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Identification of microsatellite DNA markers in *Labeo bata* for genetic variability studies

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ABSTRACT

Labeo bata, which belongs to the family Cyprinidae, has high economical importance and esteemed table fish worldwide. The present investigation identified six polymorphic microsatellite loci namely, Lr28, Lr29, Lr36, Lr38, Lro37 and Lro50 and two monomorphic loci Lro39 and MFW11. The observations showed that the primer from *L. rohita* and *Cyprinus carpio*, both belong to family Cyprinidae, could be used to identify microsatellite loci in *L. bata*. DNA sequencing of the identified microsatellite loci confirmed the presence of repeat motif.

Keywords: Microsatellite, DNA marker, Labeo bata

BACKGROUND

The species *Labeo bata* belongs to the family Cyprinidae and is a typical fresh water fish of tanks, rivers, reservoirs etc. and are not normally found in saline areas. This species of genus *Labeo* are commercially viable and an esteemed food fish.

It is a medium sized fish, which attain a length 20-25 cm. It is herbivorous in nature. The natural range of its distribution covers West Bengal, Assam, Orissa and Andhra Pradesh in India. It lives in muddy ponds as well as in clear streams of certain altitudes. These species occur in the coastal waters during monsoon**.**

It posses a cylindrical body with the dorsal profiles being more convex and a rounded abdomen. The snout is blunt and often covered with tubercles. Mouth is sub-terminal and lips are thin but not fringed. The pharyngeal teeth are plough shaped or molariform. A very short maxillary pair of barbells concealed within the lips and the scales are cycloid in shape. The characteristic bluish-green shoulder spot on the 5^{th} and the 6^{th} scales on either sides of the body gradually disappear with the advancement of age. The body colour is generally silvery and darkest along the back.

Molecular genetic markers are heritable characteristics associated with identification and characterization of specific genotype, crucial for both management and conservation programmes, including the characterization of genetic diversity both within and between populations. They have been used for various objectives, to determine species-diagnostic markers, population genetic structures, phylogenetic relationships and quantitative trait linkage studies(Utter and Ryman,1993,Carvalho and Hauser,1994,Ward and Grewe,1994 and Smith and McVeagh ,2004).

There is a wide array of molecular markers that include analysis of protein and DNA. Among DNA markers, microsatellite markers have become popular due to their wide application. Microsatellites are short tandem repeats, flanked by unique DNA sequences in non-coding part of the genome. These are loci with co-dominant alleles and inherited in Mendalian fashion(Liu and Cordes ,2004). This allows direct count on heterozygotes on the gel that may not be possible with dominant markers, and provides robustness in statistical analysis. Microsatellite markers have been used independently or in combination with other markers to determine genetic variation and draw inference on population structure in fishes. The flanking sequences in microsatellites within related taxa are highly conserved(Scribner and Pearce,2000). The potential of these markers is enhanced when primers designed for one species amplify homologous in other species(Zardoya *et al*,1996).

The present study is aimed at identification of microsatellites markers in *L. bata* that could be used to determine the genetic variation within and between the populations of *L. bata*. The outcome of this study will provide microsatellite markers to be utilized in genetic improvement programmes and planning conservation as well as management of economically important fishes.

MATERIALS AND METHODS

Sample collection: Samples of *Labeo bata* available at NBFGR, Lucknow were used. The blood samples, collected from the fishes through caudal puncture using hypodermic syringe and fixed in 95% alcohol in 1:5 blood: alcohol ratio, were used.

Isolation of genomic DNA from the Ethanol fixed blood*:* Total genomic DNA was extracted from the Ethanol fixed blood of *L. bata* with proteinase–K and phenol-chloroform method (Ruzzante *et al,*1996), which removes proteins and other cellular components from the nucleic acids and pure genomic DNA was obtained.

Determination of quality and quantity of isolated DNA: 210mg agarose was dissolved in 3ml of 5X TAE and 27ml double distilled water. Agarose solution was heated to dissolve and 0.5µl of ethidium bromide was added before cooling down to approximately 50^{o}C. Solution was poured in casting plate with already adjusted gel comb. It was left for solidification at room temperature for 30 mins for the gel to set before loading the sample in the gel. Cold 0.5X TAE as gel running buffer was used. 8µl of DNA solution with bromophenol blue was loaded in the wells along with a known quantity of DNA in adjacent wells. It was run at 70V for 15 to 20 min

and DNA band was observed with ultraviolet transilluminator.

