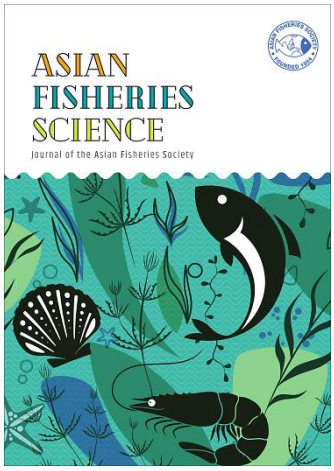

Genetic Diversity of Endangered Snakehead *Channa lucius* (Cuvier, 1831) in the Mekong Delta Inferred from ISSR Markers

WILSON SAWASAWA*, THUY-YEN DUONG
College of Aquaculture and Fisheries, Can Tho University, 3/2 Street, Can Tho, Viet Nam

*E-mail: wilsonsawasawa@gmail.com | Received: 12/06/2020; Accepted: 11/09/2020

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Abstract

Channa lucius (Cuvier, 1831) is one of the endangered and economically important species in the Channidae family with high potential for aquaculture in the Mekong Delta. Limited genetic information has hindered the conservation and domestication programs of this species. The present study used inter-simple sequence repeats (ISSR) markers to assess the genetic diversity of *C. lucius*. Samples were collected from three conservation locations (Ca Mau, Kien Giang and Long An) and one from inland fisheries areas (Can Tho) in the Mekong Delta. Eight highly polymorphic markers yielded 76 scorable bands from 93 samples (22 to 24 samples per population) with the size range from 450 to 2000 bp. Results revealed high levels of genetic diversity of *C. lucius* populations with unbiased expected heterozygosity from 0.250 to 0.271 and Shannon index from 0.364 to 0.395, in which those from Ca Mau population were lowest. Nei's genetic distance between populations ranged from 0.022 to 0.057 with low overall genetic difference G_{ST} = 0.090 and high gene flow (Nm = 5.06), indicating that *C. lucius* populations were not subdivided. This study provides useful information for conservation projects of *C. lucius* in the region.

Keywords: Channidae, conservation, dominant markers, freshwater fish, population genetics

Introduction

Snakeheads are fish belonging to the family Channidae (Rainboth, 1996). The family Channidae consists of two genera, *Parachanna* and *Channa* native to Africa and Asia, respectively (Nelson, 1994). A recently updated database has shown that genus *Channa* has 57 valid species (Fricke et al., 2020) with eight species recorded as native to Malaysia and the Mekong River Basin (Rainboth, 1996). These species are characterised by flattened and broad head, large mouth, eyes in anterior part of the head, scales present on top and sides of head, and dorsal fin longer than anal fin and beginning above pectoral fin (Rainboth, 1996). They comprise one of the most important groups of freshwater food fish in tropical Asia (Benziger et al., 2011). Snakeheads are encountered in rivers, swamps, ponds, canals, drains, reservoirs, rice fields, small streams, mining pools, roadside ditches and lakes (Mohsin and Ambak, 1983).

Among *Channa* species, *Channa lucius* (Cuvier, 1831) has a high tolerance to poor water quality and can survive in the mud during drought and flood (Courtenary and Williams, 2004). It is equipped with a highly vascularised air-breathing organ that enables this species to gulp air from the environment for its survival on land (Mohsin and Ambak, 1983). *Channa lucius* is not a typical migratory species, however, migration might still occur throughout the year during both dry and inundation periods through active and passive movements (Halls et al., 1998). Active migrations mainly occur during the wet season, which are considerably localised and strongly directional, and sometimes take place in the dry season by wriggling motions overland perhaps driven by instinctive behaviour to search for water bodies from drying habitats. Passive movements usually occur at early life stages when young fish drift passively along with water flows (Halls et al., 1998). The reproductive cycle of *C. lucius* is linked closely with seasonal flood plain habitats. Both parents build the nest and exercise

parental care to their offsprings (Adamson et al., 2010). Adult maturation takes within one year (Duong et al., 2019). It can reach a maximum size of 40 cm but is usually smaller (Rainboth, 1996). Natural populations of *C. lucius* are extensively distributed across Southern Asia, southern China, Indochina, and Sunda Islands (Mohsin and Ambak, 1983). As frequently observed in many important food-fish, over harvesting, habitat damage, and other anthropogenic factors have resulted in severe decline in the indigenous stock of *Channa* species (Nagarajan et al., 2006). In addition, *C. lucius* has not yet been successfully domesticated as such people still depend on the wild source. Consequently, this species has been listed "endangered" in some places such as Viet Nam (MARD, 2011). In order to effectively conserve and manage snakehead species, vital information on relevant population genetics is required, specifically through assessment of its genetic diversity (Jin et al., 2018).

