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Original Article

Genetic diversity analysis revealed possible long migration of Black sharkminnow *(Labeo chrysophekadion)* along the Mekong River

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Abstract

This study evaluated levels of genetic diversity of *L. chrysophekadion*, one of the important migratory cyprinid species in the Mekong River using inter-simple sequence repeat (ISSR) markers. Fish samples were collected from four populations comprising three populations in the Mekong Delta of Viet Nam (An Giang, Can Tho and Dong Thap provinces) and one population in Lao PDR. Seven ISSR primers were selected to analyze 90 individuals (19-24 individuals/ population), yielding 275 bands (sizes from 400 bp to 2,900 bp). Results showed that four populations of *L. chrysophekadion* had high levels of genetic diversity with the percentage of polymorphic loci from 81.7–85.9%, expected heterozygosity 0.276–0.306, number of effective alleles 1.47–1.54, and Shannon index 0.415–0.451. Four populations in Laos and Viet Nam had low genetic differentiation (Nei's genetic distance range: 0.016 – 0.022), suggesting a long migratory distance, approximately 1,200 km, of this species.

Keywords: genetic diversity, Inter-Simple Sequence Repeat (ISSR), *Labeo chrysophekadion*, migration, population structure

1. Introduction

Black sharkminnow (*Labeo chrysophekadion)* belongs to the family Cyprinidae, amongst the largest fish families with 1,745 valid members, representing 5% of world's fish species (Fricke, Eschmeyer, & Fong, 2020). Contrasting to great species diversity of Cyprinidae, little information on genetic diversity is available in cyprinid species, including *L. chrysophekadion*, in Southeast Asia. Black sharkminnow is native to Asia, distributing throughout from Mekong River and Chao Phraya basins to Malay-Indonesian Archipelago (Froese & Pauly, 2019). This species has migratory behavior where mature individuals migrate upstream along the river bank and start spawning at the beginning of the rainy season (Poulsen & Hortle, 2004; Rainboth, 1996). According to Rainboth (1996), spawning occurs in the shallow water and the fry at first move into the flooded grasses on the riverside and then follow floodwater rising into flooded areas. Adults also migrate widely to flooded land for feeding. During migration periods many carp species, including *L. chrysophekadion*, form schools showing social behavior (Rainboth, 1998).

Population sizes of *L. chrysophekadion* in the wild has not been updated. The species was ranked as "least concern" based on an evaluation in 2011 (Vidthayanon, 2012). However, their population sizes in nature rapidly can decrease because of overexploitation (Jutagate & Krudpan, 2004), habitat degradation, and other anthropogenic threats to freshwater species diversity in the Mekong River (Baran & Myschowoda, 2008; Ziv, Baran, Nam, Rodríguez-Iturbe, & Levin, 2012). It is one of the targeted capture fisheries species in the Mekong region. For example, a high exploitation rate (E=0.61) of *L. chrysophekadion* was reported in Mun River in Northeast of Thailand (Jutagate & Krudpan, 2004).

Despite of its importance in fisheries and aquaculture, no information on genetic diversity of *L. chrysophekadion* is available. Genetic diversity of wild populations is important information for conserving natural genetic resources (Laikre *et al.*, 2010; Neff, Garner, & Pitcher, 2011). In addition, genetic information can be used for

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understanding migratory behavior or migration pathways of fish species (Ackiss *et al.*, 2019; Bay, Caley, & Crozier, 2008; Refseth *et al.*, 1998). For instance, based on mitochondrial and multi-locus DNA data, Refseth *et al.* (1998) found that perch (*Perca fluviatilis*) that has been resident in Norway migrated before from two directions: one from the south and the other from the Baltic Sea area. In one catfish species, *Hemibagrus spilopterus*, distributing in the Mekong River Basin, genetic data revealed that this species could migrate short distances and that tributary populations isolated by dams had lower genetic diversity compared to populations in the mainstream (Ackiss *et al.*, 2019). In the same area of the Mekong River basin, *L. chrysophekadion* has been known as a migratory fish but their genetic diversity and migration distance are still in question. Populations in the Mekong Delta (Viet Nam), the downstream area of the long Mekong River, can be more vulnerable to risky factors affecting genetic diversity compared to upstream populations.