Polymerase chain reaction (PCR): A total of 12 microsatellite primers were used to screen suitable primers for *L. bata* species. The reaction mixture used, have been shown in the Table 1.

Table 1: Composition of the reaction mixture per reaction

Components	Volume/reaction	Concentration/reaction
Water	18.25µl	-
Buffer (10X)	2.5µl	1X
dNTPs	2µl	200Um
Primer	0.5µl	5p moles
MgCl	0.5µl	2.0mM
Taq DNA polymerase	0.25µl	1.5U
DNA	2µl	50ng
Total	25µl	

PCR reactions were carried out in a thermal cycler MJ PTC-200, with the cycling conditions as shown in Table 2.

Table 2: Details of the cycling conditions in a thermal cycler

Steps	Conditions		Cycles
	Temperature	Time	
Initial Denaturation	94°C	5min	1 cycle
Denaturation	94°C	1 min	24 cycles
Annealing	5-10°C below T_m value	1 min	
Extension	72°C	2 min	
Final Extension	72°C	2min	1 cycle
Soak	4°C	-	-

Seperation of Microsatellite Alleles using PAGE and silver staining for visual Scoring: 8 µl of PCR product was mixed with 1X bromophenol blue and was loaded in the wells of the gels. Standard DNA marker (Msp 1 cut pBR322 DNA) was loaded in between the samples or in the first well of the gel to estimate the size of bands. The gels were run for 5 hours at 10V/cm at 4-6°C. The bands were visualized by staining with silver stain (Silver Staining Kit, Amersham Pharmacia Biotech USA)

Study of Microsatellite Band Pattern: Gel band pattern images were captured and saved in the computer with the help of BIOVIS gel software. Molecular weight of each microsatellite band was calculated comparing the distance run by the standard molecular weight bands through the BIOVIS-ID analysis software. Each individual was genotyped for; each of the microsatellite locus either as homo or hetrozygote. The individual having single band was designated as homozygote and with two

bands hetrozygote.

Sequencing of PCR Amplified Microsatellite Markers: Individuals with polymorphic genotype, which gave dark bands, without any stutter bands, were selected for sequencing reactions. The conditions for PCR are same as that of the original PCR. 0.7 % agarose gel was prepared and 2 µl of amplified PCR product was loaded to estimate the quantity of the PCR product.

Sequencing: Sequencing was done in an automated DNA sequencer-Megabace 500 according to the protocol for sequencing given by manufacturer G E Healthcare, USA.

RESULTS AND DISCUSSION

Identification of Microsatellite markers

In cross-species amplification of microsatellite primers twelve primer pairs from two resource species were tested for the identification of homologous microsatellite loci in *Labeo bata* (Table 3). Successful amplification was observed in *Labeo bata* with eight (66.66%) primer pairs of related species i.e. seven from *Labeo rohita* out of which four were Lr28, Lr29, Lr36, Lr38 (100%) and the remaining three were Lro37, Lro39, Lro50 (42.85%) and one from *Cyprinous carpio* (100%).

The optimum annealing temperature was determined through experimental standardization for each primer pair. The optimum annealing temperatures, to obtain scorable band pattern in *Labeo bata* for all the eight primers vary within a range of 50-55^{o}C. The optimum annealing temperature of Lr36, Lro50 and MFW11 in L. bata is 50^{o}C whereas in Lro37 and Lro39 it is 52^{o}C. The annealing temperature of Lr29 in *L.bata* is 53^{o}C while of Lr28 and Lr38 in *L. bata* is 55^{o}C.

In *L. bata*, four primer pairs of *L. rohita* i.e Lr28, Lr29, Lr36, Lr38 that yielded amplified product were found to be polymorphic. Out of the remaining seven primers of *L. rohita* four primer pairs i.e. Lro33, Lro25, Lro31, Lro43 did not yield any amplified product. For cross species amplification, only Lro37 and Lro50 amplified a polymorphic locus. In L.bata, one primer pair of Common carp i.e. MFW11 and one primer pair of *L. rohita* i.e. Lro39 yielded amplified product was also found to be monomorphic (Table 4).

