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Tran Thi Minh Ly
Advanced program in Aquaculture, Course 40, Can Tho University, Viet Nam

Duong Thuy Yen
College of Aquaculture and Fisheries, Can Tho University, Viet Nam

Correspondence
Duong Thuy Yen
College of Aquaculture and Fisheries, Can Tho University, Viet Nam

Differentiation of two Pangasius species, *Pangasius krempfi* and *Pangasius mekongensis* using inter- simple sequence repeat markers

Tran Thi Minh Ly and Duong Thuy Yen

Abstract
Pangasius krempfi and *Pangasius mekongensis* are economically important species of catfish family Pangasiidae. Compared to *P. mekongensis*, *P. krempfi* has higher values and has been cultured recently in the Mekong Delta, Viet Nam. Wild catfish fingerlings (<10 grams) caught in Mekong estuaries consist of both species, which are hardly distinguishable using morphological methods. This study aimed to search for effective Inter-simple sequence repeat (ISSR) markers that can be used to quickly identify these two species. Samples were collected in different locations of the lower Mekong River. Six primers with clear and species-specific bands were selected to analyze 11 samples per species. Totally, 62 bands were detected with a size range from 500 bp to 2400 bp, in which 22 and 31 private bands were found in *P. krempfi* and *P. mekongensis*, respectively. Nei's genetic distance between the two species was 0.742 and their genetic difference G_{ST} was 0.655. Principal coordinates analysis showed that the two species clustered in two separate groups. The results indicate that six ISSR markers used in the present study can differentiate *P. krempfi* and *P. mekongensis*.

Keywords: Inter-simple sequence repeat (ISSR), migratory fish, *Pangasiidae*, species identification

1. Introduction
Pangasius krempfi Fang & Chaux, 1949 and *Pangasius mekongensis* Gustiano, Teugels & Pouyaud, 2003 are highly economic species belonging to family Pangasiidae, catfish order Siluriformes. These species have similar distribution range along Mekong River Basin, starting from Laos to Thailand, Cambodia and Viet Nam [1]. They are both migratory species that spend most of their life cycles in the Mekong River estuaries (in the Mekong Delta, Viet Nam) and migrate upstream (Khone Falls in Laos) for spawning [2, 3, 4]. Recently, in estuary areas in the Mekong Delta, fishermen catch fingerlings for grow-out. Fingerlings batches usually consist of both *P. krempfi* and *P. mekongensis*. Because they are morphologically similar, it is difficult to distinguish them, especially when body sizes are less than 10 grams. *P. mekongensis* has lower economic values compared with *P. krempfi*, therefore, it is critical to find a reliable method for correct identification of each species.

Traditionally, species of family Pangasiidae are identified based on key morphological characteristics including the number of pelvic fin rays, teeth-patch patterns, the number of gill rakers on the first gill arch [5, 6]. However, countable of traits *P. krempfi* and *P. mekongensis* are in similar ranges and other characteristics are not easy to differentiate the two species at the fingerling stages [7].

Nowadays, DNA markers have become powerful tools in fish species identification [8, 9]. Among which, amplification and sequencing a core segment of Cytochrome C oxidase subunit I (COI), or DNA barcoding, is one of the most commonly used methods for species identification is one of the most popular tools for fish identification [10, 11, 12]. The two *Pangasius* species, *P. krempfi* and *P. mekongensis* are highly divergent based on COI with their (Kimura – 2 parameter) genetic distance of 9.33% [7]. Therefore, they can be easily differentiated by using DNA barcoding in cases that morphology-based methods do not give reliable identification. However, DNA barcoding is relatively more expensive and requires a longer time for sequencing compared to PCR-ISSR (polymerase chain reaction - Inter-simple sequence repeat) method. This method is simple, quick and reliable with high reproducible species-specific bands [13, 14, 15].

PCR-ISSR have been applied to identifying many Aquatic species, such as Tilapia species [15], parrotfish [16], Mediterranean sea bream species [17], shrimp [18].

The objective of this study was to search for effective ISSR markers to distinguish *P. krempfi* and *P. mekongensis*. The results would provide a simple and quick tool for identifying these two species and thus would be useful for aquaculture and for future studies.

