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***Full Length Article***

# Genetic Variability in *Labeo rohita*, *Catla catla* and their Hybrid (*Labeo rohita* ♂ × *Catla catla* ♀) Populations Employing Randomly Amplified Polymorphic (RAPD)-Inter Simple Sequence Repeat (ISSR) Assays

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Author's Contribution: RN and SS conceived and designed the review/project/study. RN, MN and IU executed the experiment and analyzed the liver samples. SM and KAG analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version

## Abstract

The present study was conducted to estimate genetic diversity of hybrid produced by two major carps (*Labeo rohita* and *Catla catla*) using DNA fingerprinting RAPD-ISSRs assay. A total of 65 RAPD-ISSR combinations were studied to check polymorphism among the accessions. 866 loci were amplified with an average of 13.32 loci per primer and 45.26% were found informative. Primers: DDA31-ISSR1 and DDA32-ISSR2 were most informative producing 12 polymorphic bands each. A similarity of 82% and 63% was exhibited by *C. catla* and their hybrids and *L. rohita* and their hybrids, respectively. Similarity analysis resulted 92% similarity among *L. rohita* parents' group and 84% similarity for their hybrids. Cluster analysis divided all accessions into two clusters: A and B; placing *L. rohita* in-group A, while B cluster was divided into two Sub groups B1 and B2 having *C. catla* and hybrids, respectively. The low level of genetic diversity, clearly signposted the alarming need of diverse parental populations for fish breeding programs and saving hybrid gene pool losses. It is the first report molecular program for the selection of major carps for breeding of fish in seed hatcheries. © 2017 Friends Science Publishers

**Keyword:** Genetic diversity; Fish seed and parents; RAPD-ISSR

## Introduction

There is ever increasing demand for carp seeds due to quick expansions and improvements in the aquaculture practices commonly around the globe and particularly in Pakistan. The major carps seed supply trend from natural sources has been declined due to hatchery produced seeds. Although it is strong indicator of aquaculture industry growth and development, yet there are many concerns among the fish breeders that hatchery-produced seeds are not doing well in terms of commercial production and earning. This alarming scenario may be due to poor selection, inbreeding and poor brood stock management (Bondad-Reantaso, 2007).

Aquaculture and fisheries are one of the most growing areas in the food sector as compared with all other food related areas (FAO, 2009). One possible reason is the presence of genetic diversity that can be utilized to improve commercial aquaculture for improving the culture methods, domestication and captive breeding of commercially important species (Subasinghe *et al.*, 2009). The rearing of the commercially important species is based upon the maintenance of the brood stock captured from the natural freshwater bodies. It is commonly seen that breeders are not exchanged in hatcheries and these practices continued for successive generations decrease genetic variation and performances of the hatchery stocks (Hasanat *et al.*, 2014). For the establishment of efficient breeding programs in seed hatcheries, it is very important to know about the complete life cycle and genetic background of the interested species.

Genetic variation has a practical importance in stock improvement, conservation of the diverse gene pool, and efficient management of the fish breeding programs (Tassanakajon e*t al.*, 1997; Shafi *et al.*, 2016). In the capture fisheries, a poor fishery management can lead towards the

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exhaustion of the fish stocks that can have ultimately direct effects on the total gene pool of the said group (Li *et al.,* 2007). Morphological data are usually not enough to describe the genetic structure of the population either in wild environment or in the captivity. DNA markers like RAPDs were employed in various studies to determine the intra-population genetic diversity in fish (Seyoum and Kornfield, 1992; Bardakci and Skibinski, 1994; Barman *et al.,* 2003).

The uses of DNA markers are of great importance in the development and execution of genetic improvement programs. Molecular markers are exploited to improve fish brood stock through marker-assisted selection in which, along the physical traits the genes of interest. For example, high growth rate is used to locate variables for better traits. Marker assisted selection is based on the development of maker maps in which quality trait loci (QTLs) are closely located on chromosomes and can be studied for the trait of interest in segregating populations. Although the marker assisted selection through QTLs is a powerful tool for the improvement of genetic makeup, but it is hampered practically, especially in developing countries like Pakistan because there is no complete genome sequence information available for cultured species like carp.

