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Cross-species amplification of microsatellite loci in the cyprinid fish *Labeo calbasu* (Hamilton, 1822)*

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鲤科鱼蓝黑鲃微卫星位点的种间扩增*

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摘 要 本研究使用 105 对微卫星引物对 7 种鲤科鱼类进行跨越种间 PCR 扩增，共得到 14 个多态性微卫星位点。其中 9 个扩增效果较好的位点用于分析来自帕吉勒提河（Bhagirathi，$n = 20$）和戈达瓦里河（Godavari，$n = 25$）的蓝黑鲃（*Labeo calbasu*）样品的遗传多样性。结果显示，前者在每个位点的平均等位基因数为 7.33，而后者为 8.11，期望杂合度介于 0.795（Bhagirathi）和 0.801（Godavari）之间；4 个位点 *MFW*11*（Godavari）、*R*1*（Godavari）、*R*3*（Bhagirathi）和 *Lr*38*（Bhagirathi 和 Godavari）都表现出明显的杂合子缺失和哈迪温伯格平衡偏离；而任意两位点间都未观测到连锁不平衡现象；位点 *R*3* 极可能存在无效等位基因。上述结果表明这些多态性微卫星位点作为共显性标记在蓝黑鲃群体遗传学研究中有着较好的应用前景［动物学报 54（5）：937 – 940，2008］。

关键词 蓝黑鲃 跨越种间扩增 微卫星位点 多态性 遗传变异 无效等位基因

Key words *Labeo calbasu*, Cross-species amplification, Microsatellite, Polymorphic, Genetic variation, Null allele

Labeo calbasu (Hamilton, 1822) is one of the Indian major carps and inhabits deep pools of river, natural lakes and man made ponds, and is distributed in India, Pakistan, Bangladesh, Burma and Nepal. Besides constituting an important capture fishery resource, *L. calbasu* is a compatible species for polyculture with other major carps and is considered a good table fish (Chonder, 1999).

Genetic variation is an important feature for imparting capability to adapt to changing environmental conditions, and is vital for long-term survival of population or species (Ferguson et al., 1995). The polymorphic microsatellite loci, distributed across the genome are codominant and inherited in Mendelian fashion (O'Connell and Wright, 1997). Microsatellites have been extensively used for direct assessment of patterns of genetic variation for variety of vertebrates (Chistiakov et al., 2006). The conserved nature of sequences flanking the microsatellite loci provide potential application for using primers developed for one species in other close related species (Scribner and Pearce, 2000).

This study aims to test cross-species amplification of polymorphic microsatellite markers and assess their suitability for population genetic analysis in *L. calbasu*.

1 Materials and methods

1.1 Fish samples and DNA isolation

Fish specimens were collected through commercial riverine catches from two independent rivers, Bhagirathi ($n = 20$ Farakka, 24°05′N; 88°06′E) and Godavari ($n = 25$ Manthini, 18°39′N; 79°40′E) of India. A blood sample from individual fish was drawn on site by caudal puncture and fixed in 95% ethanol in 1 : 5 (blood : ethanol) ratio. The samples were transported to laboratory on ice and stored at 4℃. Genomic DNA was extracted from blood using a proteinase K and phenol : chloroform

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protocol (Ruzzante et al., 1996).

1.2 PCR amplification and Electrophoresis

PCR amplification was carried out in a 25 μl reaction mixture that included 1 × PCR buffer (10 mmol/L Tris-HCl pH 9.0, 50 mmol/L KCl and 0.01% gelatin), 0.2 mmol/L of each dNTP, 2.0 mmol/L of $MgCl_2$, 5 pmol/L of each primer, 1.5 U *Taq* DNA polymerase and ~50 ng of template DNA. PCR cycles (MJ PTC-200 thermal cycler) were as follows (i) one cycle of initial denaturation at 94℃ for 5 minutes, (ii) 25 cycles of denaturation at 94℃ for 30 seconds, optimized annealing temperature for 30 seconds, elongation at 72℃ for 1 minute, (iii) a final elongation of one cycle at 72℃ for 4 minutes and stored at 4℃. The amplified products were resolved through vertical non-denaturing polyacrylamide (19∶1 acrylamide∶bisacrylamide) gels electrophoresis (size 12 × 10 cm, Amersham Biosciences Ltd.). Electrophoresis was performed with 1 × TBE buffer for 5 hours at 10 V/cm) at 4℃. DNA samples from 12 individuals of *L. calbasu* from two different rivers were analysed in the cross-priming experiments. The optimum annealing temperature was determined through experimental standardization for each primer pair. For loci exhibiting successful amplification, gel concentration was optimized according to allele size for better resolution. All the loci yielded scorable band patterns at 10% PAGE except locus *R*3* that required 12% concentration).

