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Microsatellite Loci Based Segregation of Hybrid Varieties of the Three Important Indian Major Carps

Running title: *Microsatellite loci based segregation*

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Abstract: ***The major carps like Rohu, Catla and Mrigal are traditionally cultured freshwater varieties of fish with high commercial value in India. Production of natural and man-made hybrid fishes is very common among these varieties due to high demand and unscientific way of producing more fish-seeds in congregated manner within stagnant water of mainly private hatcheries. Conventionally, external morphological features are the basis of identification for these fish species but increasing emphasis on biodiversity issues has necessitated proper stock management through molecular genetics techniques. This has triggered genetic characterization as the true way of identifying wild and hybrid types. Present work applies molecular genetics approach facilitating microsatellite marker-based identification by polymerase chain reaction (PCR) amplification technique. It would help the fish breeders and hatcheries in discrimination of wild and hybrid species. Three sets of primers were used to cross amplify three different microsatellite markers from genomic DNA of these fishes. We report here that the PCR amplification using said markers could primarily be used as the first step in screening hybrid verities from the wild Indian Major Carps.***

Keywords: PCR, Hybrid, genomic DNA, Microsatellite marker Catla, Rohu, Mrigal

1. Introduction

An aquaculture industry can largely sustain on the availability of quality seeds. Due to habitat degradation (resulting from human interventions and other environmental issues) inadequate supply of quality seeds had been a major issue. This has triggered many studies on fisheries to be shifted in the direction of genetic characterization of natural fish sources (Reddy 1999). The genetic variation in and between populations is the outcome of several factors, such as mutation rate, breeding size of the population, breeding strategies, migration and, above all, natural selection. The genetic diversities can be judged on basis of heterozygosity, allelic diversity, and proportion of polymeric loci (Nei *et al.*, 1975; Leberg, 1992). Rohu (*Labeo rohita),* Catla *(Catla catla),* Mrigal *(Cirrhinus mrigala*) contribute 80 % of the annual production of cultivable fish in India (FAO Yearbook of Fisheries Statistics 2005). These three carps on the genetic ground are capable of producing natural hybrids (Zhang and Reddy 1991). An increase in the production of fish seeds through induced breeding, polyculture technique or composite culture under government and private hatcheries are in practice to cope up with the excessive demands for these major carps. Taking advantage of these practices many hatchery owners and seed producers supply hybrid seeds of these three species frequently in the name of 'wild ones' during young stages. At the early stages of development, hybrid of Rohu and Catla cannot be differentiated easily from each of the wild types (Figure 1). White hybrid of Mrigal also hardly can be morphologically differentiated from the wild type except its overall whitish appearance (Figure 2). To resolve bio-diversity issues properly, hybrids of Indian major carps must be segregated genetically from the wild types properly. Microsatellite DNA is the most useful molecular genetic marker that has been widely and effectively used in the genome-based evaluation of different organisms including the common carps (David et al., 2001; Lehoozky et al., 2003; Tanck et al., 2000; Bartfai et al., 2003; Kohlmann et al., 2003). The present work was undertaken with the help of PCR-based amplification of three different dinucleotide microsatellite loci from the genomic DNA to segregate hybrid types from their wild varieties. We used previously reported primer pairs for cross amplification to identify hybrid of Rohu and Catla and one morphotype/ hybrid of Mrigal from the respective wild populations.

2. Materials and Methods

Sample collection and preservation

Three fish samples each of five types of Indian major carps (including 2 hybrid varieties) studied were collected separately from 3 different markets of three different districts (South 24 Paraganas, Hooghly, Purulia) of the state West Bengal and one market from the district Ranchi of the state Jharkahnd (total 60) in India (Figure 3).

Small pieces of pectoral fins of 1 cm^2 size were collected within one hour of sacrifice from the fish markets. Collected fins were washed in water, air dried and suspended in 99% ethanol and stored at-20^0C.

Genomic DNA Isolation:

Genomic DNA from pectoral fin-cliffs was isolated using modified salt extraction protocol with NaCl to get the good quality genomic DNA.

