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Segregation of common white-morphotype and wild mrigal (*Cirrhinus mrigala*) on the molecular basis

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ABSTRACT

The recurrent formation of hybrids or morphotypes of Indian major carp is very common in eastern India. Genetic monitoring of major carp's wild and hybrid / morphotypes is important for managing genetic diversity. In Jharkhand and West Bengal, white hybrid/morphotypes of mrigal are common. Microsatellite DNA markers were used to differentiate the white morphotype from the wild mrigal in this study. Three sets of primers were used to amplify three different microsatellite markers from the genomic DNA of both carp. Comparing the presence or absence of PCR products of the three loci by analyzing through gel electrophoresis, it has become easy to identify the two varieties of mrigal. The present study looked into a rapid molecular biology technique for discriminating primarily the white hybrids from wild mrigal effectively useful for both management and conservation programs.

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1. Introduction

West Bengal and Jharkhand are known for their rich biodiversity of fish fauna. Mainly rainwater-based natural rivers, lakes, and ponds of these areas considerably contribute to national inland fish production. Mass habitat degradation in the recent past due to environmental modifications and human interventions resulted in constrained natural breeding of the major carp. This resulted in very little use of fry obtained from natural sources to produce major carp (Mondal *et al*. 2006). An increase in the production of fish seeds through induced breeding and composite culture were the few options left to cope with the growing demand. For this reason, the recurrent formation of hybrids or morphotypes among Indian major carp has become very common (Zhang and Reddy 1991). Indian major carp often crop up in ecological polymorphic populations that may differ in phenotypes like shape, colour, etc. with concomitant differences in molecular level (Moles *et al.* 2010; Allu *et al.* 2014). Morphotypes or hybrids are common in mrigal (*Cirrhinus mrigala*), rohu (*Labeo rohita*), and catla (*Catla catla*) of the eastern part of India.

Mrigal has a streamlined coppery brown-colored body with a pointed snout and obtusely round broad mouth. The high demand and pressure of import to other countries have induced the spawning of 1-year-old immature fish and artificial insemination in constrained physical conditions within stagnant water bodies with a limited resource of mating partners (Basant 2018). More than five types of intergeneric hybrid/morphotypes of mrigal were reported in Indian markets (Reddy 2000). Commercial markets of states like Jharkhand and West Bengal sell one of these hybrids as a 'White-hybrid of mrigal' with little morphometric differences from its wild types except for skin colour.

Information on the genetic structure of these fish species necessitates optimization of the identification of stocks and preservation of genetic diversity (Garcia and Benzie 1995; Dinesh *et al.* 1993). 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 common carp (David *et al.* 2001; Lehoczky *et al*. 2002; Bartfai *et al.* 2003; Kohlmann *et al.* 2003; Tanck *et al.* 2000). The present work was undertaken to help differentiate one such white-morphotype from wild mrigal taking fish samples from different markets of state West Bengal and Jharkhand of Eastern India.