The amplified loci were silver stained (Silver staining kit, Amersham Biosciences, USA). A DNA size marker (pBR322 DNA/*Msp*I digest) was used to allele size assignment by comparing the product size using software BIOVIS Gel 1D.

1.3 Screening of primers and genetic diversity analysis

Primers of 105 microsatellite loci from seven cyprinids (source species) were used for the cross-priming tests. These species include *Cyprinus carpio*, *Barbus barbus*, *Pimephales promelas*, *Barbodes gonionotus*, *Labeo rohita*, *Carassius auratus* and *Campostoma anomalum* (Table 1). Microsatellite sequences of *L. rohita* were obtained from the NCBI and were used to design primers (Primer3). All source species and test species belong to the subfamily cyprininae except *C. anomalum* (subfamily Leuiscinae).

To assess genetic variation, nine polymorphic microsatellite loci were used to genotype a total of 45 *L. calbasu* individuals from two river systems. Individual fish genotypes for each locus were analyzed using the software Genetix 4.02 (Belkhir et al., 1997) to obtain allele frequencies, mean number of alleles per locus and heterozygosity values (observed *Ho* and expected *He*). Software Genepop (ver. 3.3d, Raymond and Rousset, 1995a) was used to determine conformity to Hardy-Weinberg expectations (Probability test) and genetic homogeneity between the two populations (Genetic Differentiation Test). Genetic heterogeneity between the two populations 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). Genetic heterogeneity was tested based on the genotype rather than allele frequencies in view of the observed nonconformity to Hardy-Weinberg expectations (Raymond and Rousset, 1995b; Goudet et al., 1996). Deviation from HW equilibrium was observed at loci *MFW*11*, *R*1*, *R*3*, and *Lr*38* after the probability level was adjusted ($P < 0.003$) for Bonferroni correction (Lessios, 1992). Software Microchecker (Van Oosterhout et al., 2004) was used to assess the presence of null allele at each locus in the samples from two rivers that revealed the possibility of null alleles at locus *R*3*.

2 Results

Of the 105 primer pairs tested in *L. calbasu*, 14 loci (13.3%) were polymorphic, 30 monomorphic, while remaining 61 primers either didn't amplify or yielded unspecified products (Table 1). Out of 14 loci, nine loci (*MFW*11*, *R*1*, *R*3*, *R*12*, *Lr*28*, *Lr*29*, *Lr*38*, *Lro*23* and *Lro* 25*) yielded consistently scorable patterns. Total 45 *L. calbasu* samples from two rivers were analysed with nine microsatellite loci to test their potential in genetic variation analysis. The allele sizes for the present set of microsatellite loci in *L. calbasu* was found comparable to the allele size reported (or designed) for the source species (Table 2). The number of alleles found ranged from 6 to 11 for the nine microsatellite loci.

The mean number of alleles per locus were 7.33 (Bhagirathi) and 8.11 (Godavari). The expected heterozygosity (*He*) values ranged from 0.7951 (Bhagirathi) to 0.8010 (Godavari), where as observed heterozygosity (*Ho*) varied from 0.6945 (Bhagirathi) to 0.6946 (Godavari). No evidence of linkage disequilibrium was detected at any locus pair comparisons in any population. Probability test was used to determine, if the genotype proportions conform to that expected under the condition of Hardy-Weinberg equilibrium. Significant genetic heterogeneity ($P < 0.05$) was evident at three loci, *Lr*29, *Lro*23 and *Lro*25. The various estimates provided strong evidence that genetic variation detected at the analysed microsatellite loci can be significant in stock structure analysis of *L. calbasu*.

3 Discussion

The study demonstrates successful cross-priming amplification of microsatellite loci in *L. calbasu* and fourteen polymorphic loci were found, of which nine loci were used to assess genetic variation in samples from two riverine locations. The results are consistent with earlier reports, suggesting the possibility of cross-species

Table 1 Primers of microsatellite loci for cross-priming tests in *Labeo calbasu*