Number of alleles at microsatellite loci

A total of eight microsatellite loci were identified in *Labeo bata*. The number of alleles at polymorphic locus Lr28, Lr29, Lr36 and Lr38 were nine, four, four and seven. The number of alleles at polymorphic locus Lro37 and Lro50 were six and three. There are one alleles present respectively at MFW11 and Lro39 as they are monomorphic loci.

At Lr28 locus the size of the allele ranged from 94 to 130 and at Lr29, Lr36 and Lr38 locus the size of the allele ranged from 136 to 152, 116 to 128 and 143 to 166. At Lro37 locus the size of the allele ranged from 124 to 162 and at Lro50 the size of the allele ranged from 125 to 141. The allele sizes of MFW11 and Lro39 were

Table 3: Primers of microsatellite loci tested for cross species amplification in *L. bata*

S. No.	Resource species	Family	No. of primer pairs tested	Primer pairs	Gene bank Accession no.	References	Successful amplified loci in *L.bata*
1.	*Labeo rohita*	Cyprinidae	4	Lr28	AM231177	Patel and	4(100%)
				29	AM231178	Das,2009	
				36	AM269526		
				38	AM269528		
			7	Lro25	AM184143	Gheyas *et al.*,2008	3(42.85%)
				31	AM184147		
				33	AM184149		
				37	AM184153		
				39	AM184154		
				43	AM184158		
				50	AM184161		
2.	*Cyprinouscarpio*	Cyprinidae	1	MFW11	-	Coorjiman *et al.*, 1997	1(100%) 8 (66.66%)
Total tested			12				

Table 4: Successful microsatellite primers in *Labeo bata*

S No.	Primer	Primer Sequence	Ta(ºC)	Nature of locus amplified in *Labeo bata*
1.	Lr28	F: AAAGGAAACAGACTCCATCAGCR: CGCTAGCACTTTAATTTCACAGAG	55º C	Poly
2.	Lr29	F: CCCACGCAAACTCCTGTTR: GGAACAAGGCCAGAGCTTTA	53ºC	Poly
3.	Lr36	F: CTTGTTCACTGCACAGACACCR: AAGGTTCAGATTGCCTCCTG	50ºC	Poly
4.	Lr38	F: CTCGTAAAGCTGTGCGATTGR: TAGGAGAAGGGGTGGAAGGT	55ºC	Poly
5.	Lro37	F: TCATGCATCTGGAATGTGAAR: TCAGTATGTCAGAGCAGCACAG	52ºC	Poly
6.	Lro39	F: AAGGCAGGAATGTCGCTCTR: TCGTCATCCTCCTCTTCCTC	52ºC	Mono
7.	Lro50	F: AGCCTAAACGCTGCCTTGR: AGTTACTGGGAGGGTGTTGC	50ºC	Poly
8.	MFW11	F: GCATTTGCCTTGATGGTTGTGR: TCGTCTGGTTTAGAGTGCTGC	50ºC	Mono

Ta (ºC) - Annealing temperature in *Labeo bata*

172 and 159 respectively (Table 5).

Table 5: Number of alleles and allele size range at microsatellite loci of *Labeo bata*

S No.	Loci	No. of alleles		Allele length	
		Resource Species	*Labeo bata*	Resource Species	*Labeo bata*
Polymorphic loci					
					94
					96
					102
1.	Lr28	8	9	110	106
					108
					110
					118
					126
					130
2.	Lr29	5	4	139	136
					146
					149
					151
3.	Lr36	3	4	137	116
					120
					124
					128
4.	Lr38	2	7	154	143
					147
					152
					155
					157
					160
					166
5.	Lro37	-	6	134	124
					128
					132
					136
					142
					162
6.	Lro50	-	3	-	125
					135
					141
Monomorphic loci					
7.	Lro39	-	1	118	159
8.	MFW11	5	1	202	172

Repeat sequences in identified microsatellite loci

To confirm the nature of the amplified products in *L. bata*, the most common allele of polymorphic loci Lr38, Lro37 and Lro50 were sequenced. The perfect

microsatellite repeat $(TG)_{26}$ was present in locus Lr38 in *L. rohita*. In *L. bata* through cross-species amplification same repeat i.e. $(GT)_{18}$ was observed. In case of locus Lro37, an imperfect repeat $(CA)_3(CG)_1(CA)_9T(CT)_3(GA)_1(CA)_4$ was present in *L. rohita*, in *L. bata* also an imperfect repeat is present with same repeat motifs. While at locus Lro50 an imperfect repeat was observed whereas in *L. bata* a perfect repeat $(GT)_{13}$ was observed. Both the target and the resource species were found to have similar repeat motifs at the three microsatellite loci sequenced (Table 6).