2. Materials and methods

2.1 Fish sampling

Adult samples of two *Pangasius* species were collected from fishermen catching fish along Lower Mekong River and its estuaries in the Mekong Delta, Viet Nam. Fish species were identified based on morphological characteristics described by Rainboth [5]; Tran *et al*. [6]; Duong *et al*. [7]. Besides, large samples (weight > 500 g) were chosen in order to identify species correctly and easily. Then, a fin clip of each individual was collected and preserved in ethanol 95% for further analysis.

2.2 DNA extraction and PCR

DNA from fin clips was extracted based on the protocol of Promega SV Wizard Genomic DNA Purification kit. DNA extracts were checked for quality by running 1% agarose electrophoresis.

2.3 Primer screening and optimization

Primers were screened and optimized from ISSR libraries of 29 primers. Four samples from two species *P. krempfi* and *P. mekongensis* were used to these tests. PCR components and thermal cycles were first based on references. If primers yielded monomorphic or unclear bands, reactions were then optimized with different annealing temperatures and $MgCl_2$ concentrations. Finally, six primers with clear and species-specific bands were used to run 11 samples per species (Table 1).

PCR reaction mixture 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.

Table 1: Sequences and annealing temperatures of six primers used in the study

Primers	Sequence (5' – 3')	Annealing temperature	References
HB10	$[GA]_6CC$	45 oC	Saad *et al*., 2012 [19]
ISSR11	$[CAC]_3GC$	46 oC	Sharma *et al*, 2011 [20]
Chiu-SSR1	$[GGAC]_3A$	46 oC	Pazza *et al*, 2007 [21]
Chiu-SSR2	$[GGAC]_3C$	48 oC	Pazza *et al*., 2007 [21]
Micro11	$[GGAC]_4$	44 oC	Fernandes *et al*, 2000 [22]
EL02B	$[AG]_8T$	51 oC	Labastida *et al*, 2015 [23]

PCR thermal regimes include one cycle of initial denaturation at 94 °C for 2 minutes, 38 – 45 amplification cycles of 94 °C for 2 minutes, annealing temperature (Table 1) for 45 seconds and 72 °C for 1 and half minutes; and one final extension cycle of 72 °C for 10 minutes.

Visualization of ISSRs and scoring

PCR products were subjected to electrophoresis in 1.2% agarose gels. The gels were immersed in ethidium bromide (5 µg/mL) for staining in 15 minutes. ISSR bands were then visualized by scanning in a UV transilluminator. Band sizes were estimated based on 1 kb-DNA ladder (ABM, Canada). Each band of a sample was scored 1 if presence and 0 if absence, forming a binary data matrix for further analysis.

2.4 Data analysis

Data were analyzed by using GenAlEX 6.5 program [24]. Parameters of genetic distance between two species were estimated including Nei's genetic distance, private alleles by species, and Principal Coordinates Analysis (PcoA). In addition, genetic difference G_{ST} between the two species was estimated using Popgene [25].

3. Results

3.1. ISSR amplification

ISSR analysis of 22 samples from two species using 6 primers (Fig 1) generated 62 scoreable bands ranging from 500 bp (ChiuSSR1, Micro11) to 2400 bp (ChiuSSR2). The number of bands fosr each primer varies from 6 (HB10) to 14 (ChiuSSR2). There are 31 bands scored for *P. krempfi* and 40 bands for *P. mekongensis*, in which, 22 and 31 private bands detected in the two species, respectively (Fig 2). All bands are not rare as their frequencies are greater than 5%. Patterns of ISSRs (Fig 1) shows that two species can be differentiated. All primers have different allele frequencies between two species and five (out of six) primers yield species-specific bands, 7 bands for *P. krempfi* and 13 bands for *P. mekongensis* (Table 2).

