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Detection of Genetic Variation in the Wild Populations of Indian Major Carps Using Random Amplified Polymorphic DNA Fingerprinting

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Abstract: Genetic variations in wild populations of Indian Major Carps (IMCs) from six geographically isolated locations of Central India Rivers were examined using Randomly Amplified Polymorphic DNA (RAPD). Thirty six specimens of each species from each location were collected. Genomic DNA was isolated from the liver tissues. Out of twenty RAPD primers used, thirteen were found to be scorable on agarose gel, of which 83.33% were polymorphic for *Labeo rohita*, 80.0% for *Cirrhinus mrigala* and 75.0% for *Catla catla*. A total of 576 RAPD bands were amplified. Dendrogram generated for species-specific genetic evaluation revealed that *C. catla* was genetically dissimilar from *L. rohita* and *C. mrigala*. The present investigation is the first report on wild varieties of IMCs while the available data on farmed varieties suggest that *C. catla* and *C. mrigala* are more genetically similar to each other than to *L. rohita*. This study also revealed high intra-specific genetic variation in the wild populations of IMCs. To conclude, the present study suggest high levels of genetic variation and population differentiation required for dynamic evolution and RAPD assay therefore may have potential use for establishing genetic relationship, genome specificity and phylogeny among wild species of IMCs.

Key words: Dendrogram, genetic variation, wild population, Indian major carps

INTRODUCTION

The aquaculture and fisheries have a promising role to play in social development by providing nutritional security for the Indian population and contributing to the economic advancement of the farmers and fishery workers (Lakra *et al.*, 2007) as more than 14.66 million fishermen and fish farmers are totally dependent on fisheries for their livelihood in India. The aquaculture system in India and its neighbouring countries including Pakistan and Bangladesh mainly consists of the Indian major carps (order-Cypriniiformes) viz., rohu (*Labeo rohita*, Hamilton), catla (*Catla catla*, Hamilton) and mrigal (*Cirrhinus mrigala*, Hamilton). These carps contribute approximately 80% of the aquaculture production in India and have also gained popularity in many countries including Vietnam, Thailand, Myanmar and Philippines. Research on cultivating these species was initiated to develop suitable technologies for various farming systems including polyculture and induced breeding. The polyculture technologies are based on species manipulations and application of certain management practices while induced breeding is successfully achieved by the Gonadotropin Releasing Hormone (GnRH) method. GnRH is the best available biotechnological tool profusely used worldwide for the induced breeding of carps (Lakra and Ayyappan, 2003). Though, these technologies have increased fish production in India many folds, presently it is felt that any further improvement in fish production may not be

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possible with these technologies, so researchers gradually started realizing the importance of genetic quality and improvement of nutritive value by fully exploiting their unknown genetic potentials.

A relatively recent advancement made in the area of aquaculture genomics through developing molecular DNA markers has provided more accuracy for the identification of fish stocks. The utility of DNA-based markers is generally determined by the technology that is used to reveal DNA-based polymorphism (Bardacki, 2001). Random Amplified Polymorphic DNA (RAPD) analysis produces amplicons from anonymous genomic regions via the Polymerase Chain Reaction (PCR). This technique has captivated interest because of its simplicity and applicability for the production of genomic markers (Welsh and McClelland, 1990). Main reason for the success of RAPD analysis is the gain of large number of genetic markers that require small amounts of DNA without cloning, sequencing or any other form of molecular characterization of genome and no prior knowledge of the genetic make-up of an organism is required (Bardacki, 2001; Schlötterer, 2004), hence it is advantageous in the understanding of population genetics. Recently RAPD analysis has been widely used to evaluate genetic diversity among fishes (Barman *et al.*, 2003; Islam and Alam, 2004; Das *et al.*, 2005; Brahmane *et al.*, 2006; Nagarajan *et al.*, 2006).

The aim of present study was to shed light on intra-specific and inter-specific genetic divergence among isolated populations of riverine *L. rohita*, *C. catla* and *C. mrigala* from Central India.

MATERIALS AND METHODS

Fish Sample Collection

Geographically isolated populations of *L. rohita*, *C. catla* and *C. mrigala* were collected from six different rivers and associated water bodies from Central India during the month of November, 2007 (Fig. 1) viz., the Pench-associated water bodies at Pench (21°20'N, 79°15'E), the Kanhan river at Kanhan (21°15'N, 79°16'E), the Vainaganga river at Paoni (20°48'N, 79°4'E), the river Wardha at Wardha (21°N, 79°15'E), the river Bor-associated water bodies at Selu (20°50'N, 78°42'E) and the river Painaganga at Mukutban (19°46'N, 78°51'E). Thirty six fish specimen of each species from each location were collected for genetic variation analysis. The liver tissues were dissected out from the freshly caught and sacrificed fish and preserved at -20°C until use.

