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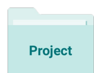

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Note

Cross-species amplification of *Catla catla* microsatellite locus in *Labeo rohita*.

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ABSTRACT

Successful cross-species amplification of microsatellites of a particular locus in *Labeo rohita* using primers of specific microsatellite loci from *Catla catla* is reported. This is the first report on amplification of microsatellites in *Labeo rohita*. The locus was found to be polymorphic ($p<0.95$) in the samples from the two rivers, Satlej and Rapti belonging to different river systems of Indus and Ganges rivers and a total of 4 alleles were found. This validates the utility of the microsatellite locus for screening genetic variability in wild stocks of *L. rohita*.

The Indian major carps, *Catla catla, Labeo rohita and Cirrhinus mrigla* (Subfamily: Cyprininae, Family: Cyprinidae) contribute over 80% of Indian freshwater aquaculture production. *Labeo rohita* is extensively cultivated as a part of polyculture system and is an esteemed table fish. Major source of its seed for aquaculture is from hatchery breeding, moreover inbreeding is reported in Indian hatcheries (Eknath and Doyle, 1990). In this species, quantification of genetic variability of wild stocks is essential for supporting programmes on management of fishery stocks, domestication and genetic upgradation. Limited attempts have been made to study the genetic characteristics of Indian major carps using different tools: cytogenetic (John *et al.,* 1993), allozymes (Gopalakrishnan *et al.,* **1997**) and DNA fingerprinting (Majumdar *et al.,* **1997**). Padhi and Mandal (1995) have reported polymorphism in mitochondrial DNA restriction fragment pattern of farm stocks. However, little is known about the intraspecific genetic variability existing in wild populations of this species. Documentation of natural genetic variability of *L. rohita* is necessary not only for stock based management and conservation but also for genetic improvement programmes. This needs development of appropriate genetic markers.

Microsatellites are a powerful DNA marker for quantifying genetic variations within and between population of species (O'Connell *et al.,* 1998) . Microsatellites are short, 2-5 base pairs (bp) tandemly repeating oligo- nucleotide sequences found to be dispersed in the genome and are highly polymorphic in nature, owing to gain

or losse of one or more repeated units (Brooker *et al.,* 1994). Microsatellites exhibit co-dominant Mendelian inheritance and display varying levels of polymorphism (O'Reilly & Wright, 1995), however no *L. rohita* microsatellite loci have yet been published.

Microsatellites can be investigated on a single locus basis using polymerase chain reaction (PCR), in which PCR primers are designed from unique sequences, flanking the repeat units and amplification products are run on the polyacrylamide / agarose gels. With this technique genotype of an individual can be ascertained at a particular locus. It is relatively simple and permits the use of small samples of tissue. Although these properties make microsatellite DNA through PCR an ideal marker system for answering many population genetic questions, the development of species - specific primers for PCR amplification of alleles can be expensive and time consuming, as it involves construction of genomic libraries, screening of clones with microsatellite sequences and designing of microsatellite primers. However there are reports which point to the fact that flanking sequences of some microsatellite loci are conserved within related taxa so that primers developed for one species can be used to amplify homologous loci in related species. The conservation of flanking regions of microsatellite sequences among closely related species has been reported by a number of groups (Moore *et al.,*1991, Schlotterer *et al.*,1991, Estoup *et al.,*1995, Zheng *et al.,* 1995, McConnell *et al.,* 1995, Presa & Guyomard, 1996, Scirbner *et al.,* 1996, May *et al.,* 1997). Thus, by using heterologous PCR primers the cost of developing similar markers in related species can be significantly reduced.

Among the Indian major carps, *L. rohita* and *C. catla* are genetically close, as indicated by their similar chromosome number (2n=50) (Ponniah & John, 1998) and the ability to produce fertile reciprocal hybrids (Jhingran, 1991), which are commonly encountered in hatcheries and farms (Padhi & Mandal, 1997). Both the species share common loci for several isozymes (K. K. Lal, personal communication) and similar DNA fingerprints using Bkm 2(8) and M13 probes (Majumdar *et al.*, 1997).

Five tetranucleotide microsatellite loci were reported in *Catla catla,* which can be amplified without stutter bands and variant alleles can be visualised using simple non-denaturing polyacrylamide gels and silver staining (Naish and Skibinski, 1998).

