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Microsatellite DNA markers for population-genetic studies of *Labeo dyocheilus* (McClelland, 1839)

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Summary

Fifty-four primers published for six cyprinid fishes were tested to amplify homologous microsatellite loci in *Labeo dyocheilus*. Fifteen primers yielded successful amplification and seven were polymorphic with 3–9 alleles. To evaluate utility of the identified loci in population genetic study, 84 samples were analysed. The samples were collected from four rivers viz. Beas, Satluj, Yamuna and Jiabharali. The four sample sets displayed different diversity levels, with observed heterozygosity from 0.34 to 0.53. Significant genotype heterogeneity ($P < 0.001$) over all loci indicated that the samples are not drawn from the same genepool. The identified microsatellite loci are promising for use in fine-scale population structure analysis of *L. dyocheilus*.

Introduction

The microsatellites DNA markers are proven tools for direct assessment of genetic variation and population level evolution (O'Connell and Wright, 1997; Neff and Gross, 2001). They reveal high levels of polymorphism and exhibit co-dominant inheritance in Mandelian fashion. One of the characteristics of most of the microsatellite loci is that the sequences flanking the microsatellites are conserved and there exists a potential to use the primers developed for one species, to characterize loci in other related species (Moore et al., 1991; Zardoya et al., 1996; Galbusera et al., 2000; Maudet et al., 2004). Scribner and Pearce (2000) reviewed cross-species amplification of microsatellite markers in various taxonomic groups. Among piscine species, cross-species amplification of the microsatellite primers was successfully demonstrated in Cichlidae (Zardoya et al., 1996), Salmonidae (Scribner et al., 1996; Smith et al., 1998; Cairnney et al., 2000) and Cyprinidae (Zheng et al., 1995; Mohindra et al., 2001; Lal et al., 2004), Percidae (Wirth et al., 1999) and in Acipenseridae (McQuown et al., 2000). Use of heterologous sequences can circumvent the extensive preliminary work and cost involved to develop microsatellite enriched genomic libraries for individual species (Zardoya et al., 1996).

Labeo dyocheilus or *kali rohu* (Family Cyprinidae) is a commercially important food fish in upland water bodies. It is native to Afghanistan, Pakistan, India, Nepal, Bhutan, Bangladesh, Myanmar, Combodia and Thailand (Froese and Pauly, 2003). In India, the species inhabits the Indus, Ganges and Mahanadi river systems. At present the fishery is supported through capture from natural resources. *Labeo dyocheilus* has been categorized as a vulnerable fish (Mahanta et al., 1994). The species is also considered as a potential candidate for aquaculture. Knowledge of genetic variation and population structure of *L. dyocheilus*, across its natural distribution, is necessary to plan effective conservation and propagation assisted rehabilitation strategies. To generate this data, identification of polymorphic molecular markers is a first crucial step (Ferguson, 1995). No information is available at present on any class of genetic markers in *L. dyocheilus*.

The objective of the present study was to test if primers developed for other cyprinids could provide amplification of homologous microsatellite loci in *L. dyocheilus*. The paper also evaluates the suitability of the identified microsatellite loci in stock structure analysis of *L. dyocheilus*.

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Material and methods

Sample collection and isolation of genomic DNA

The fish specimens were obtained through commercial riverine catches from four rivers: Beas (Pathankot, n = 21), Satluj (Nangal, n = 24), Yamuna (Yamuna Nagar, n = 26) and Jiabharali (Bhalukpong, n = 13) during September 1999 to July 2002. The sampling sites were selected to cover genetic variation on a wide geographical distribution range of the species. The rivers Beas and Satluj are part of the Indus basin. The rivers Yamuna and Jiabharali (tributary of the Brahmputra River) are associated rivers of the Ganga River system, occupying extreme west and east locations, respectively (ECAFE, 1966). The blood samples were collected through caudal puncture and fixed in 95% ethanol in 1 : 5 (blood: ethanol) ratio. The total DNA was isolated through phenol-chloroform extraction procedure following Ruzzante et al. (1996).