DNA fingerprinting studies are useful for the preservation of genetic diversity, sustainable breeding programs for high yield and for the identification of stocks (Bart 2002; Ullah *et al.,* 2011). *Labeo rohita* (rohu) and *Catla catla* (thaila) are commercially important major carp species found in Pakistan (Mcconnell *et al.,* 2001). The major carp species do not interbreed naturally, but they can produce fertile artificial hybrids in different combinations (Shah and Biswas, 2004). It is suspected that these species may have a common ancestral origin (Simonsen *et al.,* 2005). In fish hatcheries, it is usual practice to maintain a less number of brood stocks and many generations are used within the system along with the spawning of multiple species. This practice is carried out on one hand to save time and money, but on the other hand, it degrades the genetics of hatchery stock. This leads towards the inbreeding decline which is a major factor of poor seed quality. Despite the commercial importance of the major carps, the available genetic data are very limited. Especially for seed stocks, the information is scantyabout genetic relationships and diversities at the molecular level among the hybrids produced in pond cultures.

Molecular techniques like random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), simple sequence repeats (SSR), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and single nucleotide polymorphism (SNP) are being exploited for the study of genetic diversity among the populations and within the populations of different organisms (Liu and Cordes, 2004; Nawaz *et al.,* 2009; Ullah *et al.,* 2012). ISSRs are one of the effective tools to study the problems of systematics and hybridization (Wolfe and Liston 1998; Wolfe *et al.,* 1998; Maltagliati, 2002) as well as of population genetics (Abbot 2001; Luque *et al.,* 2002; Hassan *et al.,* 2003; Casu *et al.,* 2005).

The present study was designed to assess the genetic relationship among *L. rohita*, *C. catla* and their hybrids using ISSRs, from the fish seed hatchery. The ultimate aim was to apply this technique at commercial level to screen genetic diversity among hybrids and their parents in commercial hatcheries to save the gene pool of these two important fish breeds.

## Materials and Methods

The present study was conducted at Department of Zoology, Government College, University Faisalabad and Agricultural Biotechnology Research Institute (ABRI), Ayub Agriculture Research Institute, Faisalabad. Three groups, i.e., *L. rohita*, *C. catla* and their hybrids were procured from Fish Seed Hatchery, Faisalabad, Pakistan and were employed in this study.

The DNA was extracted from blood and liver tissues by the salt DNA extraction method with little modifications (Cummings and Thorgaard, 1994). The concentration of DNA samples was measured using 1 µL amount of stock by using Nanodrop Spectrophotometer with built-in software, ND 1000 3:3:1 (NanoDrop Technologies, USA). All the DNA samples were diluted to a final working concentration of 15 ng/µL and stored in 200 µL aliquots for further use in PCR reactions.

Multiplex polymerase chain reaction was performed in 0.2 mL PCR tubes. The concentrations of PCR reagents used for 25 µL final reaction volume included 2.5 µL 10X PCR buffer, 2.5 µL $MgCl_2$ (25 mmol/L), 3.00 µL dNTPs (2.5 mmol/L), 3.00 µL Primer RAPD (30 ng/µL), 3.00 µL Primer ISSR (30 ng/µL), 2.00 µL Template DNA (15 ng/ µL), 7.00 µL double distilled water and 2.00 µL gelatin. The amplification reaction was performed in Mastercycler Gradient (Eppendorf) after prior conditions optimization. The PCR profile used was; a first denaturation step of 5 minutes followed by 40 cycles for 30 Sec, and for 1 min. After the completion of 35 cycles, the reactions were kept at 72°C for 5 min. and then held at 4°C until the reaction tubes were removed. Amplified products were analyzed by electrophoresis in 2% agarose gel using 1XTBE buffer and detected by post staining with ethidium bromide. The samples were electrophoresed for approximately two hours at 80 V; the amplified products were viewed under ultraviolet trans illuminator using the NYXTECHNIK Gel Documentation system.