Genomic DNA Extraction

For the isolation of the total genomic DNA, a short procedure was applied according to the protocol modified from (Wu *et al.*, 1995). Liver tissues (150-200 mg) were placed in a 1.5 mL microcentrifuge tube (Eppendorf AG, Hamburg, Germany) and homogenized by using eppendorf micropestle (Eppendorf AG, Hamburg, Germany). In the liver homogenate 0.5 mL of lysis buffer (containing 4 mM NaCl, 0.5 mM EDTA, 0.1% SDS and 0.02% NP40) and 0.01% Proteinase K were added, mixed gently and incubated at 55°C for 45-60 min for complete lysis of liver cells. After incubation, 250 µL chloroform and phenol were added, mixed gently and centrifuged at 10,000 rpm for 5 min. The supernatant was then transferred to a new microcentrifuge tube and further, 250 µL of chloroform and 250 µL 7.5 M ammonium acetate were added, mixed and centrifuged at 10,000 rpm for 5 min. The DNA was precipitated from supernatant with two volumes of 99% ethanol (ice-cold). The DNA pellet was then washed with 70% ethanol, dried, dissolved in a Tris-EDTA buffer (containing 10 mM Tris-HCl, 1 mM EDTA, pH 7.6) and stored at -20°C. The concentration of extracted DNA was adjusted to 25 µg mL⁻¹.

PCR Primers

Twenty commercially available decamer primers (OPA-1 to OPA-20) from Operon Technologies Inc. (Alameda, CA, USA) were used in the present study for RAPD-PCR amplification.

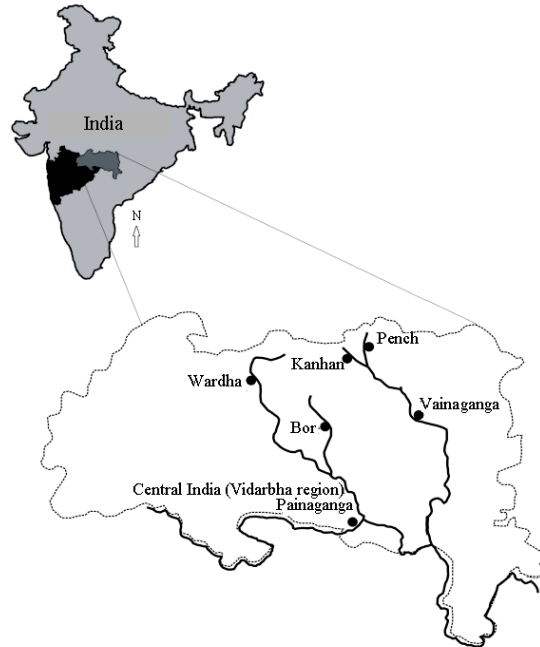


Fig. 1: The catchments areas of Indian major carps from Central India's rivers for the analysis of genetic variation

PCR Amplification

The PCR reaction was carried out in a 25 μ L reaction volume containing 25 ng of genomic DNA, 2.5 μ L of 10X Taq polymerase buffer with 1.5 mM $MgCl_2$, 200 μ M of each dNTPs (Q-Biogene, USA), 15 μ g of OPA primer and 1 unit of Taq DNA polymerase (Q-Biogene, USA). A negative control, without template DNA was included in each round of reactions. DNA amplification was performed in a Techne thermocycler, USA. PCR thermal was performed in 40 cycles. Each cycle consisted of 94°C denaturizing for 45 sec, 36°C annealing for 45 sec and 72°C extensions for 1 min. The thermal cycles were started with an initial denaturizing of 96°C for 5 min and a final 72°C extension for 10 min for polishing the ends (making smooth) of PCR products.

Agarose Gel Electrophoresis

The resulting PCR products were resolved (15 μ L PCR product mixed with 2 μ L bromophenol blue dye) on 1.5% agarose gel using submarine gel electrophoresis for 1 h in 1x TBE buffer (Tris-HCl, Boric acid, EDTA; pH 8.0). Subsequently, gels were stained with ethidium bromide according to (Sambrook and Russell, 2001) and photographed on an ultraviolet (UV) transilluminator using a gel documentation system (AlphaDigiDoc-1201 documentation system, USA). A known DNA size marker was run with every gel (100 bp DNA ladder from Bangalore Genei, India). Reproducibility of RAPD-PCR was tested by performing duplicate reactions at different times and only reproducible bands were scored. Amplicons from thirteen primers were scorable.