The objective of the present study was to quantify levels of genetic diversity of *L. chrysophekadion* in the Mekong Delta and predict its migration distance along the Mekong River. These pieces of information would provide more understanding on the migratory fish species in the high biodiversity Mekong River (Ackiss *et al.*, 2019).

2. Materials and Methods

2. 1 Fish sampling

Samples of *L. chrysophekadion* were collected from natural water bodies in three provinces of the Mekong Delta, including Can Tho, Dong Thap, and An Giang provinces (Figure 1). Samples (24-30 samples from each province) were transported to the genetic laboratory of the College of Aquaculture and Fisheries (Can Tho University, Viet Nam). Fish tissues (muscle) were collected from each sample and preserved in the ethanol 95% for DNA analysis. In addition, samples in the upstream Mekong River were collected from the fishermen in Paksan, Lao PDR.

2.2 DNA extraction

Total DNA was extracted from fish muscle by applying modified ammonium acetate protocol (Saporito-Irwin, Geist, & Gutmann, 1997). Briefly, 50 mg of tissue samples were lysed in 650 ml lysis buffer (50 mM Tris HCl, 100 mM NaCl, 50 mM EDTA, and 1% SDS) and 5 µl proteinase-K (20mg/mL) at 55 ^{o}C for overnight. Proteins were deposited from DNA by using 7.5 M Ammonium Acetate. Then DNA was precipitated by adding 3 M Sodium Acetate and cold ethanol 100%. DNA pellets were washed two times with cold ethanol 70%. Finally, DNA was diluted into TE and incubated at 55 ^{o}C for 10 minutes. DNA quantity and quality were checked by using 1% agarose gel electrophoresis.

2.3 Primers screening and optimization

Sixteen ISSR primers were screened and optimized. Annealing temperatures were initially based on references and then optimized with different temperatures. Primers often showed good results with the annealing temperatures from 44 to 48 ^{o}C. Based on the clearness and polymorphism of PCR products, seven primers (Table 1) with clear and highly polymorphic bands were chosen for analyzing all samples from the four different populations of *L. chrysophekadion*.

2.4 ISSR primer screening and amplification

ISSR amplifications were performed by using the mixture of 10 µL containing 5 µL Promega 2X Master Mix (containing GoTaq® G2 DNA Polymerase, buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM MgCl2), 0.4 µL primer (10 µM), 1 µL DNA, and 3.6 µL nuclease-free water or distilled water. Thermal regimes of PCRs were set as follows: (i) one cycle of initial denaturation at 94 °C for two minutes; (ii) repeated cycles (38-40 cycles) including denaturation: 94 °C for two minutes; annealing temperatures depending on each primer (Table 1) for 45 seconds; and extension: 72°C for 1.5 minutes; and (iii) one

