

Genetic diversity assessment of *Hemibagrus nemurus* from rivers in Java Island, Indonesia using COI gene

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Manuscript received: 21 May 2019. Revision accepted: 28 August 2019.

Abstract. Nuryanto A, Komalawati N, Sugiharto. 2019. Genetic diversity assessment of *Hemibagrus nemurus* from rivers in Java Island, Indonesia using COI gene. *Biodiversitas* 20: 2707-2717. Green catfish (*Hemibagrus nemurus*) is a popular freshwater fish that highly exploited in almost all the rivers in Java Island. The exploited population tends to have low genetic diversity. Meanwhile, separated populations might lead to a genetic difference among the river populations. This study aims to investigate the genetic diversity and population variation of *H. nemurus* collected at eleven rivers across Java Island. The analysis based on 465 bp fragment of the cytochrome c oxidase 1 gene from 140 individuals. Analysis of overall populations proved that *H. nemurus* had a high gene diversity ($h = 0.935 \pm 0.016$) and nucleotide diversity ($\pi = 0.073 \pm 0.036$). Within population analysis also showed that *H. nemurus* populations showed high levels of gene diversity ($h = 0.338 \pm 0.128$ to 1.000 ± 0.022) and nucleotide diversity ($\pi = 0.001 \pm 0.001$ to 0.071 ± 0.038). Those diversity values indicated that *H. nemurus* had high level of genetic diversity, except for the Citanduy population. Population comparison showed that significant genetic differences observed among populations ($p = 0.000$ for both variance component and *Fst*-value). However, pairwise comparison analysis indicated complex pattern of population differentiation. The high genetic diversity and complex pattern of population differentiation have important implication for *H. nemurus* conservation in Java Island.

Keywords: Genetic diversity, green catfish, *Hemibagrus nemurus*, Java, polymorphism

INTRODUCTION

Hemibagrus nemurus, known as green catfish or baung in the Indonesian Language, is among the popular freshwater species with high economic value. This bagrid species inhabits rivers, lakes, and reservoirs in Sumatra, Java, and Borneo (Muflikhah and Asyari 2007). It is among the popular raw materials for delicious cuisine serves either in traditional food stalls or big restaurants in Java. Nevertheless, in the present day, the restaurant's menu made from green catfish hardly found in the restaurants and food stalls, especially in Central and West Java (personal observations). The condition could indicate *H. nemurus* has already rarely found in nature due to overexploitation. The indication warned for more than ten years ago by Putro (2003) by providing data that natural catchment of green catfish in Klawing River Central Java decreased from 8.5 tons in 1998 to 5.35 tons in 2002. The Exploitation of *H. nemurus* is also possible to occur in most of the rivers across Java Island since high exploitation is a common feature in such area with dense communities.

In spite of the progressive decline of natural populations of *H. nemurus* across Java Island, there was limited data available on the genetic diversity and population variation of the river's population of green catfish in those areas. These data are essential for any conservation program; mainly, it is crucial in determining the choice of genetic units for conservation purposes and also for defining the source population for restoration

program (Raeymaekers et al. 2009).

The previous report proved that overexploitation caused species declines, endangerment, and extinction (Hauser et al. 2002; Frankham 2003). The natural population decline caused by human exploitation might lead to lower genetic diversity (Harris et al. 2002; Hutchinson et al. 2003; Allendorf et al. 2008). It had reported that *H. nemurus* populations in Klawing and Serayu Rivers have a low genetic diversity as observed in their allozyme markers (Nuryanto and Setyaningrum 2008). A complex pattern of genetic diversity and population variations were reported in *H. nemurus* populations as assessed by RFLP markers of D-loop regions (Nugroho et al. 2003; 2005). In one hand, low genetic diversity was also observed in *H. nemurus* from Java and Sumatera as observed by RFLP markers of D-loop region (Nugroho et al. 2003). On the other hand, moderate-high genetic diversity was observed in four dam populations of *H. nemurus* in Java (Nugroho et al. 2005). Similar phenomena were observed in population variation analysis. Significant genetic differentiation was reported by Nugroho et al. (2005) among four reservoir's *H. nemurus* in Java Island, whereas no significant genetic differentiation was also reported between *H. nemurus* populations from Jatiluhur and Wonogiri (Nugroho et al. 2003).