Statistical Analysis

Comparison of genotypes was carried out based on the presence or absence of fragments produced by RAPD amplification. The number 1 was designated for presence of fragments and 0 for the absence of fragments. Pairwise genetic similarities ($GS_{ab} = 2 N_{ab} / (N_a + N_b)$) and genetic dissimilarity ($GD = 1 - GS_{ab}$) between individuals were calculated using the data generated from RAPD profiles

according to the mathematical model for studying genetic variations given by Nei and Li (1979) and Lynch (1990), where, N_{ab} is the number of bands common in individuals 'a' and 'b', while N_a and N_b are total number of bands possessed by the individuals 'a' and 'b', respectively. Therefore, GS reflects the proportion of bands shared between two individuals and ranges from 0 (no common bands) to 1 (all bands identical). Two assumptions were made for the analysis of RAPD profile (1) marker from different loci did not migrate to the same position on the gel and (2) each band was assumed to represent the dominant genotype at the locus, whereas lack of the same band in another individual was assumed to correspond to the alternative homozygous recessive genotype in the Hardy-Weinberg equilibrium (Lynch and Milligan, 1994). The cluster analysis was performed for the molecular data by Unweighted Pair-Group Method Using Arithmetic Averages (UPGMA) (Sneath and Sokal, 1973). The dendrogram was generated with the SAHN subroutine of NTSYS-PC to yield the similarity coefficient between the genotypes (Rohlf, 1993).

RESULTS

In the present study, results were generated by examining 20 different decamer primers of OPA series with the populations of three Indian major carps which were collected from the wild. Specimens of *L. rohita* measured in average length from 33-38 cm and 1650-1970 g in their body weight; *C. catla* were of 30-34 cm in length and 2000-2300 g in body weight while those of *C. mrigala* measured 30-32 cm in length and 1200-1350 g in weight (Table 1) at the time of collection.

The number of amplified bands detected varied on the basis of type of primer, species and individual. Out of 20 primers, 13 generated reproducible data. The number of resolved amplified fragments in all the amplicons varied from 4-10 alleles, with the molecular size between 250-1800 bp. However, on an average, each RAPD-PCR amplified 14 bands for *L. rohita*, 7.4 bands for *C. catla* and 7.8 for *C. mrigala* (Table 2). As a representation of the data, only OPA-04 (5'AATCGGGCTG 3'), OPA-11 (5'CAATCGCCGT 3'), OPA-13 (5' CAGCACCCAC 3') and OPA-17 (5'GACCGCTTGT 3') RAPD amplicon profiles obtained in this analysis are shown in Fig. 2a-d, respectively.

Table 1: Biometry of three species of Indian major carps procured from geographically isolated rivers and associated water bodies of Central India

Species	Location					
	Pench	Kanhan	Vainaganga	Wardha	Bor	Painaganga
<i>Labeo rohita</i>						
Average weight (g)	1710.00±20	1921.00±51	1723.00±33	1798.00±45	1681.00±31	1671.00±71
Average length (cm)	34.17±1.1	38.36±0.67	33.66±0.6	36.00±10	33.68±1.6	33.33±1.3
<i>Catla catla</i>						
Average weight (g)	2055.00±35	2101.00±31	2100.00±30	2296.00±34	2116.00±36	2220.00±100
Average length (cm)	30.67±0.67	31.17±0.67	31.33±0.67	33.60±1.3	32.33±1.6	32.67±1.3
<i>Cirrhinus mrigala</i>						
Average weight (g)	1310.00±50	1300.00±20	1225.00±25	1220.00±35	1295.00±25	1235.00±30
Average length (cm)	32.00±1.3	31.00±0.6	30.66±0.6	30.33±1.3	30.17±1.1	30.33±0.6

Table 2: Results of RAPD-PCR amplified DNA fragments from three species of Indian major carps from Central India

Species	No. of primers amplified	Total No. of scorable bands	Average No. of bands per primer	Total No. of species -specific unique bands	Percentage of species -specific unique bands	Total No. of intra-species specific unique bands	Percentage of intra-species specific unique bands
<i>Labeo rohita</i>	13	280	14.0	63.0	22.5	40	14.3
<i>Catla catla</i>	13	147	7.4	32.0	21.8	58	39.5
<i>Cirrhinus mrigala</i>	13	149	7.8	21.0	14.1	19	12.8
Total	-	576	-	116.0	-	117	-
Mean	-	192	9.7	38.7	17.1	39	22.2