Genetic diversity of snakeheads can be affected by the biology of the species (Duong et al., 2019), environmental changes of habitats, population sizes, geographic barriers, fishing and other anthropogenic activities (Xiao et al., 2013). Previous genetic studies of snakeheads have focused on the genetic diversity of *Channa argus* (Cantor, 1842) (Zhou et al., 2015; Jin et al., 2018) and *Channa striata* (Bloch, 1793) (Jamaluddin et al., 2011; Baisvar et al., 2018; Robert et al., 2019). In the lower Mekong River, genetic work has been investigated for *C. striata* (Nguyen and Duong, 2016; Duong et al., 2019). However, no study has been conducted on the genetic diversity of *C. lucius*. Limited information exists to answer questions relevant to the conservation and management of this species.

Molecular markers are important tools for evaluating levels and patterns of genetic diversity in various fish species (Liu and Cordes, 2004). Inter-simple sequence repeat (ISSR) is a polymerase chain reaction (PCR) based technique that uses an ISSR-containing primer to amplify sequences between adjacent, inversely oriented microsatellites (Gupta et al., 1994). Like random amplified polymorphic DNA (RAPD), ISSR markers are easy to use and highly polymorphic. In addition, they have better reproducibility than that of RAPD markers because of the longer length of primers (Li et al., 2009). ISSR markers have been widely applied for studying the genetic diversity and genetic structure of many plant species (Nagaoka and Ogihara, 1997). However, reports on ISSR markers used in fish studies are limited (Ali and Haniffa, 2012). Apparently, genetic diversity studies based on ISSR markers have been conducted on some fish species such as *Channa* species (Haniffa et al., 2014), yellow catfish *Horabagrus brachysoma* (Günther, 1864) (Kumla et al., 2012), Japanese flounder *Paralichthys olivaceus* (Temminck & Schlegel, 1846) (Liu et al., 2006), and several tilapia species (Saad et al., 2012).

In this study, ISSR markers were used to assess the genetic diversity of *C. lucius* populations in conservation and inland fishing areas in the Mekong Delta. Evaluation of genetic diversity of *C. lucius* will help better understand gene flow and genetic structure amongst natural populations, as well as to establish conservation programs. Genetic information is also useful for future domestication of this species.

Materials and Methods

Fish sampling

Channa lucius samples were collected in the four locations, which included three conservation areas (in Ca Mau (CM), Kien Giang (KG) and Long An (LA) provinces) and one inland fishing area (Can Tho (CT) province) in the Mekong Delta, Viet Nam (Fig. 1). Individual fin clips (22 to 30 individuals per population) were preserved in 95 % ethanol until DNA extraction.

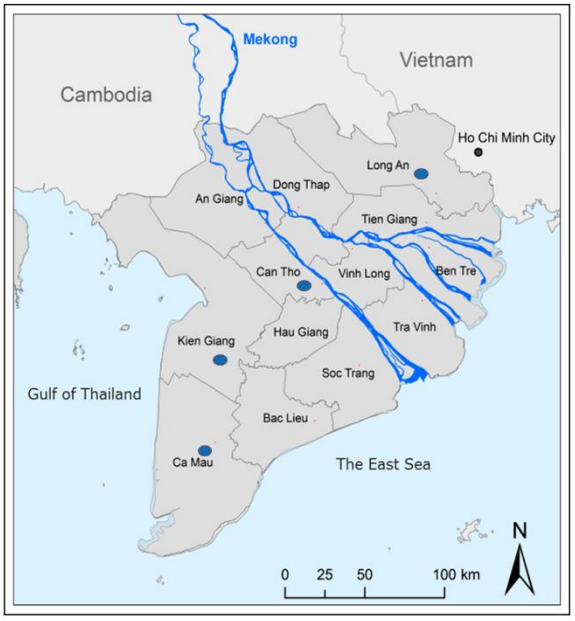

Fig. 1. Location of sampling sites ( ) of *Channa lucius* four populations in Long An, Can Tho, Kien Giang, and Ca Mau in the Mekong Delta

Genomic DNA extraction

Genomic DNA was extracted from fish fin clips based on the modified protocol using ammonium acetate (Saporito-Irwin et al., 1997). The quality of DNA extracts was checked by running 1 % agarose electrophoresis.