The objectives of the present study are (1) evaluation of these five primer sequences of microsatellite loci of *C. catla* for their cross-species amplification in *L. rohita* as well as optimization of PCR conditions (2) validation of these micro satellites as genetic markers for screening of genetic variability in wild stocks of *L rohita.*

Samples of *L. rohita* were collected from two rivers of India, namely Satlej and Rapti , which form a component of Indus and Gangetic river Systems respectively. For Satluj, samples (n = 37) were collected at Ladhowal (Punjab) and for Rapti (n = 28) at Gorakhpur (U.P.). Blood from individual live fish, sampled by caudal puncture was immediately fixed in 95 % ethanol in 1:5 (blood: ethanol) ratio, transported to the laboratory in ice and was stored at 4°C. Total DNA from ethanol preserved blood was extracted by the procedure of Ruzzante *et al.,* (1996) and concentration of genomic DNA was adjusted at 25 ng/ l TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) to be used as template for PCR. Primer sequences for PCR amplification used in the present study are of 5 microsatellite loci identified in *C. catla* (Naish & Skibinski, 1998) and the sequences are as follows:

Locus	Sequence of primers pairs
CcatA7	5' CATGCTAGTAAATGCTTTCATGCTAC 3' 5' TGAAAGCAATACAGGCTGTCAGAG 3'
CcatA12	5' GCACAATATATTGTCTCCATATCGG 3, 5 'AATGCTGGATATATGAAATGGACAG 3'
CcatC3	5' AGGCAATTCAGTCTGTTAGAG 3' 5' TAACAACATGCTAATACCTFGC 3,
CcatG1	5' AGCAGGTTGATCATTTCTCC 3' 5 ' TGCTGTGTTTCAAATGTTCC 3'
CcatG2	5' GTCCGCTGTAAAACGGAGATTCCTG 3' 5' ACCCCCATGTCTCTGTGACATTC 3'

For optimisation of PCR conditions for each pair of the above primers using genomic DNA of ten *L. rohita* as template, the conditions were (1) reaction volume (10 and 25 μl), (2) amount of genomic DNA (50 and 100 ng), (3) primer concentration (10, 15 and 20 p.moles of each forward and reverse) (4) annealing temperature 5 to 10 degree below T_m value for all primers, in addition touch down PCR for A7 locus and (5) addition of tetramethyl ammonium chloride (TMAC) for C3 locus primers was tested. The PCR reaction mix contains : 50/100 ng of genomic DNA, l x buffer (10 mM Tris-HC1, pH 9.0, 50 mM KCl, 0.01% gelatin), 2.0 mM $MgCl_2$, 0.2 mM dNTPs, primer (10, 15 or 20 pmoles of each) and 1.5 units of Taq DNA polymerase. The reaction mixes were amplified in a Perkin Elmer Cetus 480 thermal cycler and the conditions for all loci were: hot start at 94°C for 5 rain and 25 cycles of amplification (denaturing at 94°C for 30 sec, annealing at 5 to 10 degree below T_m value for 30 sec, elongation at 72°C for 1 min) and a final elongation at 72°C for 4 min. In additon, for A7 locus touch down PCR conditions from 68 to 61 °C reducing 1 °C/2 cycles were also tried. Only for C3 locus primers, addition of tetramethyl ammonium chloride (TMAC) at a final concentration of 1, 10, 20 and 60 mM (Newton, 1995) was tried for removing multiple bands. Following amplifications, the PCR products were stored at 4°C and analysed within 24 hours. Amplified products were analysed by electrophoresis on a 8% non-denaturing polyacrylamide gel (29:1) with IX TBE buffer for initial screening and for later screenings of population samples for G1 locus, 12% gel was used. The electrophoresis was carried out for 3-5 hours at a constant voltage of 10 V/cm at 4-6°C. The gels were silver stained (Silver Staining Kit, Pharmacia Biotech, USA) and a known DNA size marker (MspI cut pBR322 DNA) was run in every gel. The size of the amplified products were determined with Biovis ID gel software (Expert Vision Labs Pvt. Ltd., Bombay, India). The allele frequencies, heterozygosity (observed, H_{0bs} and expected, H_{exp}) and measure of Nei's genetic diversity (Gst) were calculated with software GENETIX (Belkhir *et al.*, 1997) and hetero zygosity levels (F_{is}) with GENEPOP version 3.1 (Raymond & Rousset, 1998).