All visible amplified fragments were counted. Amplification profiles of the species and hybrids were compared with each other and band of DNA were scored as 01 for present and 00 for absent. The data generated with primers were used to estimate the similarity based on the number of shared amplification products (Nei and Li, 1979). NTSYSpc software was utilized to generate dandrogram by

means of Unweighted Pair Group Method of Arithmetic Means (UPGMA).

**ISSR Analysis**

RAPD-ISSR combinations were surveyed to check the polymorphism among the accessions studied. The information about the primer sequence and amplification statistics is given in Table 1. The accession was first amplified by RAPD using random primers and then by Inter-Simple Sequence Repeats (ISSRs). Three ISSR primers were selected based on literature in combination with 40 RAPD primers making 65 combinations. A total number of 866 fragments amplified in all the three fish samples (*L. rohita*, *C. catla*) and their hybrids) in three replicates.

The ISSR1 produced 239 bands with a combination of 20 RAPD primers (Table 1). In this combination, around 102 polymorphic bands were amplified in both species and their hybrids with 42.67% polymorphism. The most informative combination in this group was DDA-31+ISSR1, which produced 12 polymorphic bands (Fig. 1a), while the least informative combination was DDA-40+ISSR1 with only one polymorphic band. ISSR2 with 24 RAPD combinations amplified 342 fragments in the accessions studied. In RAPD-ISSR2 combination 156 polymorphic bands were amplified in both the species and their hybrids with 45.61% polymorphism. The most informative combination in this group was DDA-32+ISSR2 with 12 polymorphic bands (Fig. 1b), while the least informative combination was DDA1-ISSR2 and DDA10-ISSR2, which produced only two polymorphic, bands each.

The ISSR4 with 21 RAPD primers generated 285 fragments. In RAPD-ISSR4 combination 134 polymorphic bands were amplified in both species and their hybrids with 47.01% polymorphism (Fig. 1c). The most informative primer pair was DDA33-ISSR4 with 11 polymorphic bands while the least informative primer pairs were DDA17-ISSR4, DDA21-ISSR4, DDA24-ISSR4 and DDA36-ISSR4 with 3 polymorphic bands each. The overall statistics of the most informative primers are given in the Table 2. The DDA32-ISSR2 remained the most polymorphic primer used in the present study.

**Similarity Analysis**

Data obtained from RAPD-ISSR analysis was used to generate similarity matrix using Nei and Lei (1979) method. Sixty five RAPD-ISSR primer combinations resulted in amplification of 866 bands; however, only unambiguous and score able loci were utilized for final analysis. The resulting similarity matrix ranged from 56 to 96% as indicated in Table 3. The maximum similarity percentage (92%) within the group was observed for *L. rohita* and the minimum similarity percentage (84%) within the group was observed for hybrids.

**Cluster Analysis**

The data generated by 65 RAPD-ISSR primer pairs were subjected to cluster analysis to reveal percentage similarities and disagreement within the groups and among the groups of fish species studied. Based on information generated a dandrogram was constructed with the help of UPGMA. Two clusters were generated which are presented in Fig. 2.

Cluster A consisting of *L. rohita* accessions, while the cluster B was subdivided into two sub-clusters B1 and B2. The sub-cluster B1 contained all the *C. catla* accessions and the sub-cluster B2 contained all the hybrids accessions. Cluster A was further split into three clusters showing inter sub cluster variation. The accession 1 and 2 are different from the third sub cluster consisting of accession 3 and 4 that are similar to one another. In accession 1, there were two distinct fragments of 580 bp and 970 bp amplified by DDA29-ISSR1.However, cluster B consisted of 10 accessions that further split into two sub clusters B1 and B2. The sub cluster B1consisted of five accessions showing inter cluster variation, accession no. 5 being different while accession 6 and 8 were more similar to one another and similar situation is true for accession 7 and 9. There was one distinct fragment of 300bp amplified by DDA8-ISSR1 for accession 5. The sub cluster B2 also consisted of five accessions showing inter sub cluster variation, accession 14 being different while accession 10 and 11 were similar to each other and similar situation is true for accession 12 and 13. There was one distinct fragment of 590bp amplified by DDA8-ISSR1 for accession 14. At a genetic distance of 0.05 all, the accessions were similar.