ISSR primer screening and optimisation

Primers were carefully screened and optimised from 29 primers of ISSR libraries. Two samples from each population (CT, LA, KG and CM) were used for these tests. PCR components and thermal cycles were first based on references and then optimised with different annealing temperatures based on observations of the clearness and polymorphic levels of bands. Finally, eight primers (Table 1) with clear

polymorphic bands were selected to run 110 samples from four populations. However, 17 samples (from 3 to 5 samples from each population) were finally removed because of missing data in 3 to 5 markers (see an example in Fig. 2). These incomplete genotyped samples were removed prior to data analysis in order to avoid errors in interpretation of the data.

PCR amplifications were carried out (using PCR max Alpha Cycler) in reaction mixtures of 10 µL volume contained 5 µL Promega PCR Master Mix (including Taq DNA polymerase supplied in a reaction buffer (pH 8.5), 400 µM dNTPs, 3 mM $MgCl_2$), 0.4 µL primer (10 µM), 1 µL DNA, and 3.6 µL nuclease-free water (Promega Corporation, USA). Amplification thermal cycles included one cycle of initial denaturation at 94 °C for 4 min, exceeded by 40 repeated cycles of denaturation at 94 °C for 45 sec, annealing temperatures (Table 1) for 45 sec and extension at 72 °C for 2 min; and one final extension cycle at 72 °C for 10 min.

Electrophoresis and visualisation of ISSR bands

Electrophoresis for PCR products was run in 1.2 % agarose gels and dipped in ethidium bromide (5 $\mu g.mL^{-1}$) for staining in 15 min. ISSR bands were then visualised by scanning in a UV transilluminator and band sizes were estimated based on 1 kb-DNA ladder (ABM, Canada). A binary data matrix for further analysis was formed from ISSR bands which were scored presence (1) or absence (0) for each of DNA sample.

Data analysis

ISSR data were first analysed by using GenAlEx 6.5 program (Peakall and Smouse, 2006). Parameters of the genetic diversity of each population and genetic distances among them were estimated, including the percentage of polymorphism, number of effective alleles (Ne), unbiased expected heterozygosity (uHe), Shannon index (I), Nei's genetic distance, and principal coordinates analysis (PCoA). In addition, the total genetic difference (G_{ST}) among populations and the number of migrant per generation (Nm) were estimated using Popgene version 1.32 (Yeh et al., 2000). A dendrogram was constructed based on UPGMA (unweighted pair-group method with arithmetic average) method using MEGA version 4 (Tamura et al., 2007). Differences in genetic diversity values among populations were tested using non-parametric Kolmogorov-Smirnov tests, which were conducted using R (R Core Team, 2017).

Results

ISSR variability

ISSR analysis of 93 samples from four populations of *C. lucius* using eight primers (Table 1) generated 76 scorable bands ranging from 450 bp (17898A, 17898B, 17899A) to 2100 bp (844B). The number of bands for each primer varied from 7 (EL02B) to 14 (844B). Electrophoresis gels representative for eight primers are presented in Figure 2. The four populations showed 66 (LA) to 71 (CM) bands, in which two and three private bands were detected in CT and CM populations, respectively. The percentage of scorable bands with frequencies greater than 5 % was 88.2 % across four populations, indicating that most bands were common. The resolving power (Rp) values varied from 6.09 for HB8 to 12.13 for EL02A, with average 9.96. Polymorphic information content (PIC) values of the primer ranged from 0.260 for 844B to 0.360 for 17899A. The average PIC value was identified as 0.26 (Table 1).