Modified Salt Extraction procedure with NaCl:
Genomic DNA (gDNA) isolation was done following a modified salt extraction procedure with Nacl (Aljanabi and Martinez 1997). Washed fish samples were suspended in Lysis buffer (50 mM Tris-HCl, pH 8.0, 50mM EDTA, 100mM NaCl) containing 1% SDS and 150 µg/mL of proteinase K (Sigma) and warmed at 50^0C for 8 to 12 hours. Samples were further treated with 100 µg/mL of RNAse (Sigma Aldrich, USA) for 1hour at 37^0C and then centrifuged at 12500 rpm. Equal volume of 5M NaCl was added with the supernatants, mixed gently by inverting the tubes and centrifuged at 12000 rpm for 10 minutes. The supernatant was mixed with 0.6 volume of previously chilled ethanol and kept for 1 hour at-20^0C to precipitate the gDNA. The solution was centrifuged at 12000rpm to get the DNA as pellet at the bottom of the tube. After discarding ethanol the pellet was washed with 70% ethanol, air dried and suspended in 150µl TE (10 mM Tris-HCl, pH 8.0, 1mM EDTA) and kept in 4^0C.

Quantification of DNA:
The quality of isolated genomic DNA samples was checked by spectrophotometer (Eppendorf, Germany) for OD_{260} and OD_{280} values and also looking through UV trans-illuminator of the ethidium bromide-stained 0.8% agarose gel after electrophoresis.

PCR Amplification:
The PCR amplification reaction was carried out using previously reported three pairs of primers (primer 1-3) for amplifying three different microsatellite markers.
1. Locus: *Cyprinus carpio* MFW1, (CA) Repeat (Croojimans et al., 1997)
5'--GTCCAGACTGTCATCAGGAG—3' and
5'--GAGGTGTACACTGAGTCACGC—3' (Ta 55-57^{0}C)
2. Locus: *Catla catla* Cc 7, (GT) 21 repeat (McConnell et al.2001)
5'--CACTCTGTGCCTAGACCTCG and
5'--CTGGAGTTTAAGCCCTGTTC (Ta 55^{0}C)
3. Locus: *Labeo rohita* Lr 22 (TG) 19 repeat (Das et al, 2009)
5'--GATCTGTGTGTGTGTGC—3'
5'--GGTGGCGACACAACAAATG—3' (Ta 58^{0}C)

Amplification reactions were carried out in 10µl containing 30-50 ng template DNA, 10 µM of each primer, 1x reaction buffer (New England Biolabs), 0.2 mM of each of dNTP (New England Biolabs) and 0.5U of Taq DNA polymerase (New England Biolabs) by using a Gradient Mastercycler (Eppendorf). Amplification conditions were 94°C denaturing step for 30s, followed by initial annealing temperature of 70°C, subsequently run down to 54°C at 1°C/cycle, 72°C extension step for 1 min), followed by a uniform three-step amplification profile (94°C denaturing step for 30 s, 54°C annealing step for 30 s, 72°C extension step for 1 min) for another 23 cycles, then 72°C for 10 min, and finally held at 4°C.

DNA sequencing:
DNA bands of the PCR product were purified from the 2% gel slice by selective binding with glass powder using a DNA isolation kit (Hi-Media). Purified DNA was subjected to sequencing by an automated DNA sequencer (Applied Biosystems 3130 Genetic Analyzer) using the same primers for PCR amplification of microsatellites.

3. Results

Modified salt extraction procedure used to isolate genomic DNA from pectoral fins of Indian major carps yielded good quality and quantity of DNA. PCR amplification of these both RNAse treated and untreated genomic DNA gave the same result.

With primer pair 1, genomic DNA of all major carps (Rohu, Catla and their hybrid, Mrigal and its morphotype / white-hybrid) gave PCR products of varying sizes (Figure 4). Sequencing of the PCR products from different carps revealed polymorphic nature of CA repeats among these closely related cyprinids. Catla was found to contain 25 tandem CA repeats while others exhibited 13 or14 repeats (Table 1). PCR amplification of other two microsatellite loci was of mixed type on the basis of presence or absence of products using other two primer pairs.