## Discussion

In the present study, the blood and the liver tissues were used for DNA fingerprinting. It was found that the genomic DNA extracted from the blood, tissue was of better quality as compared with the DNA extracted from the liver tissues, may be due to the organic metabolites stored in the liver, especially oxidative stress substances which can degrade or damage the DNA (Lazarova *et al.,* 2006). The results of DNA quantification method were in line with Garg *et al.* (2009). In this study, most of the morphometric characteristics of fishes were similar and sometime overlapped with population, exhibited a reasonable degree of genetic variation based on DNA quantification.

In the present study, different parameters as DNA template were evaluated that could influence the quality and reproducibility of ISSR fingerprints. The concentration of template (genomic DNA) could affect a lot in any PCR reaction (Huang and Sun, 2000). RAPD and ISSR were used in combination to reveal the genetic similarity between *L. rohita*, *C. catla* and their hybrids. Esselman *et al.* (1999) reported that the awareness of species relationship is a prerequisite for efficient utilization of genetic variations available to breeders.

**Table 1:** Sequence information of RAPD-ISSR primer pairs employed to fingerprint fish genotypes

| RAPD primer name | Primer Sequence 5´-3´ | Primer length | RAPD Primer Name | Primer Sequence 5´-3´ | Primer length |
|---|---|---|---|---|---|
| DDA1 | AAGCTTGTCACAC | 13 | DDA21 | AAGCTTCATAGCC | 13 |
| DDA2 | AAGCTTAGTGAGC | 13 | DDA22 | AAGCTTCTTGATG | 13 |
| DDA3 | AAGCTTCTATGGC | 13 | DDA23 | AAGCTTCCAGTAC | 13 |
| DDA4 | AAGCTTCTGTGTC | 13 | DDA24 | AAGCTTCGCATTG | 13 |
| DDA5 | AAGCTTACGGACG | 13 | DDA25 | AAGCTTCTCCGTC | 13 |
| DDA6 | AAGCTTATGCACG | 13 | DDA26 | AAGCTTTAAAGGG | 13 |
| DDA7 | AAGCTTAGCAGCT | 13 | DDA27 | AAGCTTCATGGTC | 13 |
| DDA8 | AAGCTTTGAGCGT | 13 | DDA28 | AAGCTTTGGCTCC | 13 |
| DDA9 | AAGCTTGCGCAAC | 13 | DDA29 | AAGCTTTTCGCAG | 13 |
| DDA10 | AAGCTTGAAGCGT | 13 | DDA30 | AAGCTTCTAAGCG | 13 |
| DDA11 | AAGCTTTGGTATG | 13 | DDA31 | AAGCTTCTGACAC | 13 |
| DDA12 | AAGCTTGCGAGGT | 13 | DDA32 | AAGCTTCTAACCG | 13 |
| DDA13 | AAGCTTGTCTAAA | 13 | DDA33 | AAGCTTATTGGTC | 13 |
| DDA14 | AAGCTTCTATTTC | 13 | DDA34 | AAGCTTACCAATC | 13 |
| DDA15 | AAGCTTTGAATTC | 13 | DDA35 | AAGCTTCAATCCC | 13 |
| DDA16 | AAGCTTAAATCGA | 13 | DDA36 | AAGCTTGTCATAG | 13 |
| DDA17 | AAGCTTTTATTCG | 13 | DDA37 | AAGCTTCTGACTG | 13 |
| DDA18 | AAGCTTGTTATAG | 13 | DDA38 | AAGCTTATACAGG | 13 |
| DDA19 | AAGCTTAGTTATC | 13 | DDA39 | AAGCTTAACGAGG | 13 |
| DDA20 | AAGCTTCAAGTTT | 13 | DDA40 | AAGCTTCAAGTCC | 13 |
| ISSR Primer name | Primer Sequence 5´-3´ | Primer length | ISSR Primer Name | Primer Sequence5´-3´ | Primer length |
| ISSR-1 | CTCTCTCTCTCTG | 13 | ISSR-2 | AGAGAGAGAGAGAGAGC | 17 |
| ISSR-4 | GAGAGAGAGAGAGAGA[Y]C | 18 | | | |

