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RAPD-PCR BASED BIOMARKER STUDY FOR MOLECULAR IDENTIFICATION AND POLYMORPHISM IN FISH SPECIES (CYPRINIDAE FAMILY)

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ABSTRACT
The present study focused on the genetic identification and diversity by RAPD-PCR technique among three fish species (Cyprinidae family) of Sagar Madhya Pradesh. This study was aimed at assessing the specific DNA markers for detecting polymorphism and the establishment of genetic relationship among fish *Labeo rohita, Catla catla,* and *Nile tilapia* which are commonly available all over the world. For accomplishment of this work, 5 decanucleotide primers of *RAn* primer series were used. As a result of initial RAPD analysis on pooled DNA, three primers *RAn21, RAn24* and *RAn25* were chosen for analysis, on the basis of band pattern quality, reproducibility and the presence of diagnostic bands. Number of bands obtained may be regarded as the marker bands of diagnostic value. A series of bands ranging between 150bp to 1517bp were produced by these primers. The data shows a total of 13 DNA markers (considering all DNA bands) observed in three fish species under study, out of which, 11 bands were polymorphic, may be considered as markers. Large numbers of diagnostic and species-specific bands were scored within. The results obtained out of the study shows that the primers are diagnostic and variations in three fish species under study.

KEYWORDS: Genetic differentiation, Polymorphism, RAPD- PCR, Random primers

INTRODUCTION

Biological diversity means the variability among living organisms from all sources, including terrestrial, marine and other aquatic ecosystem, the ecological complexes (Carvalho and Hauser, 1994; Klinbunga *et al.*, 2000; Lakra *et al.*, 2010; Tripathi, 2011; Askari *et al.*, 2013). It includes variety of all forms along with their genetic makeup and their all possible assemblages. Indian fisheries are an important sector of food production in the country which provides nutritional security, contributes to the agricultural exports and engage people in different activities (Yousefian and Laloei, 2011; Neekhra *et al.*, 2014). Nowadays freshwater fish species are in serious decline owing to the ecological degradation and mismanagement of natural resources and overexploitation (Was and Wenne, 2003; Shair *et al.*, 2011). The knowledge of genetic background of a species and its population structure is very essential for successful fisheries conservation and management. A number of methods have been developed to measure genetic diversity within the species (Marle-Koster and Nel, 2003; Schlotterer, 2004; Yousefian and Laloei, 2011, Kumla *et al.*, 2012). The central region of India that includes sites of Madhya Pradesh have enormous potential in terms of diverse water resources in the form of streams, rivers, reservoirs, traditional lakes and domestic ponds as well as harbors a wide variety of freshwater fishes. Common carps are spread over rivers and reservoirs of peninsular India and form an important capture and culture fishery in the central India (Garg *et al.*, 2009, 2010; Basavaraju *et al.*, 2014; Shukla and Tripathi, 2016). A number of molecular tools are available for genetic characterization commonly referred to as molecular markers, which are useful tools for the investigation and monitoring of genetic conditions. Genetic markers are important instrument for the study of fish population (Ahmed *et al.*, 2004; Alam Islam, 2005; Kumla *et al.*, 2012).

The Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) method was applied during the study. This technique is the one of the most frequently used molecular methods for the analysis of various organisms (Chauhan and Kumar, 2010; Kumar and Gurusubramanian, 2011). This technique has been widely used due to its rapidity, accessibility and high levels of polymorphism. In contrast to other types of analysis, a small amount of biological material is needed in this method. It is cost effective and can avoid sacrifice of the animals studied (Kumar *et al.*, 2007; Rahman *et al.*, 2009). RAPD and microsatellite markers are among those used to analyze genetic diversity of fish. Both of these markers may be analyzed by PCR (Yoon, 2001; Ali *et al.*, 2004b; Askari *et al.*, 2013; Wali *et al.*, 2013). The RAPD technique consists of amplification by PCR of random segments of genomic DNA using a single-short primer of arbitrary sequence. Its cost effectiveness provides an advantage in population genetic studies (Ali *et al.*, 2004a; Garg *et al.*, 2009; Shukla and Tripathi, 2016).