Table 1: Table showing presence (++) or absence (--) of PCR products from the genomic DNA of different Indian major carps and their hybrids using three different primers

Part A					Part B		
Primers / Locus	Repeat types	Rohu	Catla	Hybrid of Rohu-Catla	Mrigal	White-Hybrid of Mrigal	Comments
1/MFW1	**CA**	++	++	++	++	++	All Carp species gave PCR products; Catla has 11 extra CA repeats.
2/Cc7	**GT**	--	++	++	--	++	Catla with White Hybrids of Mrigal and Hybrid of Rohu-Catla gave products.
3 /Lr22	**TG**	++	--	++	++	--	No product from White Hybrid of Mrigal & wild Catla.

The primer pair 2 (that was reported to give PCR products for Catla) amplified the DNA segments of Catla, Rohu-Catla hybrid, and white-hybrid of Mrigal but not in wild Mrigal and Rohu. PCR-product sizes of different carps in this case had negligible difference in 2% agarose gel and found to contain 20-21 GT repeats revealed through DNA sequencing. Amplifying the carp DNA with primer 3 showed DNA products with Rohu (for which the primer was reported), Rohu-Catla hybrid and Mrigal but not with Catla or white-hybrid of Mrigal. Size of DNA bands after agarose gel electrophoresis appeared exactly the same for all the carps without any apparent changes of the number of TG (19) repeats.

To segregate the hybrids primarily from wild types a comparison can be made considering the presence or absence of PCR products (Table 1) of microsatellite DNA

sequences using three different primers of three different loci.

4. Discussion

Distinguishing the apparently similar Indian major carps under consideration from their hybrid or morpho-type needs molecular identification approach. By comparing the presence or absence of PCR products of microsatellite DNA using three primers or / and number of concerned dinucleotide repeats of different species, it is possible to discriminate the either Rohu-Catla hybrids or white morpho-type of Mrigal from their wild-types.

Specifically, Rohu-Catla hybrids gave PCR products using all three primer pairs and thus can be distinguished from either of wild-types which only gave two products under the same PCR reaction conditions with different primer pairs (Rohu with Primer pairs 1 and 3, while Catla with primer pairs 1 and 2). Moreover, Catla could be differentiated from other carps and their hybrids by looking at the number of CA repeats present (25 Repeats) using primer pair 1 as revealed by consequent sequencing step. Contrarily small-sized PCR products having 13-14 CA repeats were obtained from other carps and their hybrids with the said primer pair. The Rohu-Catla hybrids could then be separated from Rohu by using primer pair 2 that amplified only the Rohu-Catla hybrids but not the wild Rohu (considering the part A of the table 2). Of note, counting of GT and TG repeats by following DNA sequencing step after DNA amplification with primer pairs 2 and 3, respectively, were not necessary for routine discrimination process of Rohu-Catla hybrids (considering the part A of the table 2). Still we did perform the sequencing of the two types of PCR products and found 20-21 GT (Figure 5) repeats with primer 2 and 19 TG repeats with Primer 3. This sequencing step was only effective for primer pair 1-amplified DNA to select out Catla (having 25 CA repeats) from the other two (having 13-14 repeats).

On the other hand, considering the part B of the table 2 to segregate white-hybrid / morpho-type of Mrigal from the wild Mrigal, primer pair 2 was shown to amplify the DNA segments from White-hybrids of Mrigal only but not from wild Mrigal following our PCR protocol (but was previously reported by McConnell *et al.*2001 to amplify by a different PCR protocol). Amplifying the genomic DNA of these carps for TG loci with primer 3 was shown to have PCR products from wild Mrigal but not from white-hybrid of Mrigal (Part B of Table 2). Specifically, white-hybrid / morphotype of Mrigal resulted in PCR products using no 1 and 2 primers and thus can be distinguished from wild-type which showed products using only primer no 1 and 3. So, the white-hybrid / morpho-type of Mrigal could possibly be separated from its wild type comparing the results of PCR amplification with primer pairs of no.2 and no.3. Result of PCR amplification with primer pair no.1 seems not significant for routine discrimination process rather can be used as control.