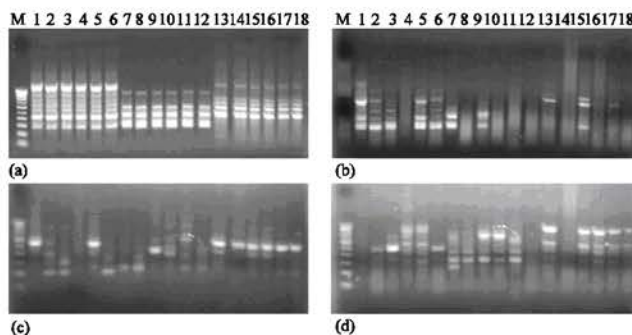


Fig. 2: RAPD banding pattern amplified with primer (a) OPA-04, (b) OPA-11, (c) OPA-13 and (d) OPA-17. M-DNA ladder, 1-6 *Labeo rohita*, 7-12 *Catla catla* and 13-18 *Cirrhinus mrigala*

Table 3: Genetic similarity coefficients among three species of Indian major carps from Central India

Species	<i>Labeo rohita</i>	<i>Catla catla</i>	<i>Cirrhinus mrigala</i>
<i>Labeo rohita</i>	0.500	-	-
<i>Catla catla</i>	0.500	0.444	-
<i>Cirrhinus mrigala</i>	0.385	0.556	0.615

The total number of scorable bands was 576, under which, species specific unique bands were 116 and intra-species specific unique bands were 117. This estimation concluded that 22.5% of bands were species specific for *L. rohita*, 21.8% for *C. catla* and 14.1% for *C. mrigala*, whereas, 14.3% of bands were intra-species specific for *L. rohita*, 39.5% for *C. catla* and 12.8% for *C. mrigala*.

Intra-species specific variation was also detected (Fig. 2b-d). The RAPD data of each species were specific in terms of number and position of bands. The average species-specific genetic similarity coefficients are mentioned in Table 3. *L. rohita* was closest to *C. mrigala* with a genetic coefficient similarity of $GS_{ab} = 0.5$, whereas *L. rohita* and *C. mrigala* were highly divergent from *C. catla* with a genetic coefficient similarity of $GS_{ab} = 0.444$. Intra-species specific genetic similarity was calculated between the three species by one-way ANOVA and was found to be highly significant at $p < 0.005$. The dendrograms (Fig. 3) were generated with the SAHN subroutine of NTSYS-PC to show the phylogenetic similarity between and within the three wild species of Indian major carps.

DISCUSSION

In the present investigation, high levels of genetic variation were detected in the wild populations of three major carps based on RAPD analysis. The presence of variability among populations and also between individuals within a population is essential for their ability to survive and successfully respond to environmental changes (Ryman *et al.*, 1995). In the present study, levels of polymorphic RAPD bands (80.0%) of *C. mrigala* were lower than that of *L. rohita* (83.33%) but greater than *C. catla* (75%). Several fixed RAPD fragments were observed in three major carp's profiles and their genomic markers were specific. The present RAPD profile was further used to test the species-specific nature of 500, 1100 and 700 bp unique bands generated from OPA-04 primer by genotyping *L. rohita*, *C. catla* and *C. mrigala*, respectively. Results indicated the specificity of this RAPD marker to these species suggesting that RAPD-PCR is a rapid and simple method for generating useful molecular type II markers (O'Brien, 1991; Liu, 2003; Lakra *et al.*, 2007) in wild populations (Bartfai *et al.*, 2003).

Unweighted pair-group method using arithmetic averages (UPGMA) analysis of the RAPD markers and pairwise genetic similarities among three wild species of Indian major carps were estimated and ranged from 0.44-0.50 indicating that the genetic distance among these species was low. During the process of evolution, *L. rohita* and *C. mrigala* genomes maintained their genetic status with respect to each other and hence have found closer proximity in the dendrogram, whereas, genome of