The studies from Nuryanto and Setyaningrum (2008) and Nugroho et al. (2003, 2005) had a narrow scope in term of geographical and ecosystem type coverage, while Dodson et al. (1995) only collected samples from Cirata Reservoir in West Java. Therefore, all those studies did not

provide a comprehensive picture of genetic diversity and population variation of *H. nemurus* across Java Island. Here, we reported genetic diversity and population variation in *H. nemurus* with broader geographical and ecosystem type coverage across Java Island using cytochrome c oxidase (COI) gene.

Numerous publications proved that partial sequence of cytochrome c oxidase I (COI) gene is a powerful and suitable molecular character for population genetic study (Luttikhuizen et al. 2003 Shefer et al. 2004; Barber et al. 2006; Kochzius and Nuryanto 2008; Nuryanto and Kochzius 2009; Diaz-Ferguson et al. 2010; Dorn et al. 2011; Sabando et al. 2011; Dung et al. 2013; Song et al. 2013; Zhang et al. 2014). It is due to that the nucleotide sequences of the COI gene have a high divergence within species because of its high mutation rate (Hebert et al. 2003). This study aims to investigate the genetic diversity and population variation of the green catfish *H. nemurus* populations among rivers across Java Island to provide data as a basis for defining genetic conservation unit and populations sources. The result of this study can be used as scientific references for setting genetic conservation units and choosing the source population for the restoration program.

MATERIALS AND METHODS

Study area and sampling

Hemibagrus nemurus samples collected from eleven different river systems; i.e., Cisadane (Csn), Ciliwung (Clw), Citarum (Ctm), Cimanuk (Cmk), Citanduy (Cty), Serayu (Sry), Klawing (Klw), Comal (Cml), Progo (Pgo), Bengawan Solo (Bso), and Brantas (Bnt) Rivers (Figure 1). Fish samples from Citarum and Bengawan Solo were collected from Cirata Reservoirs and Gajahmungkur Reservoirs. The populations represented lotic (river) and lentic (reservoir) ecosystems. Small piece of caudal fin clips was cut off from each fish for DNA examination. Fin

clip samples were preserved in 96% of ethanol and stored at room temperature.

Procedures

DNA extraction and genetic marker amplification

Total genomic DNA isolated with the GeneJET™ Genomic DNA Purification Kit (ThermoFisher Scientific, Waltham, Massachusetts, United States; www.thermofisher.com), following the protocol of the manufacturer. The molecular marker used in this study was the mitochondrial COI gene. A fragment of this gene was amplified using a pair of universal primers fwd_seq: TCAACCAACCACAAAGACATTGGCAC, and rev_seq: TAGACTTCTGGGTGGCCAAA AATCA (Ward et al. 2005). The PCR was carried out in a total volume of 50 µL. The PCR reactions contained 1 µL DNA template, 10 mM Tris-HCl (pH 9), 50 mM KCl, 2 mM of MgCl₂, 0.2 µM of each primer, 0.2 mM of each dNTP and 1 U *Taq* polymerase. The following temperature profile used for the PCR: 94 °C for 3 min, followed by 35 cycles of 1 min at 94 °C, 1.5 min at 43 °C, and 1 min at 72 °C. The final extension conducted at 72 °C for 5 min.

DNA Sequencing and sequence editing

The PCR products shipped to 1stBASE (www.baseasia.com) for sequencing. All sequences were edited with the program Bioedit (version 7.0.4.1; Hall 2005) and double-checked manually by eye to avoid mistakes. The sequences were translated to amino acids with the program ORF finder (www.cebl.auckland.ac.nz/index.php) and recheck by BLASTing the sequence to GenBank to exclude errors in sequencing and to verify if a functional mitochondrial DNA sequence was obtained and not a nuclear pseudogene. The first codon starts at nucleotide number one. A multiple sequences alignment obtained of 140 sequences by using clustalW (Thompson et al. 1994) as implemented in the software Bioedit (version 7.0.4.1; Hall 2005).



Figure 1. Maps of Java Island showing several rivers and the sampling sites of *Hemibagrus nemurus* (modified from Google maps)

Data analysis

Genetic diversity

Haplotype diversity h (Nei 1987) and nucleotide diversity π (Nei and Jin 1989) were calculated in Arlequin software version 3.5 (Excoffier and Lischer 2010). The calculation based on the Kimura 2-parameter substitution model.

Genetic variation among populations

Significance of genetic variation among populations was tested using analysis of molecular variance (AMOVA) and pairwise F_{ST} -values. Both statistical calculations were carried out using the software Arlequin version 3.5 (Excoffier and Lischer 2010). The Kimura 2-parameter (K-2P) substitution model was used during the calculation.