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In view of the above facts, the present study was aimed to develop RAPD fingerprinting in *Labeo rohita, Catla catla and Nile tilapia* (family: Cyprinidae) and also focused to identify species-specific RAPD marker as a genetic signature for species identification and characterization. Thus, the exploration will provide valuable tool in the form of diagnostic markers for fisheries conservation and supervision of these species.

MATERIALS AND METHODS

SAMPLE COLLECTION

Specimens of both the sexes of all three fish species *Labeo rohita, Catla catla and Nile tilapia* were collected locally from Sagar Lake and maintained in aquarium at laboratory conditions. Genomic DNA isolation was done from their scales (Neekhra *et al.,* 2014). In this protocol both materials and labour have been reduced to a minimum. Using this protocol, good quality high molecular weight DNA has been consistently extracted. Appropriate safety precautions were taken during all steps involving the use of phenol. Genomic DNA extraction and RAPD PCR amplification protocol was done by the following method of Neekhra et al. (2014).

GENOMIC DNA EXTRACTION

Approximately, 50 mg of scales were taken from each species and dried on a filter paper. They were then cut into small fine pieces and placed in a 2 ml-Eppendorf tube containing lysis buffer (200mM Tris-Cl (pH 8.0); 100mM EDTA (pH 8.0); 250 mM NaCl), then add proteinase K (10 mg/ml) and 20% SDS. The content in the tubes were incubated at 48°C for 45-50 min in a water bath and after incubation phenol chloroform isoamyl alcohol (25:24:1) was added to the tube containing lysed scales cells. Protein precipitation was done by gently inverting the tube for 10 min. After centrifugation, top aqueous layer was transferred to a new 1.5 ml-Eppendorf tube, leaving interface and lower phase. The DNA was precipitated out by adding isopropanol and ammonium acetate (10 M) and inverting the tubes gently for several times. The precipitated DNA was then centrifuged to form pellet and then washed briefly in chilled 70% ethanol, air-dried and re-suspended in sterile water/ TE buffer.

After confirming complete rehydration of DNA, the Optical density (A260/A280 nm) was measured by UV spectrophotometer (Cole Parmer Ins. Company, US) and its integrity was checked on 0.8% agarose gel where DNA was stained with Ethidium bromide. The extracted DNA samples were then stored at -20°C till their further use. These DNAs were used as templates in a PCR based assessment for producing RAPD markers. Finally genomic DNA was extracted from scales of all fishes.

RAPD PCR AMPLIFICATION

The DNA was amplified by using 50 µl of reaction mixture containing sterile water 39.0 µl, 10X Taq Buffer A 5.0 µl, 10mM dNTP mix 2.0 µl, RAPD primer 2.0 µl (RAn series supplied by Bangalore genei company), DNA template (10ng/µl) 1.0 µl, Taq DNA polymerase (3 U/µl) 1.0 µl. The amplification was carried out in thermal-cycler (Eppendorf pro) under the cyclic conditions provided in Table 1.

On the basis of results of initial RAPD analysis on pooled DNA, 3 out of 5 primers were chosen for analysis, based on band pattern quality, reproducibility and the presence of diagnostic bands. Finally the RAPD-PCR was done using three primers given in Table 2. The amplified products were run on 2% agarose gel (stained with Ethidium bromide) with 100bp DNA ladder and Low range DNA ruler ranges from 100 bp to 3000 bp (LRDR). Gels were photographed under gel documentation (MultiDoc-It, Labmate, UK). RAPD patterns were visually analyzed and scored from photographs.

RESULTS

In this study, the data shows that a total of 13 DNA markers (considering all DNA bands) observed in all fish species, out of which, 11 bands were polymorphic and may be considered as useful RAPD markers. The largest number of RAPD bands were observed with primer RAn 24 (5 bands), while 4 bands were scored with RAn 21 and RAn 25. Such a wide variation in the number of markers produced by these arbitrary primers may be attributed to the differences in the binding sites throughout genome of the fish species.The molecular weight of scorable bands generated ranging from 100 to 1517 bp. Some other visible polymorphic fragments were also generated but not considered because of weak or non-reproducible amplification, or inability to resolve closely migrating fragments. During the study, similar band patterns were obtained for both male and female individuals of all three species therefore only male specimens were considered for analysis.