Genetic diversity parameters among four populations of Channa lucius

Levels of genetic diversity in the four populations of *C. lucius* were quantified with the ranges of unbiased expected heterozygosity (uHe) from 0.250 to 0.271, number of effective alleles (Ne) from 1.422 to 1.458, Shannon index (I) from 0.364 to 0.395, and the percentage of polymorphic loci from 71.1 to 76.3 %. These parameters were not statistically different ($P > 0.05$) among the four populations (Table 2).

Table 1. List of sequences, optimised annealing temperatures, polymorphic bands, resolving power (Rp), and polymorphism information content (PIC) of eight ISSR primers applied in four populations (Can Tho, Long An, Kien Giang and Ca Mau) of *Channa lucius* in the Mekong Delta.

Primers	Sequence (5'-3')	Optimised annealing temperature (°C)	Number of polymorphic bands	Resolving power (Rp)	Polymorphism information content (PIC)
1. 17898A	$(CA)_6AC$	48	10	9.70	0.298
2. 17898B	$(CA)_6GT$	48	8	9.20	0.303
3. 17899A	$(CA)_6AG$	48	8	11.68	0.360
4. HB8	$(GA)_6GG$	46	8	6.09	0.197
5. HB10	$(GTG)_5GC$	46	10	10.45	0.235
6. 844B	$(CT)_8GC$	46	14	11.29	0.260
7. EL02A	$(AG)_8C$	50	11	12.13	0.298
8. EL02B	$(AG)_8T$	50	7	9.14	0.192
Average				9.96	0.258

References: Paterson et al. (2009) for primers 17898A, 17898B, 17899A and HB8; Saad et al. (2012) for HB10; Kumla et al. (2012) for 844B; Labastida et al. (2015) for EL02A and EL02B.

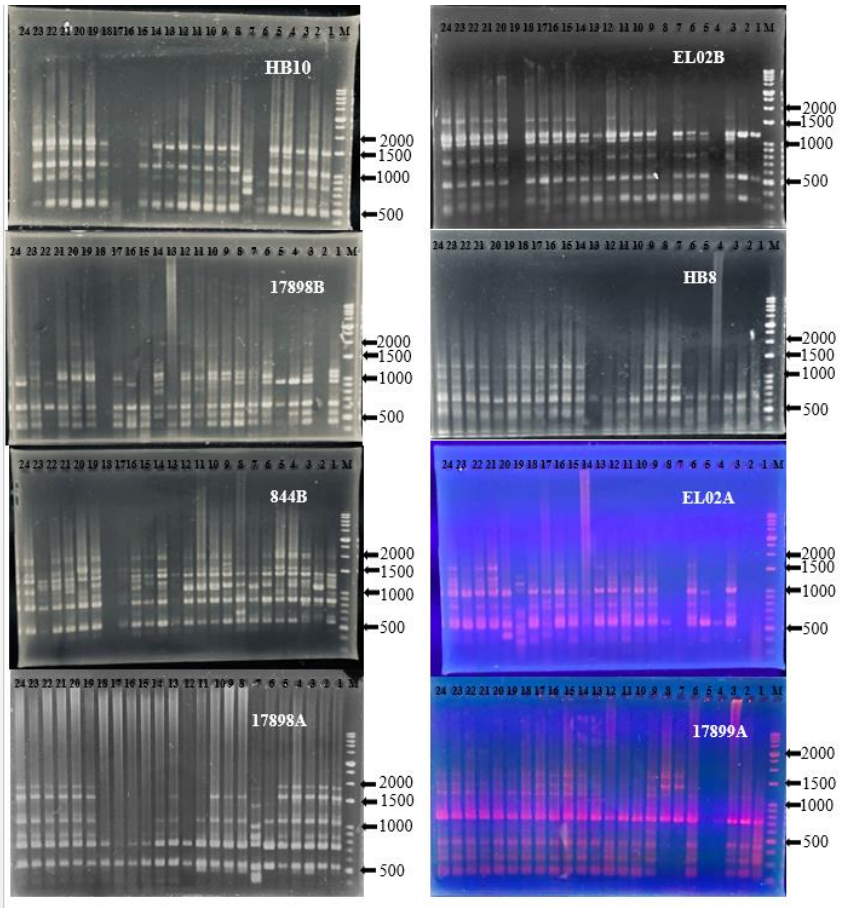

Fig. 2. Electrophoresis ISSR gel patterns of eight primers (HB8, HB10, 844B, 17898A, 17898B, 17899A, EL02A, and EL02B) applied in the study of four populations of *Channa lucius* (Lane M: ladder, L1-6: Can Tho, L7-12: Long An, L13-18: Kien Giang, L19-24: Ca Mau). Sample 19 with missing data in three primers (HB10, 17898B, and 844B) was removed from further analysis.