RESULTS AND DISCUSSION

DNA polymorphism and genetic diversity

Sequences alignment of the COI gene obtained of 465 bp length fragments from 140 *H. nemurus* individuals collected in eleven rivers across the Java Island Indonesia (Table 1). Among these 140 individuals, a total number of 85 haplotypes were observed, with 218 polymorphic sites or loci (33.798%) out of 645 loci. It is mean that the percentage of the most common or dominant loci was only 66.202%. The resulted polymorphisms value proved that the COI gene of green catfish *H. nemurus* was highly polymorphic. According to Hartl and Jones (2009), a gene referred as to polymorphic if the most common allele or site has a frequency of less than 95%.

High polymorphism in the COI gene of green catfish, as observed in this study is due to COI gene has a high mutation rate. It has been proven by Hebert et al. (2003) that COI gene has higher mutation rate compared with two other mitochondrial genes; such as 12S and 16S genes which are also commonly used in population genetic study. High level of genetic polymorphism in the COI gene was also reported from various species (Nuryanto et al. 2018; Nuryanto et al. 2017; Barber et al. 2006).

Table 1. Sample sites, number of sequences (n), number of haplotypes (nhp), haplotype diversity (h), nucleotide diversity (π) for *H. nemurus* across Java Island

Sample site	Code	n	nhp	Genetic diversity	
				h	π
Overall samples	-	140	85	0.935±0.016	0.073±0.036
Brantas	Bnt	8	8	1.000±0.063	0.006±0.004
Bengawan Solo	Bso	16	16	1.000±0.022	0.021±0.011
Cimanuk	Cmk	6	6	1.000±0.096	0.018±0.011
Cisadane	Csn	10	4	0.533±0.180	0.071±0.038
Klawing	Klw	8	8	1.000±0.063	0.069±0.038
Ciliwung	Clw	13	6	0.795±0.085	0.003±0.002
Citanduy	Cty	22	5	0.338±0.128	0.001±0.001
Citarum	Ctm	14	9	0.901±0.062	0.006±0.004
Comal	Cml	16	7	0.692±0.124	0.002±0.001
Serayu	Sry	14	6	0.604±0.150	0.002±0.001
Progo	Pgo	13	10	0.949±0.051	0.050±0.026

Note: *0.05 $\geq P \geq$ 0.01; **0.01 $> P \geq$ 0.001; *** $P <$ 0.001; NS, not significant

Polymorphisms level of the COI gene of green catfish in this study indicates that the used fragment is variable enough to be used in genetic diversity analysis of *H. nemurus* populations across Java Island. Overall, haplotype diversity (h) was of 0.935±0.016, and nucleotide diversity (π) was of 0.073±0.036 (Table 2). Both genetic diversity values proved that generally, the green catfish populations in Java Island have high genetic diversity. The obtained level of genetic diversity in present study comparable to that level in *Nothobranchius* fish (Dorn et al. 2011), *Channa striata* (Song et al. 2013), *Nodularia douglasiae* Bivalvia (Liu et al. 2017), Trochid gastropod (Diaz-Ferguson et al. 2010), tea Geometrid *Ectropis obliqua* (Zhang et al. 2014), two species of river's insects (Sabando et al. 2011), *Haplorchis taichui* (Dung et al. 2013). The present study and previous studies showed that the COI gene is a highly variable genetic marker in a wide range of animal phyla and proved that the COI gene is a suitable marker for population genetic studies.

High level of genetic diversity was also reported in *H. nemurus* populations from four different areas across Thailand (Leesa-Nga et al. 2005). A similar result also published on four reservoir's populations of *H. nemurus* in Java Island (Nugroho et al. 2005). However, the comparison to Leesa-Nga et al. (2005) and Nugroho et al. (2005) studies was not completely congruence because they used a different genetic marker to what we used in the present study. Here we used the COI gene as a genetic marker, while Leesa-Nga et al. (2005) used RAPD fingerprint and Nugroho et al. (2005) used *d-loop* PCR-RFLP markers. Nevertheless, here we provided more reliable and comprehensive genetic diversity data compared to Nugroho et al. (2005) since we used more variable and much more sampling sites across Java Island and represented more variable ecosystem types from lotic (rivers) and lentic (reservoirs) habitats while Nugroho et al. (2005) only collected samples from four lentic habitats (reservoirs).