PCR amplification

Amplification of each DNA sample was performed in a 25 μl reaction mixture containing 1 × PCR buffer (10 mM Tris HCl pH 9.0, 50 mM KCl, 0.01% gelatin), 0.2 mM of each dNTPs, 2.0 mM $MgCl_2$, 5 pmol of each primer, 1.5 units of Taq DNA polymerase and 25–50 ng of DNA. The amplifications were carried out in a MJ PTC-200 thermal cycler set with the following parameters: 5 min of initial denaturation at 94 °C followed by 25 cycles of 30 s at 94 °C, 30 s at the annealing temperature (Table 1) and 1 min at 72 °C. A final elongation of 4 min at 72 °C was added. The amplified products were stored at 4 °C. Amplified products were resolved on non-denaturing Poly Acrylamide Gel Electrophoresis (PAGE) (19 : 1, acrylamide: bisacrylamide) (size 12 × 10) with

Table 1
Detailed characteristics of amplified microsatellite loci in *L. dyocheilus*

Amplified Locus		Characteristics			
		Resources species		*L. dyocheilus*	
Range No. of alleles	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	T_a (°C)	Size (bp)
MFW 1	GTCCAGACTGTTCATCAGGAG CA GAGGTGTACACTGAGTCACGC	55	57	174–196	4
MFW 2	CACACCGGGCTACTGCAGAGCA GTGCAGTGCAGGCAGTTTGC	55	59	157–178	5
MFW 9	GATCTGCAAGCATATCTGTCG CA ATCTGAACCTGCAGCTCCTC	55	55	90–96	3
MFW 15	CTCCTGTTTTGTTTTGTGAAA CA GTTCACAAGGTCATTTCCAGC	55	51	156–190	6
MFW 17	CAACTACAGAGAAATTTCATC CA GAAATGGTACATGACCTCAAG	55	51	196–222	5
MFW 19	GAATCCTCCATCATGCAAAC CA CAAACTCCACATTGTGCC	55	49	150	1
MFW 24	CTCCAGATTGCACATTATAG CA TACACACACGCCAGAGCCTTT	55	51	161	1
MFW 26	CCTGAGATAGAAACCACTG CA CACCATGCTTGGTGCAAAAG	55	51	96	1
Bgon 17	CAATTACAAGGGGTTACATACTGA AC CATCTAACATTGCCTTGGG	–	55	156	1
Bgon 22	TCTTGTTGATCACACGGACG CCT ACAGATGGGGAAAGAGAGCA	–	55	109	1
Ca12	GTGAAGCATGGCATAGCACA $(TAGA)_{10}$ CAGGAAAGTGCCAGCATACAC $(CAGA)_4$ $(TAGA)_2$	57	55	168–205	8
R-1f	CGAGACACCAGAGAAAAGAC TG GGGACATAATGTTGGGATAA	–	50	134	1
R-3r	TATTCACCCCAAATCCATTA GT GACCCTTGTGCATAAGACC	–	50	222	1
R-6f	TATCCTGGCTGAAAACTTTG GT CTACAGGAACAACCATCACC (2 loci)	–	50	146,157	1
R-12F	CTATTCCTGTGCAGACCTTC AC GATACACGTCCAGTTTCACC		–	55	9

1 × TBE buffer for 5 h at a constant voltage of 10 V/cm. at 4 °C. The gels were silver stained (silver staining kit, Pharmacia Biotech, USA). A DNA size marker PBR 322 DNA/MSPI digest was used to designate the pattern of alleles comparing the product size to the relevant size marker. The size of the amplified products was determined with ID ELIGHT Version 3.01 (VDS, Amersham Biosciences, USA).

Screening of PCR primers and genetic diversity analysis

Microsatellite sequences (54) published or available for six cyprinid species (resource species) were used for the cross-species experiment (Table 2): *Cyprinus carpio* (Croojimans et al., 1997), *Barbus barbus* (Chenuil et al., 1999), *Catla catla* (Naish and Skibinski, 1998), *Barbodes gonionotus, L. rohita* and *Campostoma anamolum* (Dimsoski et al., 2000). Except for *C. anamolum* (subfamily Leuiscinae), the other five resource species and test species (*L. dycheilus*) belong to the subfamily Cyprininae. The six species are termed as resource species in the study (Table 2). This cross-species amplification experiment was done with ten specimens of *L. dyocheilus*. The optimum annealing temperature, to obtain a scorable band pattern, was determined through experimental standardization for each primer pair.