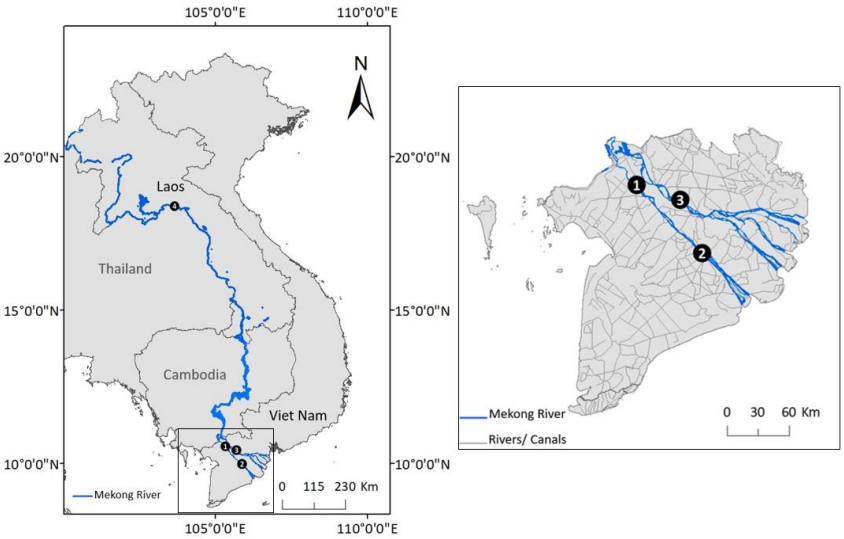

Figure 1. Sampling sites of *L. chrysophekadion* in the Mekong River (1-An Giang, 2-Can Tho, 3-Dong Thap (Viet Nam), and 4-Paksan, Laos) and a close-up map of three sites in the Mekong Delta

Table 1. ISSR primers with annealing temperatures from references (Ref. Ta) and after optimization (Ta) in this study

Primers	Sequence (5'-3')	References	Ref. Ta	Ta (°C)
1. 17898A	$(CA)_6AC$	Paterson, Downie, and Hill, (2009), Wolfe, Xiang, and Kephart (1998)	55	48
2. 17898B	$(CA)_6GT$		55	48
3. 17899A	$(CA)_6AG$		55	48
4. UBC811	$(GA)_8C$	Casu, Casu, Lai, Cossu, and Curini-Galletti (2006)	48	45
5. .Micro11	$(GGAC)_3$	Fernandes-Matioli, Matioli, and Matioli (2000)	48	46
6. Chiu-SSR1	$(GGAC)_3A$	Pazza *et al.* (2007)	48	46
7. ISSR11	$(CAC)_3GC$	Sharma, Kumaria, Tandon, and Rao (2011)	48	46

cycle of final extension: 72 °C for ten minutes. In addition, for testing DNA contamination, negative controls (without DNA template) were applied.

All amplified products were separated by using electrophoresis on 1.2% agarose gels in 1X TBE buffer. Thereafter, gels were visualized with 0.01% of ethidium bromide solution staining for 15 to 30 minutes and were digitally photographed under a UV scanner for bands recording.

2.5 Data analysis

Gel pictures of PCR products were used for data scoring. Bands were estimated for their sizes based on 1kb-DNA ladder (ABM, Canada) and scored as present or absent (1 = present; 0 = absent). Thereafter, the binomial data were analyzed using GENAlEX (Peakall & Smouse, 2006) and POPGENE package version 1.31 software (Yeh, Yang, & Boyle, 1999). Parameters were estimated including the observed number of alleles (Na), effective number of alleles (Ne), Shannon's (I) index, expected heterozygosity (He), unbiased heterozygosity (uHe), coefficient of differentiation (G_{ST}), and gene flow (Nm). Differences in genetic diversity parameters among populations were tested using univariate analyses, conducted in SPSS 20.0. Nei's genetic distances between populations were calculated (Nei, 1972). A dendrogram based on the unweighted pair-group method with arithmetic average UPGMA (Unweighted Pair Group Method with Arithmetic Average) method was constructed by using MEGA7 (Kumar, Stecher, & Tamura, 2016).

3. Results

3.1 Levels of genetic diversity of *L. chrysophekadion* populations in the Mekong Delta

In total, 71 bands were amplified from 90 samples using seven primers. The percentage of the polymorphic bands varied from 81.7% to 85.9% with the average polymorphism of 83.8±0.9% (Table 2). The amplified PCR fragment sizes ranged from 400 bp to 2,900 bp. The number of bands generated by each ISSR primer varied from 7 to17 with the average of 10.1 bands per primer. Genetic data analysis showed that the four populations of *L. chrysophekadion* had high levels of the genetic diversity with expected heterozygosity range 0.276-0.306, the number of the effective alleles 1.47-1.54, and Shannon index range 0.415-0.451.

Comparing genetic diversity parameters among populations showed that An Giang population had the highest level, and Dong Thap population was lowest. However, the magnitude of differences in all parameters was small and not significant among four populations (P>0.05).