Overall genetic diversity value of the COI gene of green catfish resulted in our study was higher compared to that value reported in *Tor tambroides* fish (Esa et al. 2008), mussel *Epioblasma torulosa rangiana* (Zanatta and Murphy 2007) and in *Bactericera cockerelii* (Liu et al. 2006). The genetic diversity patterns in the present study and the studies from Esa et al. (2008), Zanatta and Murphy (2007) and Liu et al. (2006) indicate that genetic diversity of the COI gene showed a complex pattern of genetic diversity level across phyla. Complex pattern of genetic variation even might be observed within species that are collected from several local populations, such as observed in *Capoeta trutta* fish (Parmaksiz and Eksi 2017) and *Macarorchestia renyi* (Pavesi et al. 2011) and other studies in various animals (Sugama et al. 2002; Baber et al. 2002, 2006; Luthikuizen et al. 2003; Kochzius et al. 2009).

The present study was also showed higher genetic diversity of *H. nemurus* population to that observed by Nuryanto and Setyaningrum (2008). However, the comparison was not congruence since Nuryanto and Setyaningrum (2008) used a different genetic marker. Nuryanto and Setyaningrum (2008) used allozyme markers which are well known as a stable marker that is less

variable compared to the COI gene that we used in the present study which is famously known as highly variable molecular marker. A not parallel comparison has also resulted when we compare present study to earlier studies although all the earlier studies also proved a complex pattern of genetic diversity. For example, a complex pattern of genetic diversity was observed in other bagrid species *Horabagrus brachysoma* from three different locations in the Western Ghats region, Kerala, India (Muneer et al. 2009). Similar results were reported to occur in *Aorichthys seenghala* (Garg et al. 2009), *Arapaima gigas* (Hrbek et al. 2005) and in *Clarias batrachus* (Garg et al. 2010). But again, since all of the previous studies used different markers with our present study, the comparison does not mean congruent.

Analysis within-population indicated that variable genetic diversity values were variable depending on the population. Haplotype diversity within population ranges from 0.338 ± 0.128 in the population from Citanduy River to 1.000 in Bengawan Solo, Brantas, Cimanuk, and Klawing Rivers. Nucleotide diversity ranges from 0.001 in Citanduy population to 0.071 in Cisadane population (Table 1). Those genetic values proved that the genetic diversity of the green catfish *H. nemurus* in Java Island ranged from low to high genetic diversity. Low-level genetic diversity on *H. nemurus* Citanduy River could be due to overexploitation. This argument was proposed based on a fact that during sample collection, we found that fishers at Citanduy River use electric shocker to catch the fish. Electric fishing was also a common phenomenon in Brantas River, but Brantas River is far broader than Citanduy River. According to Nuryanto et al. (2012) proved that the broader and bigger river has more and variable microhabitat. The wider river has more variable riverscape for a specific allele to adapt. Therefore, the impact of electric fishing was not yet visible in the Brantas River compared to Citanduy River. It has been known that there were significant correlation genetic diversity and variable riverscape (Davis et al. 2017; Brauer et al. 2016; Cooke et al. 2014; Cook et al. 2011; Olsen et al. 2010).

Electric fishing kills individuals from all life stages from eggs up to mature individuals and lead to mass mortalities even not targeted size, which might speed up the reduction of the population size of green catfish population in Citanduy River. A small population might lead to inbreeding depression. A continuous effect of inbreeding depression would decrease genetic diversity. Reduction of genetic diversity on small population might be caused by increasing of inbreeding probability with the potential of inbreeding depression (Hartl and Jones 2009). Population decline due to overexploitation may cause a loss of genetic diversity (Allendorf et al. 2008).

Moreover, overexploitation is suggested to affect population size and produce a genetic effect that contributes to extinction risk (Frankham 2003). The role of genetic factors on conservation includes the impact of genetic drift and inbreeding depression. Genetic drift has caused long term of losing genetic diversity magnitude on overexploited populations (Hauser et al. 2002).

The ten remaining populations in our present study had

a high haplotype diversity of the COI gene. Great haplotype diversity within populations of the COI was also reported in numerous animal phyla spanning from an arthropod (Barber et al. 2002), Gastropod and *Linckia laevigata* (Kochzius et al. 2009) up to fish (Hrbek et al. 2005). High genetic diversity of *H. nemurus* was also observed in the population from Java, Sumatera, and Kalimantan (Dodson et al. 1995). However, the comparison to Dodson et al. (1995) was not balanced since they used PRC-RFLP marker of mitochondrial DNA and was only collected sample from a single location in Java Island. Therefore, their sample size cannot be used as a reference in the conservation of green catfish in Java Island.