Table 2. Genetic diversity parameters; polymorphism (%), effective alleles (Ne), Shannon index (I), unbiased expected heterozygosity (uHe) of four *Channa lucius* populations from Ca Mau, Can Tho, Kien Giang, and Long An.

Population	Number of samples	Polymorphism (%)	Ne	I	uHe
Ca Mau	24	71.1	1.422 ± 0.044	0.364 ± 0.032	0.250 ± 0.024
Can Tho	22	75.0	1.451 ± 0.042	0.395 ± 0.031	0.271 ± 0.023
Kien Giang	24	76.3	1.458 ± 0.043	0.394 ± 0.031	0.271 ± 0.023
Long An	23	73.7	1.457 ± 0.045	0.388 ± 0.032	0.268 ± 0.024
P-value*			0.584	0.776	0.691

Ne: effective alleles, I: Shannon index, uHe: Unbiased expected heterozygosity.
*Statistical tests for differences in diversity parameters among populations (significant level of 0.05).

Genetic difference and identity of Channa lucius *populations*

The overall genetic difference of *C. lucius* populations G_{ST} was 0.090 and the number of migrant per generation (Nm) was 5.06. Nei's genetic distance ranged from 0.022 to 0.057 and genetic identity ranged from 0.945 to 0.979 (Table 3). Genetic differentiation was highest between CM and LA (0.057) and lowest between KG and LA (0.022) (Table 3).

UPGMA dendrogram (Fig. 3) constructed using Nei's genetic distance indicated the genetic relationship among the four populations of *C. lucius*. The dendrogram was divided into two clusters holding CM population in one cluster and CT, KG and LA in the other. KG and LA with the smallest genetic distance were in the same group of the phylogenetic tree.

Molecular variance analysis revealed that the majority of genetic variation (96 %) existed within populations, whereas only a small amount of genetic variation (4 %) was present among populations. Variations within and between populations were further explored in principal coordinates analysis (PCoA), which showed no clusters among the four populations of *C. lucius*, with coordinates 1 and 2 explaining 16 % and 9 % of genetic variation, respectively (Fig. 4).

Table 3. Nei's unbiased genetic distance (below diagonal) and identity (above diagonal) among four populations of *Channa lucius* from Ca Mau, Can Tho, Kien Giang, and Long An estimated from ISSR marker.

Populations	Ca Mau	Can Tho	Kien Giang	Long An
Ca Mau	****	0.955	0.952	0.945
Can Tho	0.046	****	0.969	0.974
Kien Giang	0.049	0.032	****	0.979
Long An	0.057	0.026	0.022	****

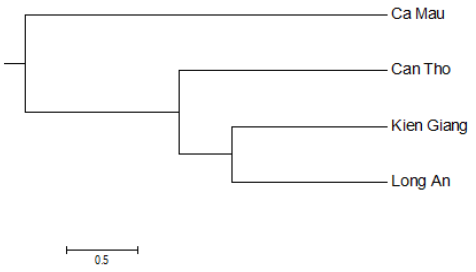

Fig. 3. UPGMA dendrogram constructed using Nei's unbiased genetic distances among four populations of *Channa lucius* in Ca Mau, Can Tho, Kien Giang and Long An.