Table 6: Repeat motif in identified microsatellite loci in *L. bata*

S No.	Locus	Repeat sequence	
		Resource species	*L. bata*
1.	Lr38	$(TG)_{26}$	$(GT)_{18}$
2.	Lro37	$(CA)_3(CG)_1(CA)_9(CT)_2T(CT)_3(GA)_1(CA)_4$	$(GA)_2(CA)_4TCG(CT)_5(AC)_7$
3.	Lro50	$(GT)_4AT(GT)_9$	$(GT)_{13}$

Microsatellite loci are abundant and distributed throughout the eukaryotic genome and each locus is characterized by a conserved DNA sequence. These sequences consist of both unique DNA and a repetitive DNA motif. Many microsatellite loci, despite their high rates of repeat evolution, are quite conserved in their flanking regions and hence can persist unchanged over a long evolutionary time. High levels of conservation of such flanking sequences have been reported in a large variety of fishes(Scribner and Pearce,2000).

The success of cross-species amplification across genera observed in this study agreed with the reports that microsatellite loci can be obtained by using primer sequences developed for related species(Zheng *et al*,1995,Zardoya *et a*l ,1996 and Yue *et al* ,2002).

The present study demonstrated successful amplification of scorable loci in *L.bata* with primer pairs Lr28, Lr29, Lr36 and Lr38 from *L. rohita* and is found to be polymorphic in target species. The numbers of alleles were nine, four, four and seven respectivly. In resource species, *L. rohita*, the numbers of alleles were eight, five, three and two at the respective loci. The other primer pairs also from *L. rohita*, which were found to be polymorphic on *L. bata*, were Lro37 and Lro50. The numbers of alleles were six and three. Successful amplification achieved in *L.bata* was 100% (in case of Lr primers) and 42.85% (in case of Lro primers) which is much comparable with 66.7% reported for the species of genus within *Labeo*(Mohindra *et al* ,2001).

In *L. bata*, at locus Lr28, Lr29, Lr36 and Lr38, the allele sizes generated by cross-species amplification, were varying from the resource species. Similar observations were made by at several loci, when the primers isolated from *Clarius batrachus* were cross primed with congeneric species and species from different family.

One primer pair of *Cyprinus carpio* i.e. MFW11 and one of *L. bata* i.e. Lro39 were used which amplified as a monomorphic locus.

In the present study 66.66% out of 27 primer pairs were found to be successful in amplifying microsatellite loci in *L. bata* and primers were from the same family as target species, *L. bata*. The success of cross-species amplification of the primer pairs of the family Cyprinidae in *L. bata* shows the evidence of remarkable evolutionary conservation of microsatellite flanking sequences within the family. Similar results were also reported in fishes and other organisms(Watanabe *et al*,2001, Sukmanoman *et al* ,2003 ,Revaldaves *et al*,2005 and Megleez *et al* ,2007).

The repeat motif identified in *L. bata* was quite similar to the resource species showing conservation of microsatellite loci. These data indicated that the cross species amplification might be locus specific(Watanabe *et al*,2001).

CONCLUSION

The present study identified six polymorphic loci namely Lr28, Lr29, Lr36, Lr38, Lro37 and Lro50 and two monomorphic loci namely Lro39 and MFW11 for an important Cyprinidae fish species *L. bata*. The observations showed that the primer from *L. rohita* (which belongs to same family Cyprinidae) and *Cyprinus carpio* (family Cyprinidae) could be used to identify microsatellite loci in *L. bata*. DNA sequencing of the identified microsatellite loci confirmed the presence of repeat motif.

Our results suggested that these identified microsatellites can be used for population genetic analysis and to assess or monitor genetic variation. The availability of conserved microsatellite markers is important for gene mapping, marker assisted selection and evolutionary studies.

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