To ensure that the pattern of population genetic of *H. nemurus* obtained in the present study describe a general pattern for that species, further study using other markers such as microsatellite and D-loop regions would be very interesting. There is a general acceptance that microsatellite and D-loop are a highly polymorphic marker and suitable marker for a population study. However, the choice of both markers for population study in freshwater fish should be done with carefulness since several studies also proved both markers also showed a complex pattern of genetic diversity and population variation in various animals, including freshwater fish.

Previous studies proved that more than 14 freshwater fish species has low genetic diversity in their microsatellite loci (Cheng et al. 2017; Esa and Rahim 2013; Meldgaard et al. 2003; Esa et al. 2011; Nguyen et al. 2008; Mohindra et al. 2004; Costello et al. 2003; DeWoody and Avise 2000). Complex pattern of microsatellite markers were also reported in fish with gene diversity range from very low to very high depend on the locus (Abbas et al. 2017; Achrem et al. 2017; Gouskov et al. 2016; Jungker et al. 2011; Wang et al. 2007; Castric et al. 2002; Hauser et al. 2002; Castric et al. 2001). Low genetic diversity of certain microsatellite loci in river Sculpin *Cottus gobio* was observed although the utilized loci were considered to be highly polymorphic in an earlier study (Hanfling and Weetman 2006). Even in freshwater Bivalvia, it has been proven that the COI gene sequences of *Nodularia douglasiae* (Bivalvia) were highly more diverse than that in microsatellite marker (Liu et al. 2017) and low diversity of microsatellite markers was also reported in *Crassostrea ariakensis* (Bivalvia) which is comparable to the value obtained from PCR-RFLP COI and COIII (Zhang et al. (2005).

A complex pattern of genetic diversity was also reported when analyzed using D-loop markers. Low to medium genetic diversity pattern on RFLP D-loop control region was observed in two species of catfish with the haplotype diversity range from 0.295 up to 0.674 (So et al. 2006). A similar pattern of genetic diversity ($h = 0.00$ to 0.80) was also observed in giant tortoises when observed using the control region or D-loop marker (Beheregaray et al. 2003). Medium-high genetic diversity of RFLP D-loop marker was observed in bigeye tuna, *Thunnus obesus* (Chow et al. 2000). The remaining studies showed high genetic diversity in various animal phyla based on D-loop control region markers (Zhong et al. 2013; Santos et al. 2007; Chiang et al. 2006; Aboim et al. 2005).

Table 2. Analysis of molecular variances among *H. nemurus* populations

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among populations	10	17.541	0.109 Va	22.62
Within-population	129	48.380	0.375 Vb	77.38
Total	139	65.921	0.484	
Fixation Index (F_{ST})	0.226	Remark: Va and F_{ST} P-value= 0.000 ± 0.000		

Genetic differentiation among populations

Genetic differentiation among the population can be inferred from variance component (Va) and average fixation index (F_{ST}) values on AMOVA result. The result shows that the populations of *H. nemurus* in Java Island have Va value of 0.109 and F_{ST} value of 0.226 with the probability value (p-value) was of 0.000 (Table 2).

The observed p-value of 0.000 ± 0.000 for both Va and average F_{ST} values indicated that significant genetic variation occurred among *H. nemurus* populations across Java Island. Significant genetic differences among rivers on this study were expected because such pattern, theoretically, is a typical case since rivers are fundamentally fragmented ecosystem and considered as 'closed' ecosystem. Even in the same island, such as in Java Island, the connectivity among river ecosystems is also inhibited by a physical barrier as distribution barrier. There was a general acceptance that rivers are separated or closed ecosystems. Therefore, freshwater species tend to exhibit higher levels of genetic structuring than those inhabiting estuarine or marine environments (Hughes et al. 2009). Significant population variation can be observed even among tributaries within a river system without the presence of physical barrier (Kano et al. 2011). Restricted dispersal results in low levels of gene flow among rivers and creates fragmented populations such as in *H. nemurus* population in Java Island, as shown in the present study. According to Unmack (2001), the distribution or movement of freshwater species between drainages depends on the connectivity of these freshwater systems. Genetic differentiation among rivers is also affected by the magnitude of social pressure among waterways. It had reported that anthropogeny activities improve source-sink

population differentiation in the river population of *Cottus gobio* (Hanfling and Weetman 2006).