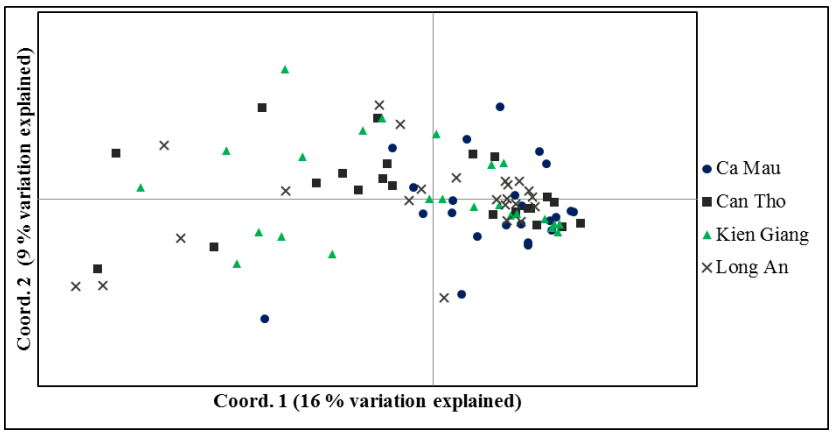

Fig. 4. Principal coordinates analysis (PCoA) of *Channa lucius* from Ca Mau, Can Tho, Kien Giang and Long An populations in the Mekong Delta.

Discussion

ISSR variability

The high-quality bands generated in this study fall within the size range of 450 to 2100 bp with the percentage of polymorphic range from 71.1 to 76.3 % (Table 2). The annealing temperatures optimised in this study (46 to 50 °C) are lower than those reported in references but still correspond to the normal range of annealing temperatures (45 to 60 °C) for many ISSR primers (Ng and Tan, 1994). Haniffa et al. (2014) applied annealing temperature range from 42.0 to 48.2 °C for nine ISSR markers, yielding 63.2 % polymorphic bands (lower than the current study) for five Indian channid species. In Japanese flounder *P. olivaceus*, Liu et al. (2006) used 12 ISSR markers with annealing temperatures from 48 to 52 °C, producing 27.5 to 33.9 % polymorphic bands. Tran and Duong (2019) found annealing temperatures from 45 to 51 °C for similar ISSR markers applied in the present study to differentiate two *Pangasius* species including *Pangasius krempfi* Chaux and Fang, 1949 and *Pangasius mekongensis* Gustiano, Teugels & Pouyaud, 2003. Lower annealing temperatures in this study than those reported in the above mentioned references would be anticipated to the differences in the ISSR primers and fish species. The polymorphic levels of ISSR markers depend on the annealing temperatures, nature of primers, and types of species (Labastida et al., 2015).

Resolving power (Rp) provides a modest indication of the ability of primers to distinguish between genotypes (Provost and Wilkinson, 1999). The average resolving power (Rp) of the eight ISSR primers applied in this study (9.96; Table 1) was informative. Tank et al. (2014) reported high informative of ISSR markers with

resolving power range from 0.13 to 6.00 with an average of 2.34 in assessing genetic diversity of triticale genotypes. Apparently, resolving power (Rp) has been mostly used for evaluating effectiveness of primers in plants (Majid et al., 2012; Singh et al., 2014).

Genetic diversity of Channa lucius *populations*

Data obtained from eight ISSR primers revealed high levels of genetic diversity in four wild populations of *C. lucius* in the Mekong Delta, indicated with the number of effective alleles (Ne) from 1.422 to 1.458, unbiased expected heterozygosity (uHe) from 0.250 to 0.271 and Shannon index (I) from 0.364 to 0.395 (Table 2). Genetic diversity based on ISSR markers has also been reported in other studies on different species. Kissing gourami *Helostoma temminckii* Cuvier, 1829, populations in the Mekong Delta were found with genetic parameters such as uHe: 0.180 to 0.245 and I: 0.269 to 0.386 (Duong et al., 2018) comparable to those of *C. lucius*. Native populations of grass carp *Ctenopharyngodon idellus* (Valenciennes, 1844) in Hanjiang and Shishou sections (China) had lower genetic diversity (uHe: 0.078 to 0.132; I: 0.118 to 0.195) (Chen et al., 2009). In addition, lower levels of genetic diversity (uHe: 0.080 to 0.197; I: 0.077 to 0.197) were reported among four natural populations of *Arapaima gigas* (Schinz, 1822) based on ISSR markers (Vitorino et al., 2015).