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Table1: Cyclic conditions for PCR machine having 50 µl mastermix per PCR tube.

	STEP	T(°C)	Time	Cycles
1	Denaturation	94°C	5.0 mints	X 1
2	Denaturation	94°C	45 sec	X 10
	Annealing	35°C	1 mint	
	Extension	72°C	1.5 mints	
3	Denaturation	94°C	45 sec	X 40
	Annealing	37°C	45 sec	
	Extension	72°C	1 mint	
4	Final extension	72°C	10 mins	X 1
5	Hold	4°C	∞	-

*Tempareture: T(°C); Times: X.

Table 2: Total number of amplified band fragments and number of selected species specific fragments with three primers in three fish species (bands pattern of male were considered for analysis).

Primer	Oligonucleotide sequence 5' to 3'	Size range of amplified bands (bp)	Total number of bands			Number of selected species-specific RAPD fragments characteristic for:		
			Lr	*Cc*	*Nt*	*Lr*	*Cc*	*Nt*
RAn21	TGGCGTCCTC	100->1200	1	1	2	1	1	2
RAn24	TGTCGTGGTC	400->1200	1	3	1	1	3	1
RAn25	TGTGTGCAAC	250-900	2	1	1	1	0	1
Total			**4**	**5**	**4**	**3**	**4**	**4**
				13		**Monomorphic bands: 1** **Polymorphic bands : 11**		

**Lr*: *Labio rohita*; *Cc: Catla catla* & *Nt: Nile tilapia*; Monomorphic band means two common bands between *Labio rohita* & *Catla catla.*

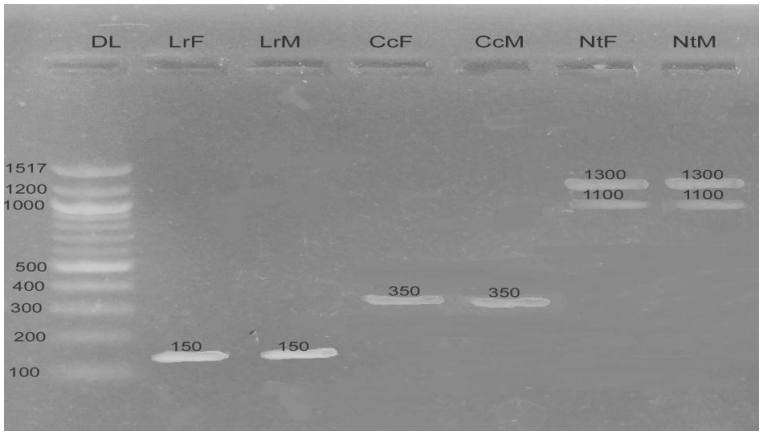

Figure 1: RAPD banding pattern amplified with Primer RAn 21 (TGGCGTCCTC)
Lane 1: 100bp DNA ladder (DL), Lane 2: *Labeo rohita* female (LrF), Lane 3: *Labeo rohita* male (LrM), Lane 4: *Catla catla* female (CcF), Lane 5: *Catla catla* male (CcM), Lane 6: *Nile tilapia* female (NtF), Lane 7: *Nile tilapia male* (NtM)