3.2 Genetic difference among *L. chrysophekadion* populations

The overall genetic difference (G_{ST}) and the number of migrants per generation (Nm) among populations were 0.075 and 6.17, respectively. The Nei's pairwise genetic distance (the difference between a pair of populations) ranged from 0.019 to 0.053, while, the genetic identity varied from 0.938 to 0.974. Results revealed that the Can Tho population has slightly higher genetic distance (Nei's unbiased genetic distance: 0.053) or lower genetic similarity (Nei's unbiased genetic identity: 0.938) compared to the other populations. On the other hand, the genetic distances between the remaining populations were lower, from 0.016 to 0.022 (Table 3). As a result, in the phylogenetic tree, Can Tho population was in one cluster, different from the others (Figure 2). Molecular variance among the four populations was 5.3% and a higher portion of molecular variance was from within populations, accounting for 94.7% (Table 4). Genetic relationships among and within populations were also indicated by principal coordinates analysis (PCoA), where the first and the second coordinates explained 23.8 and 21.3% of total molecular variance (Figure 3).

4. Discussion

4.1 Genetic diversity of *L. chrysophekadion* in the Mekong River Basin

Results based on ISSR markers showed a high level of genetic diversity in four populations of *L. chrysophekadion* including three in the Mekong Delta and one in Laos, with polymorphic mean of 83.8%, heterozygosity (He) of 0.300 and Shannon index (I) of 0.436. Compared to a study on *Mystus nemurus* using ISSR markers, a higher polymorphic value of 92.6% was obtained but He (0.153) and I (0.230) were lower than *L. chrysophekadion* (Kumla, Doolgindachba porn, Sudmoon, & Sattayasai, 2012). Lower levels of genetic diversity (polymorphic ratios: 55.4-90.4%, He: 0.180-0.245, and I: 0.269-0.386) were observed in kissing gourami (*Helostoma temminckii*) in the Mekong Delta based on similar markers (Duong, Nguyen, Vo, & Tran, 2018). Given that genetic diversity levels increase with the increase of population sizes (Alcala, Streit, Goudet, & Vuilleumier, 2013; McCusker & Bentzen, 2010), high genetic diversity in *L. chrysophekadion*'s populations compared to the above studies

Table 2. Genetic diversity parameters among four populations of *L. chrysophekadion*

Populations	N	P (%)	Ne	I	uHe
An Giang	24	85.9	1.54±0.05	0.451±0.045	0.312±0.023
Can Tho	19	83.1	1.50±0.04	0.428±0.030	0.295±0.023
Dong Thap	24	81.7	1.47±0.04	0.415±0.030	0.282±0.022
Laos	23	84.5	1.53±0.04	0.448±0.030	0.309±0.022
Overall	90	83.8±0.9	1.51±0.02	0.436±0.015	0.300±0.011

N: sample size, P: polymorphic ratios, Ne: number of effective alleles, I: Shannon index, and uHe: unbiased heterozygosity

Table 3. Nei's unbiased genetic difference (below diagonal) and genetic identity (above diagonal) of *L. chrysophekadion* populations

Populations	An Giang	Can Tho	Dong Thap	Laos
An Giang		0.939	0.974	0.974
Can Tho	0.053		0.939	0.938
Dong Thap	0.019	0.053		0.970
Laos	0.016	0.053	0.022	

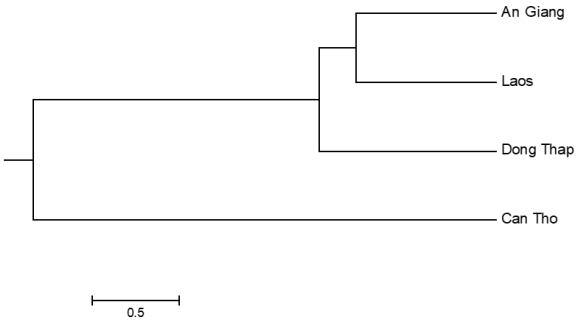

Figure 2. *UPGMA* dendrogram using Nei's unbiased genetic distances of *L. chrysophekadion* populations