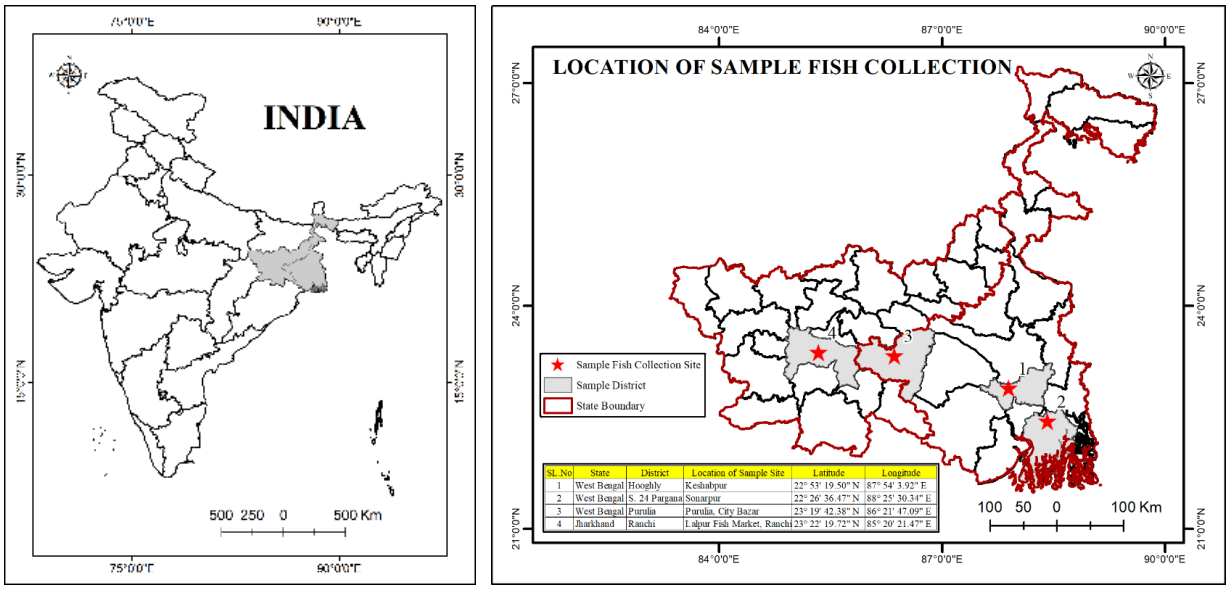
2. Materials and Methods

2.1. Sample collection and preservation

Five fish samples of both the mrigal varieties were collected separately from 4 different markets located in three districts of West Bengal namely South 24 Paraganas, Hooghly, Purulia, and one from Ranchi district of Jharkhand. (Fig. 1). Small pieces of 1 cm^2 size pectoral fins were collected within one hour of sacrifice. Collected fins were washed in water, suspended in 99% ethanol, and stored at -20^{0}C. The fish collection and downstream processes were done in 2022-23 (Fig. 2).

2.2. DNA isolation

Genomic DNA was isolated by a slightly modified phenol-chloroform-isoamyl alcohol extraction procedure with the following protocol. Fin samples were dissolved in the lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 100 mM NaCl) containing 1% SDS and 150 µg ml^{-1} of proteinase K (Sigma) and incubated at 50^0 C for 8 hours. Samples were then treated with 100 µg mL^{-1} of RNase (Sigma) for 1 hour at 37^0 C and centrifuged at 12500 rpm. Added equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) with the removed supernatant. Mixed the solution slowly by repeated inversion. Centrifuged at 12000 rpm for 10 minutes, the top aqueous layer was collected in a fresh microfuge tube without disturbing the intermediate layer. Equal volume of chloroform: Isoamyl alcohol was added in the ratio 24:1 and mixed slowly and thoroughly by inversion of the tube. After that, the tube was centrifuged



Sl. No	State	District	Location of Sample Site	Latitude	Longitude
1	West Bengal	Hooghly	Keshabpur	22° 53' 19.50" N	87° 54' 3.92" E
2	West Bengal	S. 24 Pargana	Sonarpur	22° 26' 36.47" N	88° 25' 30.34" E
3	West Bengal	Purulia	Purulia, City Bazar	23° 19' 42.38" N	86° 21' 47.09" E
4	Jharkhand	Ranchi	Lalpur Fish Market, Ranchi	23° 22' 19.72" N	85° 20' 21.47" E

Fig. 1. Map of India on the left and Map on the right showing the magnified Eastern part of India that includes two states of Jharkhand and West Bengal and the red asterisk marks denoting the marketplaces from where the fish samples were taken for this experiment.

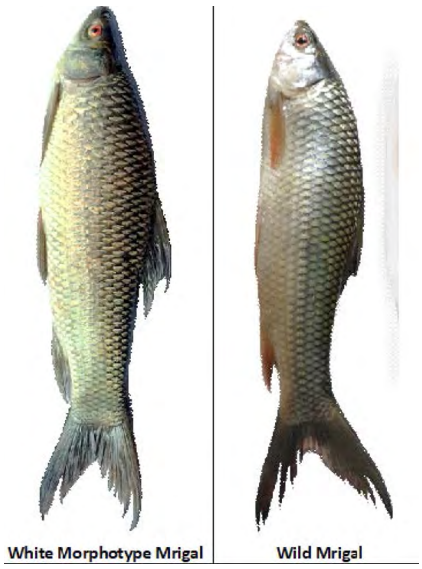


Fig. 2. Comparative morphology of the two different types of Mrigal taken for this experiment showing no apparent dissimilarities except the whitish color of the hybrid morphotype of mrigal in contrast to the normal copperish color of the wild type