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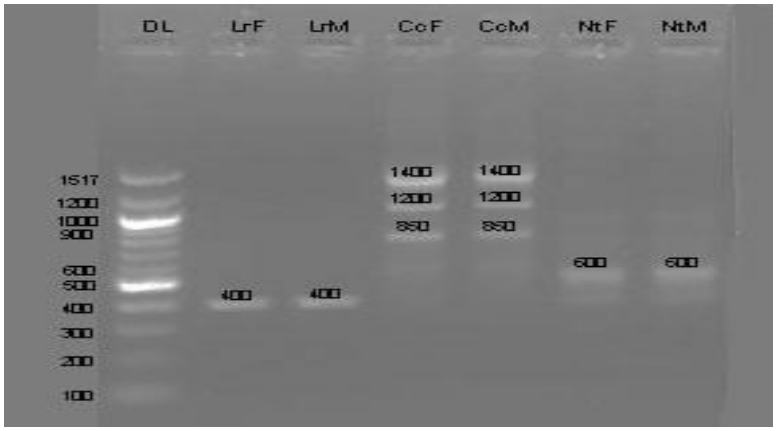

Figure 2: RAPD banding patterns obtained with Primer RAn 24 (TGTCGTGGTC)
Lane 1: 100bp DNA ladder (DL), Lane 2: *Labeo rohita* female (LrF), Lane 3: *Labeo rohita* male (LrM), Lane 4: *Catla catla* female (CcF), Lane 5: *Catla catla* male (CcM), Lane 6: *Nile tilapia* female (NtF), Lane 7: *Nile tilapia male* (NtM)

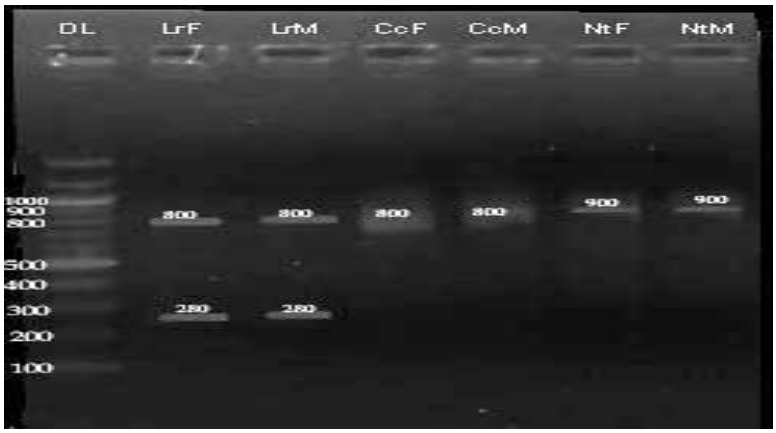

Figure 3: RAPD banding pattern obtained with Primer RAn 25 (TGTGTGCAAC)
Lane 1: 100bp DNA ladder (DL), Lane 2: *Labeo rohita* female (LrF), Lane 3: *Labeo rohita* male (LrM), Lane 4: *Catla catla* female (CcF), Lane 5: *Catla catla* male (CcM), Lane 6: *Nile tilapia* female (NtF), Lane 7: *Nile tilapia male* (NtM)

Primer RAn 21 (TGGCGTCCTC**):** This primer showed four polymorphic diagnostic bands as well as species specific bands for fish species under study. Polymorphic bands suggested genetic difference between species belonging to other genus of the same family. The size of amplified fragments ranges from 150 bp to 1517 bp. Primer RAn 21 successfully amplified two high molecular weight band (>1000bp) for *Nile tilapia* while single band of 150 bp and 350 bp for *Labeo rohita* and *Catla catla* respectively.

Primer RAn 24 (TGTCGTGGTC**):** This primer exhibited multiple band pattern for *C. catla* but unique band pattern for *Labeo rohita* and *Nile tilapia*, and displayed a variety of species specific marker. RAn 24 produced largest number of polymorphic bands (five) than other primers. In *Catla catla* three high molecular bands were amplified but for *Nile tilapia* and *Labeo rohita* only one unique band was amplified of 600 bp and 400 bp respectively.

Primer RAn 25 (TGTGTGCAAC**):** Obtained amplified bands by this primer were 250bp and 800bp for *Labeo rohita*, 800bp for *Catla catla* and 900bp for *Nile tilapia.* One 800bp band which is monomorphic between *Labeo rohita* and *Catla catla* was obtained. This common band is suggested as molecular marker to identify genetic relatedness. While two bands of 250bp and 900bp are species-specific and dominant revealed precise differentiation between *Labeo rohita* and *Nile tilapia* species at DNA level. In the present study the frequency of diagnostic primers is quite high in comparison to other fish species. Out of 13 RAPD markers, 11 are species specific in three fish species under study i.e.