Table 2: Species-specific bands (bp) in six primers

Species	Primers					
	HB10	ISSR11	ChiuSSR1	Chiu2	Micro11	EL02B
P. krempfi		700	500, 1000, 1250	1400	600	550
P. mekongensis	1800	650, 850	450, 600, 750, 900, 1400, 1800	1450	650, 2000	750

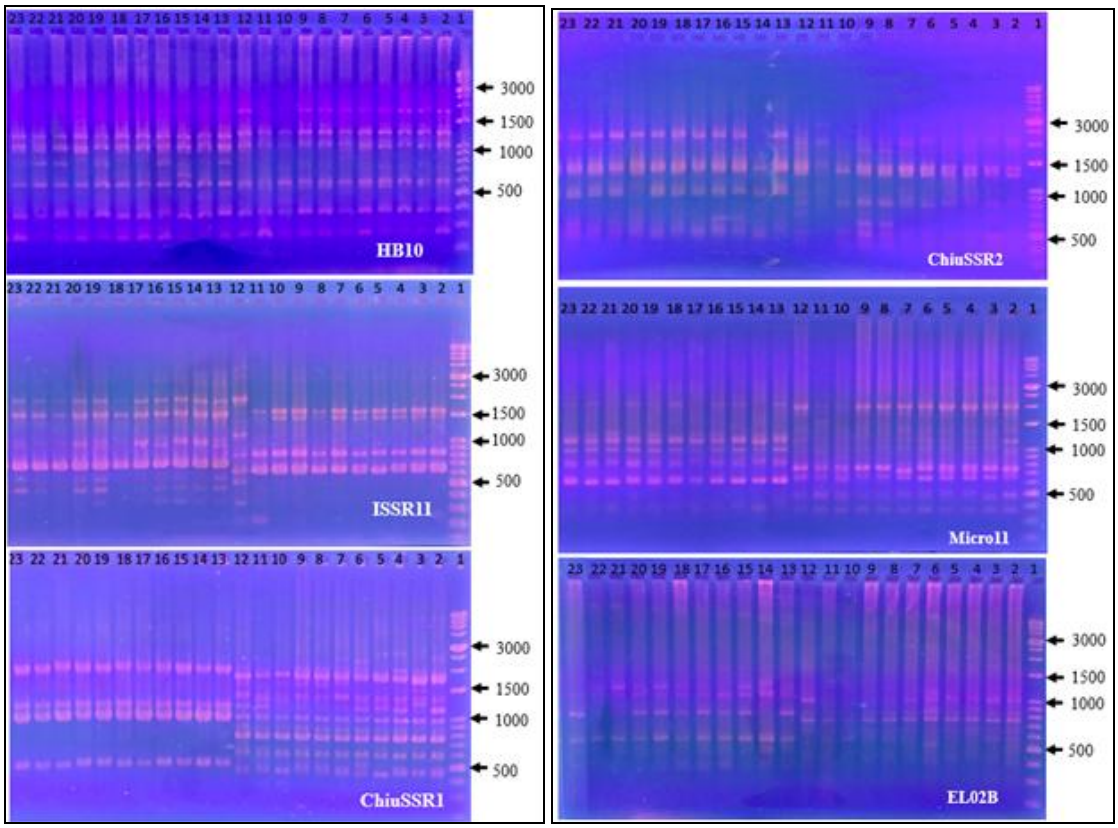

Fig 1: ISSR patterns of *P. krempfi* and *P. mekongensis* (Lanes 1: ladder, L2-12: *P. mekongensis*, L13-23: *P. krempfi*)

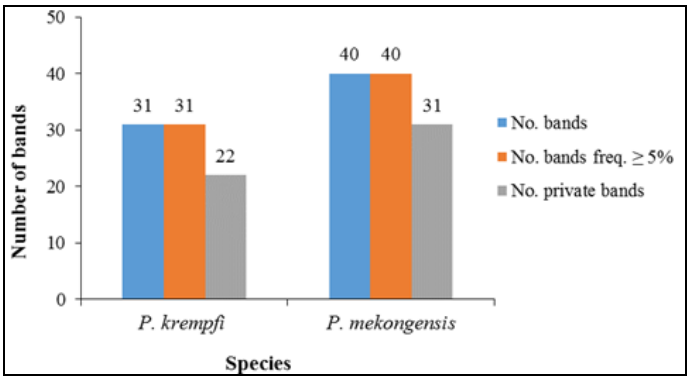

Fig 2: Band patterns between two species, *P.krempfi* and *P. Mekongensis*

3.2 Genetic difference between two *Pangasius* species

Nei's genetic distance between *P. krempfi* and *P.mekongensis* is as high as 0.742, while their genetic identity is 0.258%, and G_{ST} genetic difference between the two species is 0.655. The differentiation between the two species is also shown by another method, Principal Coordinates Analysis (PCoA). The two species are separated into two different groups, mainly in PC1, which explains 72.3% genetic variation (Fig 3). Therefore, these results indicated that *P. krempfi* and *P. mekongensis* can be distinguished by ISSR markers.