The suitability of the identified polymorphic loci was evaluated for population substructuring of *L. dyocheilus* on a heterogenous collection of 84 samples from four different riverine sites, as given above. Individual fish genotypes for each locus were determined. The data was analysed using software Genetix 4.02 (Belkhir et al., 1997) to obtain allele frequencies, mean number of alleles per locus, heterozygosity values, expected (H_e) and observed (H_o). Tests for conformity to Hardy–Weinberg expectations were performed by the Markov chain method with parameters dememorization = 1000, batches = 100 and iteration = 100 (Genepop Version 3.3d, probability test, Raymond and Rousset, 1995a). Genetic homogeneity of four sample sets was determined through an exact test (*G* based test) that assumes random samples of genotypes (Genepop Version 3.3d, Genotype differentiation test, Raymond and Rousset, 1995a). This test is performed on genotype tables and possible non-independence of alleles within genotypes that will not affect test validity (Raymond and Rousset, 1995b; Goudet et al., 1996).

Results and discussion

Primer sequences and the specific annealing temperature (T_a °C) in the resources species and *L. dyocheilus* are given in Table 1. The optimum annealing temperatures to get the scorable bands in *L. dyocheilus* differed from that reported for the resources species. Fifteen (27.7%) out of the 54 primer pairs tested yielded successful amplification in *L. dyocheilus*. All primer pairs amplified only a single locus, except R6f that amplified two loci. It is evident from Table 1 that amplification success was higher when primers were from the resource species within the subfamily Cyprininae than

Table 2
Primers of microsatellite loci tested for cross-species amplification in *L. dyocheilus*

S. No.	Donor species (Subfamily)	No. of primer pairs tested	Loci/Primer	Genbank Accession. No.	Successful amplified loci in *L. dyocheilus* number (%)
1	*C. carpio* (Cyprininae)	21	MFW1,2,6,7,9,11,14,15,16,17,18, 19, 20,23,24,26,28,29, 30,31,32	–	8 (33.3)
		2	Cc72,Cc80,	AY169249-50	
		1	Cca30	ABO43469	
2	*B. barbus* (Cyprininae)	5	Barb37,54,59,62,79	–	0
3	*C. catla* (Cyprininae)	4	CcatG1,G2, A12,C3	AF045378-80	0
4	*B. gonionotus* (Cyprininae)	5	Bgon22,69,75,79,17	AJ291680-84	2 (40)
5	*L. rohita* (Cyprininae)	6	R-1F,R-2F, R-3R, R-5F,R-6F,R-12F	AJ507518-24	4 (66.7)
6	*C. anomalum* (Leuciscinae)	10	Ca3,5,6,8,10,11,12,15,16,17	AF277575,77, 78,80,82–84,87–89	1 (10)
Total tested		54			15 (27.8)
Total primers from Subfam. Cyprininae		44			14 (31.8)

from other subfamilies of the family Cyprinidae. Results suggested that certain sequences flanking tandem repeats are conserved within the subfamily Cyprininae and, to some extent, also between the subfamilies of Cyprinidae. It is interesting that a sequence (Ca12) could be found in the subfamily Cyprininae, homologous to the locus in subfamily Leuiscinae. Zheng et al. (1995) demonstrated the possibility of using primers interspecifically among cyprinids. Mohindra et al. (2001) reported successful amplification of homologous microsatellite locus in *L. rohita* using primer developed for another cyprinid, *C. catla*. Tong et al. (2002), using primers developed for *C. carpio*, could amplify microsatellite loci in silver and bighead carps. Successful identification of polymorphic microsatellite markers for *Cirrhinus mrigala* was

Table 3
Parameters of genetic variation for seven microsatellite loci in *Labeo dyocheilus* from four locations (B = Beas, n = 21; Y = Yamuna, n = 26; J = Jiabharali, n = 13; S = Satluj, n = 24). Observed (H_o) and Hardy–Weinberg expected (H_e) heterozygosity values with associated probability (P); probability (P) of genotype homogeneity between samples are given. Significant probability values are marked *Significant at ($P < 0.05$), **Significant after critical significance level is adjusted for Sequential Bonferroni correction (Lessios, 1992)