5. Conclusion

In conclusion, using all three different primer pairs mentioned in this study, primary screening of either of the natural/man-made hybrid of Rohu and Catla from their wild types or white hybrid / morphotype of Mrigal from wild Mrigal can be performed. These findings provide a rapid, accurate, and powerful molecular biology technique for primary segregation of common wild Indian major carps from their commonly available hybrid varieties / morpho-types.

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Conflict of interest: There was no conflict of interest as admitted by each authors of this article.

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Captions to the figures:

Figure 1: Comparative morphology of apparently similar well grown wild Catla (Left), Rohu (Right) and their hybrid (Middle) carp populations.

Figure 2: Comparative morphology of the two different types of Mrigal taken for this experiment showing no apparent dissimilarities except little whitish color of the hybrid / morphotype in contrast to the normal dark coppery brown color of the wild type.

Figure 3: Map of India on the left and Map on the right showing magnified Eastern part of India that includes two states, Jharkhand and West Bengal. The red asterisk marks denoting the places from where the fish samples were taken for the experiment.

Figure 4: Lane no.10, 12, and 13 of the 2% Agarose gel electrophoretogram showing PCR products of primer 1 of Catla with a little bigger size (with 25 CA repeats).100 bp DNA ladder as the molecular-size marker was at lane no.11. Rests of the lanes are PCR products of different Indian carps included in this experiment; all were found to give PCR products of approximately same size.

Figure 5: Total sequence (157 bp) of the PCR amplification product with Primer 2 of the genomic DNA from White Hybrid of Mrigal showing 21 GT repeats (Red Color) from 90th bp to131st bp. Blue (1-20 bp) and Green (138-157 bp) colored sequences are of forward and backward primers respectively.