at 12500 rpm for 10 minutes, and transferred the aqueous layer into a fresh microfuge, and add 0.1 volume of 3M sodium acetate and an equal volume of ice-cold ethanol (100%). Mix the solution thoroughly with gentle inversion until the DNA pellet is formed as a clump. Kept at -20°C for 1 hour. Then the tube was centrifuged at 10000 rpm for 10 minutes. After discarding the supernatant (ethanol) the pellet was washed with 70% ethanol, centrifuging at 10000 rpm for 10 minutes, and then decanting the ethanol carefully without losing the pellet. The air-dried pellet of the genomic DNA was then resuspended in 150 µl TE.

2.3. 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 in ethidium bromide-stained 0.8% agarose gel electrophoresis.

2.4. PCR Amplification

PCR amplification of three different microsatellite markers using 3 pairs of primers (previously reported) was performed with the isolated genomic DNA from all the fish samples (Table 1).

Table 1. List of primer pairs used for PCR amplification of genomic DNA of mrigal and related fish

Primer No.	Reference	Primer Sequence (5'-3')	Locus	Repeat Motif	Tm (^{0}C)
Primer 1	Crooijmans *et al.*,1997	GTCCAGACTGTCATCAGGAG GAGGTGTACACTGAGTCACGC	MFW 1	$(CA)_{13,14}$	56
Primer 2	McConnell *et al.*, 2001	CACTCTGTGCCTAGACCTCG CTGGAGTTTAAGCCCTGTTC	Cc 7	$(GT)_{20-21}$	55
Primer 3	Patel *et al.*, 2009	GATCTGTGTGTGTGTGTGC GGTGGCGACACAACAAATG	Lr 22	$(TG)_{19}$	58

PCR reactions were carried out in 10 µl containing 30-50 ng template DNA, 10 µM of each primer, 1x reaction buffer (New England, Biolabs), 0.2 mM of each dNTP (New England, Biolabs), and 0.5U of Taq DNA polymerase (New England, Biolabs) using a thermal cycler (Eppendorf) with following time and temperature selections: 94^{0}C enzyme-activating step for 5 min, followed by a touchdown program (94^{0}C denaturing step for the 30s, followed by initial annealing temperature of 70^{0}C, subsequently run down to 54^{0}C at 1^{0}C/cycle, 72^{0}C extension step for 1 min), followed by a uniform three-step amplification profile (94^{0}C denaturing step for 30s, 54^{0}C annealing step for 30s, 72^{0}C extension step for 1 min) for another 23 cycles, then 72^{0}C for 10 min, and finally held at 40^{0}C.

2.5. DNA sequencing

Sliced DNA bands of the PCR products were purified using a DNA isolation kit (Hi-Media) and sequenced by an automated DNA sequencer (Applied Biosystems 3130 Genetic Analyzer, USA) using the same primers used for PCR amplification.

3. Results and Discussion

The phenol-chloroform-isoamyl alcohol method with slight modification was tested for genomic DNA isolation from fish fins, giving good quality DNA yield. Genomic DNA amplification for the CA locus using primer pair 1 (Croojimans *et al.* 1997) was successful for both the mrigal varieties taken for this experiment. Sequencing of the PCR products exhibited a polymorphic nature of CA repeats among these two varieties of mrigal exhibiting 13-14 repeats (Fig. 3).

The primer pair 2 (McConnell *et al.*, 2001) amplified the DNA segments only in white-hybrids of mrigal but not in wild mrigal, following our PCR protocol (Fig. 4).

PCR-product sizes of two different carps, in this case, contain 20-21 GT repeats. Amplifying the genomic DNA of both these carps for TG loci with primer 3 (Patel *et al.* 2009) showed PCR products from wild mrigal only (for which the primer was reported) but not from white hybrids of mrigal. The size of DNA bands appeared the same for both the carps without any apparent changes in the number of TG (19) repeats (Fig. 5).

