.09 respectively, while that between *M. singaringan* from Klawing river and Thailand was 0.14. The genetic distance between *M. singaringan* of the present study and *M. nigriceps* was 32.80 (Table 2). No genetic distance was observed between *M. singaringan* from each sampling station (Table 2). The sequencing results indicate no level of polymorphism. There were three haplotypes distinct to the three regions, and *M. singaringan* from Klawing river showed no gene flow, no habitat fragmentation isolating the fish, and no speciation. Those factors will result in a very limited stock and population size. Gene flow can occur when a species flows from one habitat to a new habitat, although this occurs very slowly (Farrag et al 2015). However, Jang et al (2017) reported that ecophysiological differences from river branches can inhibit gene flow between populations.

Table 2

Genetic distance of senggaringan fish (*M. singaringan*)

	1	2	3	4	5	6	7	8	9	10
MS1										
MS2	0.00									
MS3	0.00	0.00								
MS4	0.00	0.00	0.00							
MS5	0.00	0.00	0.00	0.00						
KU692660.1	0.09	0.09	0.09	0.09	0.09					
KU692662.1	0.07	0.07	0.07	0.07	0.07	0.18				
KU692661.1	0.00	0.00	0.00	0.00	0.00	0.09	0.07			
KU692659.1	0.00	0.00	0.00	0.00	0.00	0.09	0.07	0.00		
JQ281946	0.14	0.14	0.14	0.14	0.14	0.26	0.24	0.14	0.14	
KU692658.1	32.80	32.80	32.80	32.80	32.80	31.82	34.34	32.80	32.80	34.97

The geographical distance will reflect the genetic distance value of a distant population. The genetic distance between 12 Siluriformes fish species from various countries based on the *CO1* gene has been found to be 0.455 (Punhal et al 2018). Geographic distance is an important factor which influences gene flow and increases genetic differences in several separate locations (Kusmini et al 2011). Radona et al (2016) reported that within the Tengadak fish population (*Barbonymus schwanenfeldii*), which originated from Java and Sumatra, the genetic distance was 0.4826, while the genetic distance between Tengadak fish populations from Java and Sumatra and populations from Kalimantan was 0.5495. In the present study, genetic distance within one population or between populations did not show gene flow.

Unlike the results of Bahiyah et al (2013) which revealed high genetic variation in *Barbonymus balleroides* in the upstream of Serayu River in Central Java. There were two genetic distance clusters from six sampling stations, with the distance between clusters reaching 2%. The first cluster was above Soedirman Reservoir, while the second cluster was below it. This high genetic variation of *B. balleroides* suggests habitat fragmentation in the sampling zone due to the construction of reservoirs within 25 years (1988-2013).

The levels of M. sangaringan genetic variation in one population in Klawing river and fish resource status. Genetic diversity is very important for the sustainability of future fish resource management plans. Information on genetic variation can also be used as selection criteria for restocking programs (Nuryanto et al 2007) and conservation strategies (Jang et al 2017). The absence of genetic diversity in *M. singaringan* indicated by the absence of polymorphism and genetic distance is very concerning. Loss of genetic diversity will reduce the ability of the species to adapt to environmental changes and ultimately affect the stability of the population (Frankham 1996; Hughes et al 2008). Genetic diversity is also an indicator of the fitness of a population, adaptability, and reproductive capacity of fish in natural and artificial selection (Han et al 2011; Lorenzen et al 2012).

Conclusions. Senggaringan fish from the Klawing river were identified as *M. singaringan*. The fish showed 99.84% sequence identity and shared the same gene cluster as *M. singaringan* from Mojokerto and Thailand. The genetic distances between fish from the Klawing river and Mojokerto populations are 0.07 and 0.09, while that between Klawing river and Thailand is 0.14. Inter-population genetic distance and polymorphisms of *M. singaringan* from Klawing river were not observed. In future, efforts to conserve and develop the domestication of fish will be essential. Mixing diverse genetic stock will increase genetic variation and help increase production for restocking programs, domestication, and fish farming.

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