Significant variation in the COI gene among populations was also observed in *Channa striata* (Song et al. 2013), lanternfishes (Pappalardo et al. 2015), *Oephroneurus goramy* (Nuryanto et al. 2018), *Tor tambroides* (Esa et al. 2008), *Haplorchis taichui* (Dung et al. 2013), *Macarorchestia remyi* (Pavesi et al. 2011), and in *Amblema plicata* (Elderkin et al. 2007). The significant population variation that resulted in the present study and previous studies proved that the COI gene is a reliable marker for population genetic differentiation in a broad range of animals. However, no significant genetic variation was observed in other fish

More detail information on the genetic variation among pair-populations obtained from a significant p-value of pairwise Φ_{st} comparison is summarized in Table 3. It seems that the complex pattern of genetic variation among populations has occurred. This phenomenon is very complicated to explain. A similar pattern of complex genetic differentiation among river populations had reported in numerous studies using various marker and species (Kamarudin and Esa 2009). Specific in *H. nemurus*, previous studies from Nugroho et al. (2003) showed significant genetic differences among the reservoir's populations in Java Island. In another study, Nugroho et al. (2005) stated that *H. nemurus* populations from Jatiluhur and Wonogiri reservoirs had less genetic distance compared to the population from Jambi (Sumatera Island).

Further studies based on mtDNA RFLP and morphological divergences Dodson et al. (1995) divided South-east Asian *H. nemurus* populations into three different regional groups, i.e., Indochina, Sundaic, and Sarawak groups with the haplotype mean differentiation of 2.22. Similarly, the high genetic structure was also revealed on two other riverine species *H. guttatus* populations (Yang and He 2008) and *H. macropterus* (Yang et al. 2009). Nevertheless, all those previous studies were used a different marker to our research. Hence, the comparison is not congruent. Our present study enriches, comprehended, and updating the available data on genetic differentiation in river population. However, some other publication on different organisms and ecosystem using COI gene showed strong genetic structure among populations (Barber et al. 2002, 2006; Kochzius and Nuryanto 2008; Nuryanto and Kochzius 2009; Kochzius et al. 2009).

Table 3. Matrix of significant Φ_{st} p-values (significance Level=0.0500)

sample site	Bnt	Bso	Cmk	Csn	Klw	Clw	Cty	Ctm	Cml	Sry	Pgo
Bnt		-	-	+	-	+	+	+	+	+	
Bso	-		-	+	-	+	+	+	+	+	
Cmk	-	-		+	-	+	+	-	+	+	
Csn	+	+	+		+	+	+	+	+	+	
Klw	-	-	-	+		+	+	+	+	+	
Clw	+	+	+	+	+		+	+	+	+	
Cty	+	+	+	+	+	+		+	+	-	
Ctm	+	+	-	+	+	+	+		+	+	
Cml	+	+	+	+	+	+	+	+		+	
Sry	+	+	+	+	+	+	-	+	+		
Pgo	-	+	-	+	-	+	+	+	-	+	

A complex pattern of population variation was also observed in various animal populations, even when it is analyzed using genetic markers that accepted as highly polymorphic markers, such as D-loop and microsatellite markers. In one hand, no significant population variation was reported in several fish species though high genetic diversity within populations was observed, such as in Bluefin tuna (Ely et al. 2002); freshwater fish *Colossoma macropomum* (Santos et al. 2007) and in wild populations in *Laporinus elongatus* (Martins et al. 2003). No significant genetic structure was also reported even in distantly separated bigeye tuna (*Thunnus obesus*) based on D-loop control region (Chiang et al. 2006). A similar result was reported in *Pan paniscus* (Hominidae) (Erikson et al. 2004). In the second hand, significant genetic structure was observed in earlier studies when analyzed using D-loop control region (Zhong et al. 2013), even among populations with a low level of genetic diversity within population, a significant population variation was reported (So et al. 2006; Davey et al. 2003; Fauvelot et al. 2003).