**Table 2:** Amplification statistics of most informative primer combinations employed on two fish species (*L. rohita* and *C. catla*) and their hybrids

| Primer name | Band Size | | Total no. of bands | No. of polymorphic bands |
|---|---|---|---|---|
| | Minimum | Maximum | | |
| DDA31-ISSR1 | 250bp | 1200bp | 17 | 12 (70.59%) |
| DDA32-ISSR2 | 300bp | 1400bp | 16 | 12 (75%) |
| DDA33-ISSR4 | 250bp | 1300bp | 16 | 11 (68.75%) |

**Table 3:** Average estimates of genetic similarity among *L. rohita*, *C. catla* and their hybrids based on RAPD-ISSR DNA fingerprints. *L. rohita* (01-04), *C. catla* (05-09) and Hybrids (10-14)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | | | | | | | | | | | | |
| 2 | 0.89 | | | | | | | | | | | | | |
| 3 | 0.89 | 0.94 | | | | | | | | | | | | |
| 4 | 0.90 | 0.95 | 0.96 | | | | | | | | | | | |
| 5 | 0.58 | 0.61 | 0.60 | 0.64 | | | | | | | | | | |
| 6 | 0.56 | 0.60 | 0.57 | 0.62 | 0.83 | | | | | | | | | |
| 7 | 0.59 | 0.62 | 0.62 | 0.65 | 0.83 | 0.83 | | | | | | | | |
| 8 | 0.60 | 0.63 | 0.61 | 0.65 | 0.83 | 0.93 | 0.91 | | | | | | | |
| 9 | 0.60 | 0.63 | 0.62 | 0.67 | 0.83 | 0.81 | 0.88 | 0.85 | | | | | | |
| 10 | 0.56 | 0.59 | 0.60 | 0.63 | 0.75 | 0.80 | 0.82 | 0.81 | 0.85 | | | | | |
| 11 | 0.57 | 0.58 | 0.59 | 0.63 | 0.80 | 0.81 | 0.85 | 0.84 | 0.84 | 0.87 | | | | |
| 12 | 0.64 | 0.64 | 0.64 | 0.68 | 0.82 | 0.80 | 0.86 | 0.85 | 0.84 | 0.80 | 0.85 | | | |
| 13 | 0.61 | 0.64 | 0.63 | 0.68 | 0.82 | 0.80 | 0.83 | 0.82 | 0.85 | 0.83 | 0.84 | 0.86 | | |
| 14 | 0.63 | 0.65 | 0.64 | 0.68 | 0.81 | 0.82 | 0.82 | 0.83 | 0.82 | 0.83 | 0.82 | 0.86 | 0.85 | |

Usually RAPD markers have been employed to reveal genetic similarity among the wild populations of major carps but ISSR has the advantage over RAPD due to its high reproducibility and high power for polymorphism detection. As compared with RAPD markers, a larger number of loci/ fragments can be produced more rapidly with a limited number of primers to estimate the genetic diversity in a population. In a recent, study of *Trachidermus fasciatus*, it is reported that ISSRs are good DNA markers for diversity studies and furthermore the genetic differences in the hatchery populations are low as compared to wild populations (Li *et al.*, 2009). These results were although for endangered fish species from China, but they verified the present finding about the genetic diversity of *L. rohita*, *C. catla* and their hybrids from hatchery stocks.

In this study, 65 RAPD-ISSR primer combinations generated 866 fragments out of which 45.26% fragments were found polymorphic in both the major corps.