Locus	River	Alleles	Allele size range (bp)	HW Equilibrium (P)	H_e	H_o	Allelic Homogeneity (P)
MFW-1	B	3	176–183	1.0000	0.2166	0.2381	0.0455*
	S	3	176–183	1.0000	0.3130	0.3636	
	Y	3	176–183	1.0000	0.1102	0.1154	
	J	4	176–192	0.1721	0.5237	0.3846	
MFW-2	B	3	159–166	0.5303	0.5512	0.4500	0.0002**
	S	3	156–166	0.6860	0.4081	0.3636	
	Y	5	156–174	0.3737	0.6510	0.7083	
	J	2	156–159	–	0.0950	0.1000	
MFW-9	B	3	90–96	0.2913	0.5499	0.7143	0.0017**
	S	3	90–96	0.8537	0.5486	0.5000	
	Y	3	90–96	0.8286	0.5141	0.5000	
	J	2	90–96	1.0000	0.2604	0.3077	
MFW-15	B	6	156–201	0.0104*	0.6259	0.5238	0.0000**
	S	6	156–201	0.0182*	0.7266	0.5417	
	Y	5	159–189	0.1651	0.4786	0.3846	
	J	4	159–182	0.6459	0.6716	0.6923	
MFW-17	B	3	212–222	0.5586	0.3220	0.2857	0.2856
	S	3	212–222	1.0000	0.3336	0.3913	
	Y	3	212–222	1.0000	0.4268	0.5000	
	J	2	212–215	1.0000	0.4012	0.3333	
Ca12	B	5	169–198	0.0490*	0.6950	0.5238	0.0002**
	S	6	163–187	0.4151	0.7221	0.6364	
	Y	8	163–198	0.7899	0.7722	0.8462	
	J	3	172–181	0.0010**	0.6531	0.0000	
R12	B	3	141–156	1.0000	0.1769	0.1905	0.0000**
	S	6	130–159	0.8415	0.4931	0.6250	
	Y	6	130–156	0.4348	0.5391	0.6250	
	J	7	118–159	0.0749	0.7813	0.5833	
Mean	B	3.71			0.4482	0.4180	0.0000**
	S	4.42			0.5064	0.4888	
	Y	4.7			0.4988	0.5256	
	J	3.42			0.4837	0.3430	

achieved through use of primers of other cyprinid fishes (Lal et al., 2004).

Seven microsatellite loci, MFW1, MFW2, MFW9, MFW15, MFW17, Ca12 and R-12F were polymorphic and had 3–9 alleles (Table 1). A locus was considered polymorphic when the frequency of most common allele was less than 0.99 (Hartl and Clark, 1997). The observed heterozygosity values ranged from 0.34 (Jiabharali) to 0.53 (Yamuna) samples. The mean number of alleles was found to be from 3.42 (Jiabharali) to 4.71 (Yamuna). The probability test did not detect any significant deviation in allele frequencies from that expected under ($P < 0.001$) Hardy–Weinberg equilibrium, except at locus Ca12 in the Jiabharali sample (Table 3). A significant deficiency of heterozygotes was observed at this locus. One of the reasons could be the null allele at this locus that are not amplified due to mutation in primer site and which contribute to an excess of homozygotes (Paetkau and Strobeck, 1995). Therefore it was of interest to perform the Hardy–Weinberg test with the H1 heterozygote deficit (Raymond and Rousset, 1995c). The test yielded the probability value of 0.001, the same as found with the probability test for locus Ca12 (Jiabharali sample), indicating significant deficiency of heterozygosity. Besides null alleles, another possibility is a serious concern that the assumptions underlying the Hardy–Weinberg equilibrium relevant to natural population of *L. dyocheilus* could be violated (Ferguson, 1995).

A test for genetic differentiation was performed to test the hypothesis that the sample sets had genetic heterogeneity. The genetic heterogeneity was tested based on the genotype rather than on allele frequencies The combined probability over all loci and the sample sets was found to be significant ($P > 0.0001$), indicating that sample sets differ significantly in their genotype frequencies. Genotype frequencies at five loci: MFW2, MFW9, MFW15, Ca12 and R12, displayed highly significant heterogeneity in all the sample sets. A global test for pair-wise comparison clearly indicated the highly significant values for each population pair made with the population of Jiabharali, showing its distinctiveness from the rest of the population. *Labeo dyocheilus* was obtained from Gst value for a small sample size (Nei and Chesser, 1983) and was estimated to be 0.112.

The present study successfully identifies seven polymorphic microsatellite loci through cross-species amplification. The genetic variation detected at these loci exhibit promise for use in fine level population structure analysis of *L. dyocheilus*.

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