Similar phenomena were observed when the microsatellite marker is utilized in a population study. Moderate to significant population variation was observed in several fish species, such as in *Channa argus* (Yan et al. 2017), *Misgurnus anguillicaudatus* (Abbas et al. 2017), *Tor tambroides* (Esa and Rahim 2013), two species of notothenioid fish (Damerau et al. 2012), *Nothobranchius furzeri* (Bartakova et al. 2013), *Labeo dero* (Chaturvedi et al. 2011), littoral fish *Tripterygion delaisi* (Carreras-Carbonell et al. 2007), *Cottus gobio* (Junker et al. 2012), and in gorilla (Bergl and Vigilant 2007). However, numerous studies also proved no significant microsatellite variation among fish population, such as in fish *Wallago attu* populations (Basharat et al. 2016), *Tor duoronensis* (Esa et al. 2011), five species of notothenioid fish (Damerau et al. 2012), two coral reef fish (Purchel et al. 2006), among most populations of *Theragra chalcogramma* (O'reilly et al. 2004), and among some populations of *Pagellus bogaraveo* (Stockley et al. 2005). Indeed, a study in freshwater mussel *Cumberlandia monodonta* results in a similar pattern of population variations from the COI gene and microsatellite data (Inoue et al. 2014).

Based on the result of the present study and all previous studies; the COI sequences, RFLP, D-loop sequences and microsatellite markers did not show an absolute level of genetic variability and population variation. The variability levels and significance of population variation based on those three different markers depend on the species or even local population of certain species. The best thing would be to use more than one genetic marker to obtain a general pattern of population genetic of certain species. However, if one maker can answer the hypothesis, the utilization of that marker would be sufficient.

Genetic conservation unit and restoration scheme

Hemibagrus nemurus populations in Java Island have a high genetic diversity level, except the Citanduy River. High genetic diversity of the COI gene as observed in this

study proved that the *H. nemurus* populations across Java Island are stable. However, for conservation purpose, recent data on catchment is needed to obtain an overview or estimate of the current population size in each river. For Citanduy River with low genetic diversity, it could indicate population bottleneck after over-harvesting in the past periods. It has reported that over-harvesting cause low genetic diversity of a certain population (Kochzius and Nuryanto, 2008). Low genetic diversity in Citanduy River might be caused by founder effect due to re-colonization of the river after hot lava from Galunggung mountain eruption in 1982 (Ariwibowo 2017) dismissed habitat lost in Citanduy River. According to Kochzius and Nuryanto (2008) re-colonization of small founder population might lead to low genetic diversity. However, mismatch distribution analysis could not be performed to check bottleneck event in Citanduy River. Therefore, to support the argument of bottleneck event in that river, a serial catchment data is also vital to get a more factual picture about the present condition of *H. nemurus* population in Citanduy River.

Mismatch distribution analysis could not be conducted in several other populations; i.e., Bengawan Solo, Cisadane, Klawing, Ciliwung, and Serayu. Nevertheless, these five populations showed medium (Cisadane, Ciliwung, and Serayu) to high (Bengawan Solo and Klawing) genetic diversity. Mismatch distribution analysis for overall samples indicated that the river populations of *H. nemurus* in Java Island indicated several bottleneck events (blue line Figure 2). Population declines of *H. nemurus* in Java Island also indicated by simulated and model frequency lines (Figure 2). However, some populations had high genetic diversity. This high genetic diversity might indicate population expansion population growth after population decline. It is shown by not significant sum of square deviation (SSD) and Harpending's Regardless index (HRI) values (p -value > 0.05) (Roger and Harpending 1992; Rogers 1995).

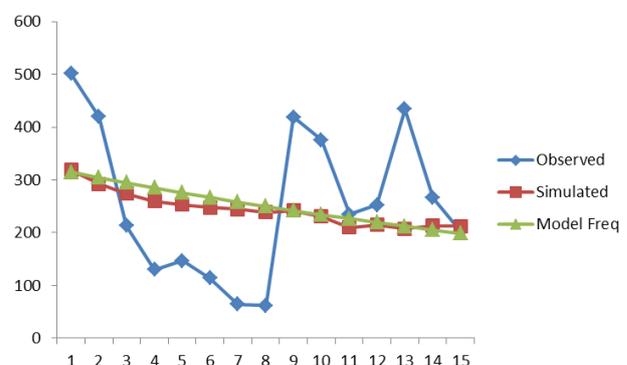


Figure 2. Mismatch distribution analysis indicate several bottleneck events in the river populations of *H. nemurus* in Java Island

Table 4. Sampling site grouping based on significance pairwise $\Phi_{st}p$ -values and geographic position

Group	River
I	Brantas and Bengawan Solo
II	Citanduy and Serayu
III	Citarum-Cimanuk
IV	Comal-Progo
V	Cisadane
VI	Ciliwung
VII	Klawing

A careful examination of the comparison significance values as presented in Table 3 and considering the geographic position of the sampling sites (rivers) resulting in a logic grouping instead. This grouping is expected to use as a scientific basis for defining genetic conservation units and source populations for the restoration program. The rivers grouping presented in Table 4 and illustrated in Figure 3.