In *L rohita, CcatG1* primers yield clear scorable PCR products (single or double bands, Fig. 1) *with* 50 ng DNA, 10 pmol of each primer in 25μl reaction mix at annealing temperature of 55°C (Table 1), where as in *C. catla,* the optimal conditions for amplification were 0.2 pmol

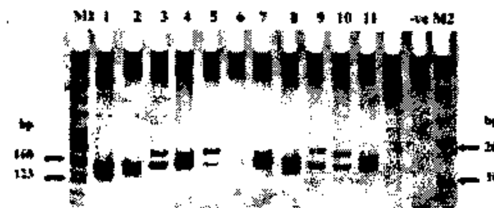

Fig. 1. Microsatellite locus in L. rohita obtained by cross-amplification with *Catla catla* microsatellite primers of Ccat G1 locus, observed on polyacrylamide gels. Molecular weight markers : M1-PBR 322, Msplcut, M2-100 bp ladder, I-II : Different individulas, Genotype of individual 1 : 135/123, 2: 135/135, 3 : 135/187, 4 : 135/171, 5 : 135/187, 6 : 135/135, 7 : 135/171, 8 : 135/135, 9 : 135/187, 10 : 135/187, 11 :135/171

TABLE 1. *Cross species amplification in* L. rohita *using 5 pairs pf* C.catla *microsatellite primers.*

Locus in *C. catla**	**Amplification in *L. rohita***
Ccat A7	Diffused products
Ccat A12	No product
Ccat C3	Multiple products
Ccat G1	Clear scorable product
Ccat G2	No product

*Naish and Skibinski, 1998

TABLE 2. *Allele frequency and heterozygosity at G1 locus from two wild populations of* L. rohita

	Allele frequency in populations	
Allele (bp)	Satlej (n = 37)	Rapti (n = 28)
G1 Locus		
123	0.0405	0.0000
135	0.6892	0.6607
171	0.0405	0.1250
187	0.2297	0.2143
H exp.	0.4690	0.5019
H obs.	0.6216	0.6786
Fis	-0.313	-0.319
Hs = 0.4854	Ht = 0.4879	Gst = 0.0050

H exp = Expected genetic heterozygosity
H obs. = Observed genetic heterozygosity
Fis = Heterozygosity level
Ht = Total genetic diversity of both populations,
Hs = Mean genetic diversity per population, calculated from average expected heterozygosities.
Gst = Nei's gene-diversity statistic (Nei, 1973)

primer and 25 ng DNA in 10 µl final volume (Naish & Skibinski, 1998). It seems that unique flanking sequences at this locus are found to be conserved between these species under study. Diffused PCR products bands were observed for *CcatA7* locus and touch down PCR conditions did not help in improving the resolution of the bands. With *CcatC3* locus primers, multiple bands appeared and these became more intense with the addition of TMAC, at the concentrations given above. Absence of *Ccat12* and *CcatG2* product may be due to sufficient sequence divergence at one or both target sites to prevent the primers from annealing during PCR. Redesigning of primers may be needed to detect the product at these loci. Previously no success was obtained when these primers were tested in *L. rohita* for cross-amplification by Naish & Skibinski (1998). The cross-species amplification of microsatellite loci has been reported in many species; e.g. in salmonids (Scribner *et al.*, 1996), in *Clarias*

species Na-Nakorn *et al.*, 1999), in rock fishes (Wimberger *et al.*, 1999), in sunfishes (Neff *et al.*, 1999) and sturgeon (May *et al.*, 1997).

The amplified microsatellite *CcatG1* locus is polymorphic (p<0.95) in *L. rohita* and can be used as a genetic marker for differentiation of population structure. The DNA extracted from blood samples from two rivers of Satlej and Rapti (N=65) belonging to Indus and Ganges basins respectively is analysed in the present work. Total four alleles of approximately 123, 135, 171, and 187 bp (Fig.1) are detected. The 135 bp allele is most common in both the sample sets. The observed heterozygosity range from 0.6216 to 0.6786 (Table 2) which is higher than that observed for *Catla* for this locus (range 0.15 to 0.58, Naish and Skibinski, 1998). The Gst revealed low level of genetic differentiation in the *L. rohita* populations studied.

This study has clearly brought out the feasibility of cross species amplification of microsatellite loci among closely related species of Indian major carps and its use as one of the molecular markers in population genetic studies.

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