Table 4. Analysis of molecular variances (AMOVA) based on ISSR markers

Source	df	SS	MS	Est.Var.	%
Among populations	3	77.6	25.9	0.64	5.3
Within populations	86	988	11.5	11.5	94.7
Total	89	1066		12.1	100

df: degree of freedom, SS: sum of squares, Est. Var: estimated variance, %: percentage of genetic variance

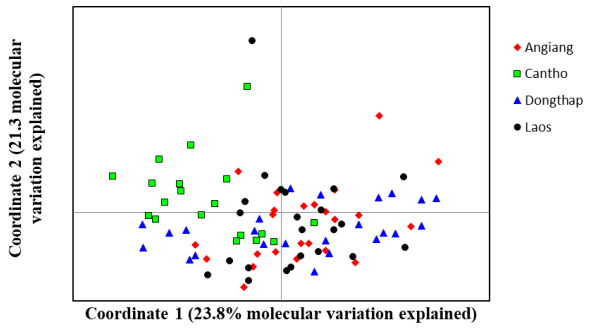

Figure 3. Principal coordinates analysis (PCoA) of *L. chrysophekadion* among four populations

might be due to the species' abundance in the Mekong River Basin. Further investigations can increase sample sizes in a wider geographic ranges to test if more genetic diversity is quantified for this species. In addition, levels of genetic diversity among four populations can be attributed by gene flow among populations, as indicated by high number of migrants per generation (Nm = 6.17). Leonardi *et al.* (2012) reported that migration behavior which connects among populations leads to the increase of genetic diversity through gene flow and minimizes negative effects of inbreeding and genetic drift within each population.

4.2 Genetic differences and migration among populations of *L. chrysophekadion*

The present study found that genetic differentiation were low among four populations of *L. chrysophekadion*, indicated by Nei's genetic distances, a low value of G_{ST} (0.075), and a scatter pattern of PCoA diagram (Figure 3). The low genetic differentiation among populations can result from a high level of gene flow (Nm=6.17) due to migration behavior of *L. chrysophekadion*. As other riverine cyprinid species, *L. chrysophekadion* migrates upstream of the Mekong River for spawning and downstream for feeding and nursing (Poulsen & Hortle, 2004; Rainboth, 1996). However, the migration distance of the species has not been reported. Low genetic distances between Laos (Paksan) and Vietnamese (Mekong Delta) black sharkminnow populations imply that the species can migrate long distances, approximately 1,200 km. Migration of fish sepcies can be disrupted by hydropower dams. Between sampling locations of *L. chrysophekadion* used in this study, there was no existing dam but some have been under construction or in plan (International Rivers, 2017). Recently built dams might not have been affected genetic differences among populations distributing along the river. However, Can Tho population was relatively different from the others when Lao population is the furthest site from the rest. Possible explanation for this observation is that Can Tho site is further downstream with more affluents and canals where some black sharkminnow individuals can reside and thus they have limited gene flow with upstream populations. This prediction about the migration pattern of black sharkminnow should be further tested by using other markers and more populations. The result on low genetic differentiation among *L. chrysophekadion* populations along Mekong River is similar to the findings from other cyprinid species. In another cyprinid, *Henicorhynchus lobatus* in the Mekong river Basin showed a low genetic variation (non-significant F_{ST} based on mtDNA ATP*ase* 6 and 8 genes) among sites along the main river (Hurwood, Adamson, & Mather, 2008). Besides geographic barriers, differences in

species' migration behaviors explain levels of genetic structure among species.

5. Conclusion and Implications for Conservation of *L. chrysophekadion*

The present study showed that *L. chrysophekadion* has high levels of genetic diversity and gene flow among populations. Thus, it is very crucial to maintain and conserve this current genetic diversity by sustainable fishing and preventing migration barriers along the Mekong River. Overfishing is considered as one of the main factors which lead to the reduction of the population size and cause the depletion of genetic diversity in many fish species (Kenchington, 2010; Smith, 1994; Smith, Francis, & McVeagh, 1991). In addition, some dams that have been planned to be constructed in the Mekong River (International Rivers, 2017) will be potential risks to fish migration and thus to genetic diversity of the species. Countries along the Mekong River should work together to conserve genetic resources of *L. chrysophekadion*.

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