As shown in Table 4 that sampling sites divided into seven genetic groups. The first group consisted of Brantas and Bengawan Solo Rivers and considered as a single genetic conservation unit. It is mean that both populations could be genetically managed together. It was because the green catfish populations from both rivers were genetically not different, and both rivers are geographically nearby and also empties in the Java Sea in the North of East Java. In case of the restoration program, Bengawan Solo population referred to as a source population because it has higher nucleotide diversity than Brantas population. Citanduy and Serayu Rivers placed together in a single genetic unit separated from other populations because both populations are a genetically similar one to another, while they are

genetically different from the remaining populations. Within this group 2, Serayu suggested as a source population for restoration program since it has higher genetic diversity than that Cintanduy River. The third group formed by Citarum and Cimanuk Rivers, these two rivers could also be managed as a single genetic conservation unit because genetically not different. Both populations could be referred to as to source population for them because the two populations have high genetic diversity. Comal and Progo Rivers placed in group number 4 and it can be managed as a single genetic conservation unit. Klawing Rivers, though were genetically similar to specific other populations, it could not be managed together as a single unit with its genetically similar populations because of technical difficulties due to their geographic location. Therefore, Klawing River stands alone as a single genetic conservation unit. However, it can be used as a source population for other genetically similar population such as for Brantas, Bengawan Solo, and Cimanuk due to its high genetic diversity. Different to other population which showed genetic similarity to specific populations, Cisadane, and Ciliwung populations were different to all green catfish populations under study. Therefore, each population should be managed separately as a single conservation genetic unit. Schematic diagram of the proposed genetic conservation units is presented in Figure 2. According to Kochzius and Nuryanto (2008) and Nuryanto and Kochzius (2009), genetically similar populations can be managed together as a single conservation unit. Nevertheless, river grouping for useful application of conservation management should also consider the ecology of each river and other genetic data from other markers, such as D-loop sequences and microsatellite.

**Figure 3.** Map of Java Island showing a proposed seven genetic conservation units of *Hemibagrus nemurus* (I-VII)

Klawing River was unique. In one hand, it was different from Surayu River though it empties in Serayu River. In the second hand, it was genetically similar to Progo River while they are geographically distantly separated. This phenomenon explained in two ways. Firstly, genetic differences between Klawing and Serayu Rivers populations could be due to the nature of *H. nemurus* as potamodromous species with limited migration activities. They are only moving to other parts of the river with high vegetation to spawn (Froese and Pauly 2019). It assumed that there was little possibility for *H. nemurus* to move to other streams, while fish samples collected at the upper of meeting point between Klawing and Serayu River.

In some cases, genetic differentiation among river populations does not necessarily need a physical barrier. Kano et al. (2011) had proven that *Salvelinus fontinalis* shows significant genetic differentiation among populations in the absence of physical barrier within the tributary. Secondly, genetic similarity between Klawing and Progo while they are geographically separated populations could be due to convergent evolution between both populations caused by similar environmental conditions. However, this argument needs additional ecological data from both rivers (Klawing and Progo). According to Stern (2013), convergent evolution in genetic level molecular results in the same characteristic two different lineages. Convergent evolution was also reported to occur in cichlid fishes (Muschick et al. 2012).

The populations of green catfish *H. nemurus* in Java Island have high genetic diversity, except the population in Citanduy River. This fish also showed a complex pattern of genetic differentiation among the rivers and led the rivers separated into seven genetic conservation units. Both data have important implication for the conservation of *H. nemurus* in Java Island. In the case of the reintroduction program, population with high genetic diversity can be chosen as a source population to increase genetic diversity in the population with low genetic diversity by considering the pattern of genetic differentiation among rivers. However, to obtain a general picture on population genetic of *H. nemurus* populations in Java Island, the utilization of other genetic markers, such as D-loop and microsatellite markers should be considered.

ACKNOWLEDGEMENTS

We would like to thank Nagao Environment Foundation (NEF) Japan, which provided the funding to make this study was possible. Thank you very much also to Universitas Jenderal Soedirman (Unsoed) and Faculty of Biology of Unsoed for the facilities that we can utilize during the study. We also thank peoples for their help to the researcher in collecting fish samples and even some students such as Tofan, Sani, and Nanang who were become guides during the field trips. Finally, our high appreciation is delivered to reviewers for valuable suggestion and inputs to raise the scientific value of this manuscript.

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