84.61%. Therefore *Labeo rohita, Catla ctla, and Nile tilapia* can be identified by the RAPD markers. Primer RAn 24 produced five species specific markers, highest in the present study. Highest and lowest molecular weight range was exhibited by RAn 21(150bp- >1200bp) and RAn 25 (250bp- 900bp) respectively. Average number of bands produced per primer has been 4.33.

100% sexual genetic similarity: A similar band pattern for both the sexes indicates that there is no sexual differentiation in male and female of the same species. Also there is no such evidence that any primer distinguish sex differentiation.

The observations of this study indicates the effectiveness of RAPD markers in detecting the ratio of polymorphism, monomorphism and estimating genetic distance among three fish species under study**.** Both monomorphic and polymorphic DNA bands were identified based on their presence or absence that could be used for species differentiation. We observed 11 polymorphic bands among these species by using three primers. No monomorphic band obtained by using the primers RAn 21 and RAn 24, indicates 100% polymorphism between them. One unique monomorphic band produced for two species *Labeo rohita*, *Catla catla* by **RAn 25** primer. The diverse nature of DNA bands clearly indicates the genetic distance but the presence of common bands suggests evolutionary relationship among fish species (Brahmane *et al.*, 2006; Vasave *et al.*, 2014).

Many RAPD-PCR studies analyzed in fisheries at molecular level and can be applied efficiently for variation analysis of populations with differential degrees of geographic isolation (Mojekwu *et al.*, 2013; Wali *et al.*, 2013). A little but significant difference was detected in genetic diversity between two feral populations of *Aorichthys seenghala* (Skyes) of Madhya Pradesh, India by Garg *et al.* (2009). RAPD-PCR based biomarker study in fish species of central India also analyzed by Neekhra *et al.* (2014). The Random Amplified Polymorphic DNA (RAPD) was also applied to analyze the genetic variation in three stocks of *Labeo fimbriatus* representing Cauvery, Vedavathi and Tungabhadra river streams of peninsular India by Basavaraju *et al.* (2014). Genetic diversity of *Clarias batrachus* was assessed using RAPD markers in three water bodies of Bhopal by *Garg et al.* (2010). In Nile tilapia (*Oreochromis niloticus*), pollution was recently detected from Lake Qarun, Wadi El-Rayan and Fish Farm at Fayoum Governorate, Egypt using RAPD PCR by Bakr *et al.* (2016).

RAPD fingerprinting offers a rapid and efficient method for generating a new series of DNA markers in fish (Ward and Grewe, 1994; Bartfai *et al.*, 2003; Mishra *et al.*, 2012). Barman *et al.* (2002) evaluated the RAPD assay as a source of genetic markers to generate species-specific RAPD profiles for four species of Indian carp and to estimate genetic variation among them. Data obtained out of present study is useful in genetic diversity assessment and studying taxonomic relationship at molecular level. This technique can also be used to examine the genetic variability in endangered fish species.

CONCLUSION

Due to advances in molecular biology techniques, large numbers of highly informative DNA markers have been developed for the identification of genetic polymorphism. RAPD technique based on the polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers. The technical simplicity, low cost, efficiency in developing a large number of DNA markers in a short time with simple and high equipment's has made this technique valuable and facilitated its use in detecting polymorphism among different fish population, their applicability in population studies, and the establishment of their genetic relationships. RAPD marker has the greatest advantage of its capability to scan across all regions of the genome thus highly suitable for phylogeny studies at species level. The present study may serve as a reference for future examination of genetic variations within the populations of fish which are commercially important. Data obtained out of RAPD assays can be extended to further detect traits in a more refined way. There is also the opportunity and the need to study sequences of specific polymorphic bands to determine the genes detected by RAPD experiments. Further studies with other molecular methods are essential to study and confirm genetic relationships among fish species.

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