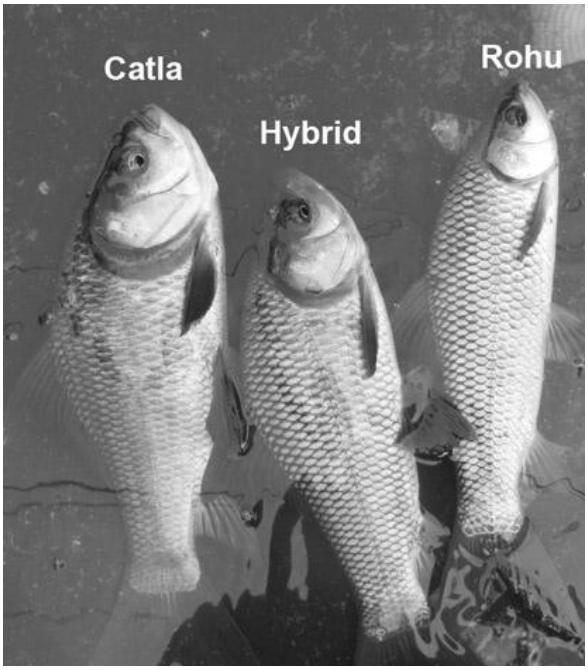

Figure 1

Wild Mrigal **White Hybrid of Mrigal**

Figure 2

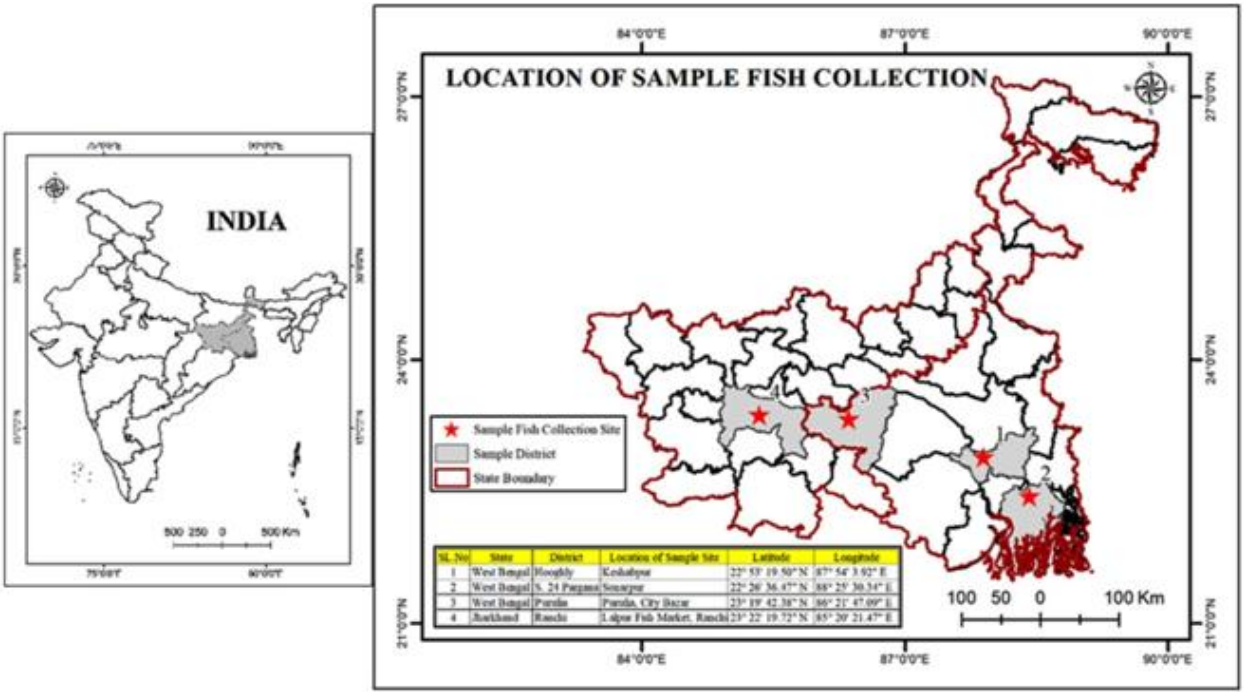

Figure 3

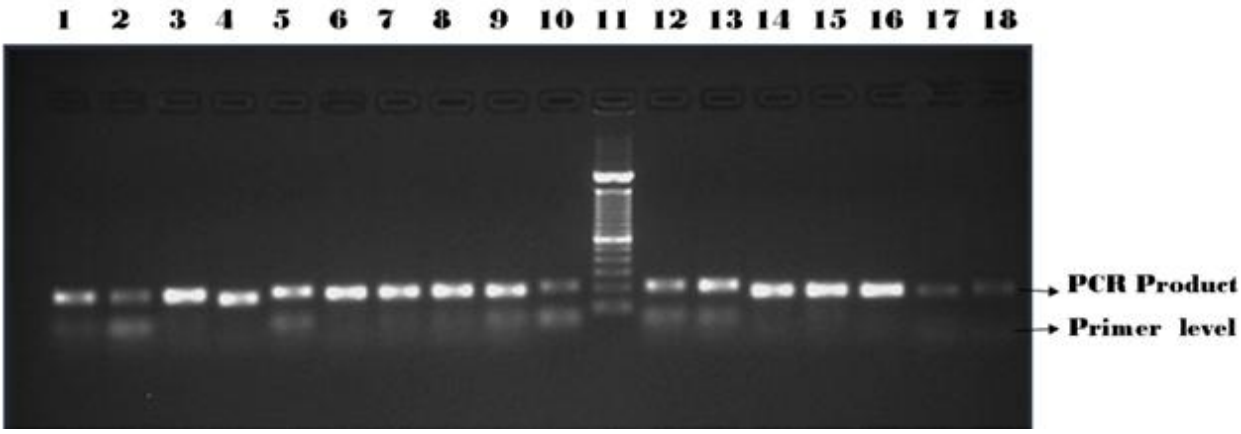

Figure 4

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1   cactctgtgcctagacctcgaaaaaaagaagaaaaactataggaaaaaaagcttttacag
61  gcacttgggataaaataaagatacgggtcgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtg
121 tgtgtgtgtgtkcgggggaacagggcttaaactccag
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Figure 5