Table 2 was constructed by comparing the presence or absence of PCR products using three different primers (1, 2, and 3) to amplify three different dinucleotide-repeat loci from two varieties of mrigal carp, and can be used to differentiate them genetically based on the amplification results.

Genomic DNA isolation by modified phenol-chloroform extraction was tried, and it gave a good yield regarding the fruitful downstream process of PCR amplification. RNase treatment made the DNA free from RNA, which was not essential in this study as the RNase untreated genomic DNA also gave PCR products. We checked PCR amplification using both RNase untreated and treated DNA samples with only microsatellite primer 1 of *Cyprinus carpio* (MFW 1) and so concluded that the RNase treatment step could reasonably be omitted to cut down the price for bulk extractions of fish DNA for molecular genetic studies of stock management.

According to Brown and Epifanio, (2003) for proper stock management in fish farming and to achieve proper conservational strategies of natural fish populations, molecular biology techniques like assessing sequence variation of Nuclear DNA have no comparison. Molecular genetic markers are heritable characteristics associated with the identification and characterization of a specific genotype. These markers like microsatellite loci have wide use in aquaculture genetics for the characterization of genetic diversity both within and between populations to determine the species-diagnostic markers, population genetic structures, phylogenetic relationships, etc. (Smith and McVeagh 2004; Faria and Miyaki 2006).

To distinguish the morphologically similar two Indian major carps under consideration, one of them being a morphotype/hybrid form (Fig. 2), a molecular genetics approach of distinction must be followed. Focussing on Fish genetics a few studies have evaluated genetic diversity among carp populations in some wild and hybrid/morphotype varieties using RAPD (Xue et al., 1998; Zhang et al., 2004), AFLP, mt DNA (Zhang et al., 2002), microsatellites (Liao et al., 2005).

As Firas and Abdulkareem (2015) discussed in their review paper on molecular markers, microsatellite markers have several advantages as they are considered robust, and more variable and informative than RFLP, RAPD, and AFLPs. (Senan et al, 2014; He et al, 2003 and Lee et al,2004). High polymorphism and PCR-based analysis has made microsatellite marker one of the most popular genetic markers (Duran et al. 2009; Boris et al., 2011). For this reason, microsatellite-based segregation was initiated via PCR amplification in this study.

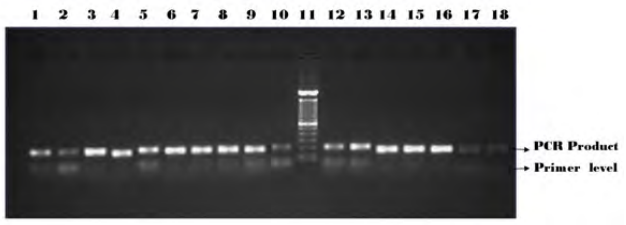


Fig. 3. Lane no. 1 to 10 of the 2% Agarose gel electrophoretograms showing PCR product of primer 1 of wild mrigal. A 100 kb DNA ladder as the molecular-size marker was on lane no. 11. Rests of the lanes are PCR products of morphotype/hybrids of mrigal

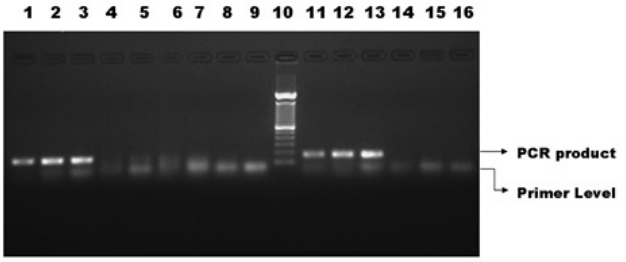


Fig. 4. 2% Agarose gel electrophoretogram showing PCR products of primer 2 (having GT repeats) of genomic DNA from white hybrid morphotype of mrigal (lane nos.1, 2, 3, 11, 12, 13). 100 kb DNA ladder as the molecular-size marker was at lane no. 10. No products were found in lane nos. 4, 5, 6, 7, 8, 9, 14, 15, and 16 containing amplified genomic DNA of wild mrigal