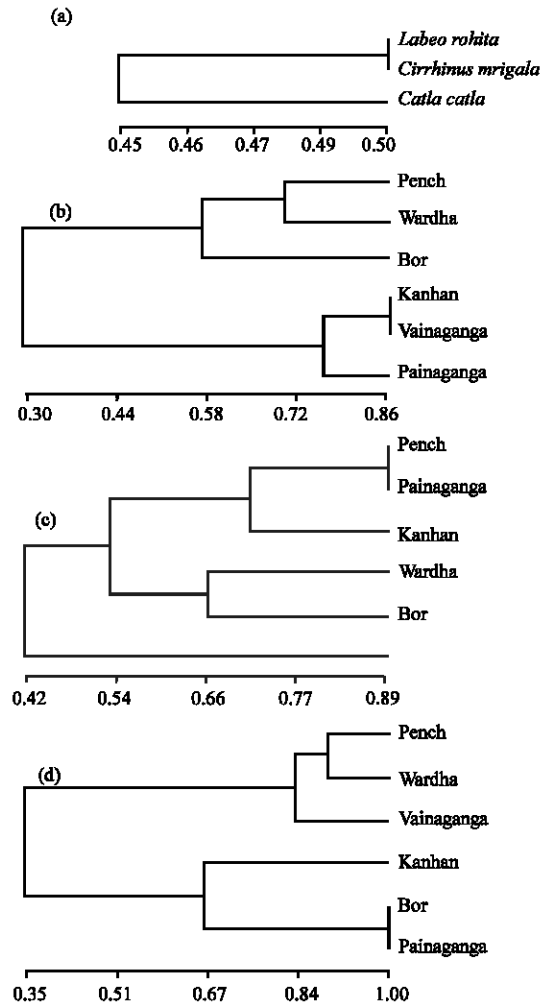


Fig. 3: Unweighted pair-group method using arithmetic averages (UPGMA) (a) Dendrogram for three species of Indian major carps, (b) For *Labeo rohita*, (c) For *Catla catla* and (d) For *Cirrhinus mrigala*, of Central India based on average linkage cluster analysis summarizing data on variation between Indian major carps population according to RAPD-fingerprinting

C. catla finds the place at the end of dendrogram indicating that *C. catla* is genetically dissimilar from *L. rohita* and *C. mrigala*. However, Barman *et al.* (2003) concluded that *C. catla* and *C. mrigala* were more genetically similar to each other than to *L. rohita*; possibly due to their sampling from farmed stocks (and not from the wild) that they have maintained and propagated for long period of time (since 1970s), hence the present study findings are more crucial in the understanding of major carp diversity in the wild.

Intra-specific genetic diversity in six wild and isolated populations of *L. rohita* were estimated and ranged from 0.30-0.86 indicating that the genetic distance within the species was high. Dendrogram generated for *L. rohita* did not show genetic variation between Kanhan and Vainaganga populations whereas Pench, Wardha and Bor populations were arranged in the hierarchical fashion. Painaganga population is placed at the end of the same dendrogram. This analysis revealed significant genetic

variation between Bor and Kanhan/Vainaganga populations. In the dendrogram of *C. catla*, Pench and Painaganga populations were placed at the same level whereas Kanhan population is placed near them and formed a cluster. Wardha and Bor populations of *C. catla* made another cluster and placed near to each other while the population of Vainaganga is far away from both the clusters and found the place at the end of dendrogram. These findings suggest that genetic variation within the group of wild variety of *C. catla* ranged from 0.42-0.89 indicating that the genetic distance was high and genetically distinct from each other. Pairwise genetic similarities and UPGMA analysis of RAPD markers of *C. mrigala* were estimated and ranged from 0.35-1.00 indicating that the genetic distance within the species was high. Intra-specific dendrogram of *C. mrigala* reveals that Pench, Wardha and Vainaganga populations were arranged in the hierarchical fashion and formed a cluster whereas Kanhan, Bor and Painaganga made another cluster and linked with each other to form a major cluster at the distance of 0.35. It is also observed that populations of Bor and Painaganga were placed at the genetic distance of 1.00 from each other indicating both the isolated populations are genetically identical. The RAPD technique that was demonstrated in other groups of fishes exhibited low levels of polymorphism among channel catfish, *Ictalurus punctatus* and blue catfish *I. furcatus*, but polymorphism between them was shown to be higher (Liu *et al.*, 1999). The results of this study overall suggests that intra-specific genetic diversity among the isolated populations of *C. catla* is higher than the populations of *L. rohita* and *C. mrigala* in Central India.

The present investigation is the first report that provides baseline information of genetic polymorphism within and among the three wild and isolated populations of Central India's major carps. Studies utilizing microsatellite, allozyme and mitochondrial markers will further enhance the understanding of genetic variation in wild populations of three Indian major carps. This information can be used to design suitable management guidelines for the wild stock of Indian major carps.

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