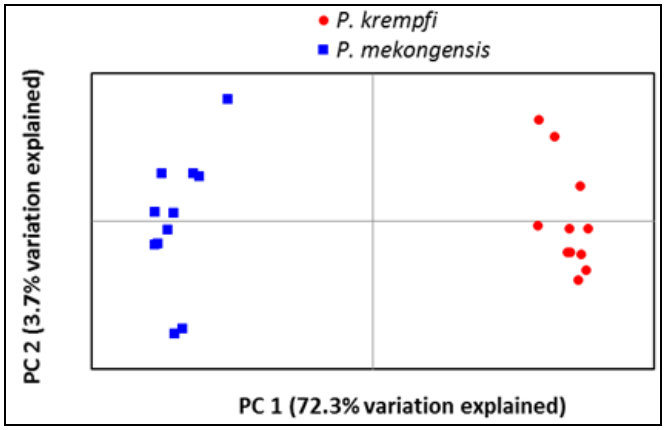

Fig 3: Principal Coordinates Analysis (PCoA) of ISSR data of *P. krempfi* and *P. mekongensis*

4. Discussion

The present study screened successfully six ISSR markers that can be used to distinguish two *Pangasius* species, *P. krempfi* and *P.mekongensis*. A large genetic divergence between the two species is indicated by differences in allele frequencies, species-specific bands, Nei's genetic distance or G_{ST} genetic difference, and two distinct groups in PCoA. Qualitatively, the two species are differentiated by 7 species-specific bands for *P. krempfi* and 13 bands for *P. mekongensis*. With these numbers of "diagnostic bands", the probability of correct species identification is very high. Values of Nei's genetic distance (0.742) between the two species is higher than those reported in other species. Using ISSR markers to study phylogenetic relationships among five species of Channidae in India, Haniffa *et al.* [26] reported that genetic distance among species varied from 0.270 to 0.593. Compared to within species, values of Nei's genetic distance based on ISSR or RAPD are often less than 0.04, which is much smaller than that of two *Pangasius* species. For example, genetic distance of *P. krempfi* (based on the same ISSR markers used in the present study) between two localities in the Mekong Delta is 0.034 [27]. Similarly, another research using ISSR showed that genetic distance among three Japanese flounder (*Paralichthys olivaceus*) populations were low at 0.012 – 0.034 [28]. The present research indicates that ISSR is an effective tool to distinguish two *Pangasius* species. Similarly, other studies used ISSR markers for fish species identification. Saad *et al*., [16] reported that eight parrotfish species were successfully differentiated by 11 ISSR markers that yielded 132 bands with 21 species-specific bands. ISSR markers were also used to distinguish an invasive species (red lionfish- *Pterois volitans*) from a native species (devil firefish- *Pterois miles*) in Cuba [23]. Other studies reported that ISSR markers yielded a large number of species-specific bands resulting in reliable identification fish species such as Mediterranean *Diplodus spp*. and *Dentex dentex* [17], and cyprinodontiform aquarium fish [29].

In addition, ISSR markers were also used to reconstruct phylogenetic relationships among four shrimp species of genus *Penaeus*, including *P. latisulcatus, P. semisulcatus, P. monodon, and P. indicus* [18]. In phytoplankton Bornet *et al.* [30] employed six ISSR primers that could clearly differentiate 12 strains of micro-algae. The study also showed that phytoplankton genomes contain a large number of simple sequence repeats, therefore, ISSR can be a powerful tool to investigate genetic variation within and between species.

5. Conclusion

Six ISSR markers used in the study showed higher numbers of species-specific bands (22 and 31 bands) and a large genetic difference between two *Pangasius* species, *P. krempfi* and *P. mekongensis*. Therefore, ISSR markers can be a quick and effective tool to distinguish these two species.

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