Table 2. Comparing the presence or absence of PCR products

Primers	Repeat Types	Mrigal	White Hybrid	Comments
1	CA	++	++	PCR products obtained from both white hybrid of mrigal and wild mrigal
2	GT	- -	++	White hybrids of mrigal gave PCR product; No product obtained from wild mrigal and is confirmatory for white hybrids of mrigal. 21 GT repeats were found after sequencing the PCR products.
3	TG	++	- -	No product obtained from white hybrids of mrigal and was confirmatory for wild mrigal

Showing presence (++) or absence (- -) of PCR products from the genomic DNA of different Indian major carps using three different type primers

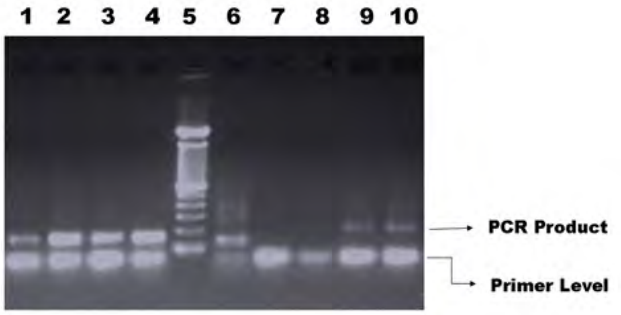


Fig. 5. Lane nos. 1, 2, 3, 4, and 9, 10 of the 2% Agarose gel electrophoretograms show PCR products of primer 3 (having TG repeats) of genomic DNA from wild mrigal only. 100 kb DNA ladder as the molecular-size marker was at lane no.5. No PCR product was found for the white hybrid of mrigal in lane nos. 7 & 8

Comparing either the presence or absence of PCR products of different microsatellite DNA marker sequences given in Table 2, it was possible to discriminate the white morphotype/hybrid of mrigal effectively from wild types of mrigal carp.

When amplified with primer 1 of *Cyprinus carpio* (MFW 1), both the mrigal varieties studied in this experiment gave PCR amplification products and could not be utilized as a means of discrimination for them.

The hybrid morphotype of mrigal can easily be separated from wild mrigal by looking at the results of the other two loci as PCR products of primer pairs 2 and 3 that have GT and TG repeats respectively. The mere presence or absence of PCR products using primers 2 and 3 could reveal the type of mrigal fish involved without needlessly doing the subsequent sequencing steps. Still, we did the sequencing of the PCR product of the white hybrid/morphotype of mrigal using primer 2 which revealed 21 GT repeats in them (Fig. 6). The white morphotype of mrigal gave PCR product using primer 2 and not by using primer 3; while the wild mrigal showed just the opposite result.

4. Conclusion

In conclusion, preliminary separation of the natural/man-made hybrid or morphotype of mrigal from its wild-type can be performed by using two primer pairs (viz. 2 and 3) and checking the presence and absence of products by comparing them with Table 2. Further accurate way of differentiating the two types needs genotyping of more samples of diverse geographical areas with more molecular markers to be checked. Despite that, this finding provides a rapid molecular biology approach for the primary separation of wild-type mrigal from white-hybrid/white-morphotype of apparent similar morphology.

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Author contributions

SG conceived and designed the analysis; NS Collected the data; SG and NS performed the analysis; SG and NS wrote the paper.

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1   CACTCTGTGCCTAGACCTCGAAAAAAAGAAGAAAAACTATAGGAAAAAAAGCTTTTACAG
61  GCACTTCGCGATAAAATAAACATACGCGTCCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTC
121TGTGTGTGTGTKCGGGGGAACAGGGCTTAAACTCCAG
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Fig. 6. Total sequence (157 bp) of the PCR amplification product with Primer 2 of the genomic DNA from the White hybrid of mrigal showing 21 GT repeats (Red Color) from 90th bp to131st bp. Blue (1-20 bp) and Green (138-157bp) colored sequences are forward and backward primers respectively.

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