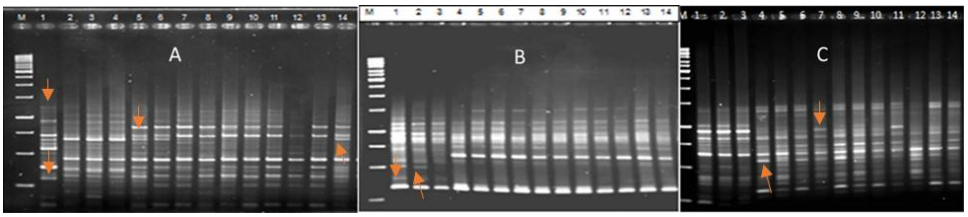


**Fig. 1:** DNA fingerprints of *L. rohita* (01-04), *C. catla* (05-09) and their hybrids (10-14) amplified by a DDA31-ISSR1 (A), DDA32-ISSR2 (B) and DDA33-ISSR4 (C) primer pairs respectively. M is (1Kb marker)

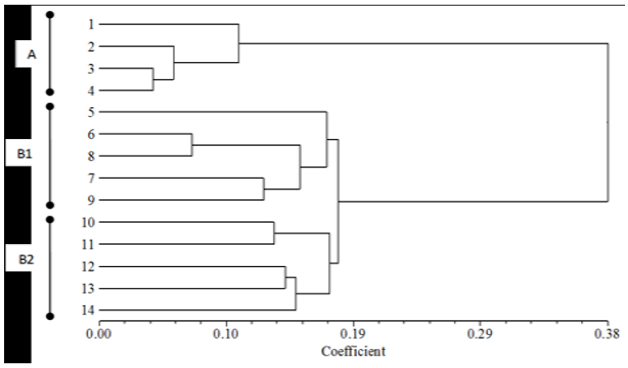


**Fig. 2:** Dendrogram constructed for *L. rohita*, *C. catla* and their hybrids based on genetic distances using 65 RAPD-ISSR primer pairs. Legend. A=(1,2,3,4) B1=(5,6,8,7,9) B2(10,11,12,13,14)

The size of the amplified loci ranged from 250bp to 1500bp which are in good agreement of previous ISSR studies. Li *et al*. (2009) reported that eight primers produced polymorphic results, 31.88, 31.15, 31.03, 27.27 and 26.92%, respectively in five populations of *Hyriopsis cumingii* from five largest lakes in China. The genetic variation within *L. rohita* was only 15%, within *C. catla* only 08% and within hybrids, only 16% (Table 1). This low genetic variation may be due to inbreeding practices usually carried out at fish seed hatcheries. Rahman *et al*. (2009) reported the genetic variation studies among the wild and hatchery stock populations in Bangladesh. High genetic diversity was reported in wild type population and high similarity within the hatchery population. Inbreeding is a common practice in hatcheries with the mass stocking of genetically inferior fry into open water bodies as a result the *C. catla* may be subjected to low genetic diversity (Simonsen *et al.*, 2005).

The similarity matrix results based on the data generated by RAPD-ISSR primers, indicates that the range of similarity is from 56% to 96% among the groups and the maximum similarity mean (82%) was observed between *C. catla* and their hybrids (Table 3). A similar trend was depicted in cluster analysis. *C. catla* and hybrids were sub-grouped in the same cluster showing that they are more closely related to each other. The similarity means between *C. catla* and hybrids was 85%, while similarity means between *L. rohita* and hybrids was only 63%. This suggests that the hybrid population does not represent both the parents equally, but hybrids are more like *C. catla.* The results of genetic similarity among the two major corps *L. rohita*, *C. catla* and their hybrids revealed by RAPD-ISSR markers can be confidently used for the development and execution of molecular breeding programs in fish seed hatcheries. It may be concluded that DNA markers can display better picture of fish genetic background in individuals or mixed populations.

## Conclusion

There was a low level of genetic diversity in rohu and its hybrid, clearly signposted the alarming need of diverse parental populations for fish breeding programs and saving hybrid gene pool losses. A greater inclination of hatchery hybrids towards *L. rohita* and maximum similarity within the parent groups indicates that there is a dire need of exploring the diverse population of *L. rohita* and *C. catla* to be used as parents in fish seed hatcheries.

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