The present study found that CM population has the lowest genetic diversity than the other three populations in the Mekong Delta (Table 2). We found this interesting as opposed to our expectation. CM is one of the conservation areas (as KG and LA), thus this population was anticipated to have high genetic diversity than CT population in an open inland with intensive fishing activities. Low genetic diversity in CM population could be attributed to changes in the habitat conditions due to high levels of brackish water resulting from salt intrusion, affecting the population growth of this freshwater species. In the Mekong Delta, salinity intrusion occurs in the dry season and this problem has become severe recently. The salinity concentration of 4 $g.L^{-1}$, a level that is threatening agricultural production and aquaculture, has reached further inland (60–70 km in 2016 and 82–85 km in 2020) and coastal provinces including Ca Mau (Fig. 1) are heavily affected (Viet Nam Disaster and Management Authority, 2020). Environmental changes in habitats affect the levels of genetic diversity and adaptation (Ebied et al., 2014). In addition, restricted gene flow can lead to low genetic diversity of an isolated population (Allendorf and Luikart, 2007). *Channa lucius* can migrate actively for short distances (Halls et al., 1998) and fish in CM population might have limited migration opportunities due to geographic barriers without river network connecting CM with other populations (CT and LA) in the upper region. So, geographic and habitat conditions could explain for the slight variation in genetic diversity among *C. lucius* populations in the region. Similarly, differences in geographical locations and seasons affected genetic diversity estimated by ISSR markers of *A. gigas* in the Araguaia-Tocantins basin (Vitorino et al., 2015)

Genetic difference and identity of Channa lucius *populations*

Low overall genetic difference (G_{ST}: 0.090) associated with high gene flow (Nm: 5.06) and a small portion of between-population genetic variation (4.0 %) indicate that *C. lucius* populations have not been subdivided. Nei's genetic distance values among *C. lucius* populations (from 0.022 to 0.057, Table 3) are comparable to those found in other fish species in the Mekong Delta. For example, in the kissing gourami *H. temminckii*, a non-migratory fish, genetic distances among four populations varied from 0.023 to 0.102 (Duong et al., 2018). In a migratory pangasiidae catfish *P. krempfi*, two populations collected in two estuaries of the Mekong River had the genetic distance of 0.034 (Duong and Nguyen, 2019). Relative low genetic differences among four populations of *C. lucius* as well as in other fish species in the Mekong Delta can be attributed by the highly connected hydrological system in the region. However, CM population showed higher genetic distance compared to the others (Table 3 and Fig. 3). This finding is in concordance with an investigation on striped snakehead *C. striata*, in which CM population was more genetically different from other wild and cultured populations in the Mekong Delta (Duong et al., 2019). When comparing among wild populations of striped snakehead along the Mekong River basin in Cambodia and Viet Nam, Duong et al. (2019) found that genetic differences increased with the increase of hydrological distances among populations. *Channa lucius* is a non-migratory fish although it can actively migrate for short distances (Halls et al., 1998), thus its passive migration depends on water flow. In addition, geographic and habitat conditions that might negatively affect the genetic diversity of *C. lucius* in CM (mentioned above) could also cause this population more genetically different from the others. Similarly, the distributions of the river systems and ecological conditions resulted in the population structure of striped snakehead in Thailand (Hara et al., 1998). Moreover, other anthropogenic factors such as overfishing and agricultural activities can intensify genetic differences among populations of *A. gigas* in Araguaia-Tocantins basin in Brazil (Vitorino et al., 2015). These factors probably also affect levels of inter-population variation in genetic diversity of wild *C. lucius* in the Mekong Delta, where the species has been ranked "endangered" (MARD, 2011).

Conclusion

ISSR data from eight primers indicated high levels of genetic diversity and low genetic differentiation of *C. lucius* populations in the Mekong Delta. In terms of fisheries management, the fish population in CM

conservation area with low genetic diversity, conservation strategies should be intensified. In addition, management programs should be implemented in order to maintain the gene pool and high levels of genetic diversity in other populations (i.e., CT, LA and KG). Short-term strategies can focus on controlling fishing pressure and implementing protective measures in the conservation area. In the long term, artificial propagation of this species for aquaculture can be a feasible solution to reduce fishing pressure on *C. lucius* for food, and establishing co-management measures with the fishing communities. The present study helps understand the genetic relationship among different *C. lucius* populations inhabiting the Mekong River, and implications for conservation and management of the species. This study is also useful as a reference investigation for the future molecular studies on *C. lucius* and other snakehead species.

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