Source species	No. of primer pairs tested	Loci/Primer	GenBank Accession No.	References
Cyprinus carpio	21	*MFW*1, 2, 5, 6, 7, 9, 11, 12, 14, 15, 16, 17, 18, 19, 22, 23, 24, 26, 29, 31, 32	–	Croojimans et al., 1997
	2	*cc* 80, 72	AY169249 – 50	
Barbus barbus	5	*Barb*37, 54, 59, 62, 79	–	Chenuil et al., 1999
Barbodes goniotus	5	*BGO*80, 81, 82, 83, 84	AJ291680 – 84	–
Labeo rohita	12	*R*1, 2, 3, 5, 6, 12, 26, 23, 14a, 14b, 21, 24	AJ507518 – 22, 24, AJ831434, 36 – 39	Das et al., 2005
	11	*Lr* 27, 28, 29, 30, 32, 33, 36, 38, 44, 45, 46	AM269523, 26, 28, 34 – 36, AM231176 – 79, 81	Patel et al., 2006
	21	*Lro* 14, 23, 25, 26, 29, 30, 31, 32, 33, 34, 35, 36, 37, 39, 40, 41, 43, 44, 47, 49, 50	AM184142 – 48, 50 – 56, 58 – 63	Gheyas et al., 2006
Campostoma anomalum	10	Ca3, 5, 6, 8, 10, 11, 12, 15, 16, 17	AF277575, 77, 78, 80, 82 – 84, 87 – 89	Dimsoski et al., 2000
Pimphales promelas	7	*Ppro*48, 80, 118, 126, 132, 168, 171	AY254350 – 254357	Bessert et al., 2003
Carassius auratus	11	*J*1, 2, 7, 9, 12, 20, 50, 58, 60, 68, 69	AY115092 – 93, 95 – 97, 99, 102 – 106	Yue et al., 2002
Total tested	105			

Table 2 Characteristics of *Labeo calbasu* microsatellite loci

Locus (Donor species)	Primer sequence	Allele size in source species (bp)	Optimum Ta (℃)	River	Na	Size range in test species (bp)	*He*	*Ho*	P_{HW}	P_G
*MFW*11*	F: GCATTTGCCTTGATGGTTGTG	180	57	Bha	7	156 – 176	0.7368	0.6842	0.1083	0.178
(*Cyprinus carpio*)	R: TCGTCTGGTTTAGAGTGCTGC			Goda	8	156 – 176	0.8290	0.6667	0.0007#	
*R*1*	F: CGAGACACCAGAGAAAAGAC	116	53	Bha	8	106 – 120	0.7763	0.6500	0.0663	0.750
(*Labeo rohita*)	R: GGGACATAATGTTGGGATAA			Goda	7	106 – 118	0.7400	0.6000	0.0005#	
R3*	F: TATTCACCCCAAATCCATT	115	50	Bha	7	92 – 112	0.8125	0.6000	0.0030#	0.346
(*Labeo rohita*)	R: ACCCTTGTGCATAAGACC			Goda	6	94 – 112	0.7977	0.7083	0.0589	
*R*12*	F: CTATTCCTGTGCAGACCTTC	124	55	Bha	7	112 – 128	0.7675	0.7500	0.5330	0.468
(*Labeo rohita*)	R: GATACACGTCCAGTTTCACC			Goda	8	112 – 134	0.8096	0.7200	0.4388	
*Lr*28*	F: AAAGGAAACAGACTCACATCAGC	110	53	Bha	7	96 – 108	0.7525	0.6500	0.0678	0.718
(*Labeo rohita*)	R: AAAGGAAACAGACTCACATCAGC			Goda	7	96 – 108	0.7622	0.7600	0.8534	
*Lr*29*	F: CCCACGCAAACTCCTGTT	139	53	Bha	7	141 – 153	0.8356	0.6667	0.0155	0.022*
(*Labeo rohita*)	R: GGAACAAGGCCAGAGCTTTA			Goda	7	141 – 153	0.7775	0.7000	0.6929	
*Lr*38*	F: CTCGTAAAGCTGTGCGATTG	154	53	Bha	8	148 – 166	0.8413	0.7000	0.0003#	0.127
(*Labeo rohita*)	R: TAGGAGAAGGGGTGGAAGGT			Goda	9	148 – 166	0.8392	0.6800	0.0003#	
*Lro*23*	F: GCACTCGCACACACATTCAC	149	53	Bha	7	152 – 220	0.7800	0.8000	0.5258	0.041*
(*Labeo rohita*)	R: CAGCCGCCTGTCAGTAATCT			Goda	10	152 – 220	0.7917	0.6667	0.2462	
*Lro*25*	F: GTTGCACTGTCAGCATTGGT	94	51	Bha	8	94 – 112	0.8537	0.7500	0.0428	0.005*
(*Labeo rohita*)	R: GTTCTGCAACTACTGCAACCTG			Goda	11	88 – 112	0.8568	0.7500	0.0854	

F (forward primer), R (reverse primer), Ta (annealing temperature), Na (alleles observed), *He* (expected heterozygosity), *Ho* (observed heterozygosity), Bha (Bhaghirathi), Goda (Godavari), P_{HW} (Probability of conformity to HW expectations, significant after Bonferroni correction # $P < 0.003$), P_G (Probability of genetic homogeneity between the populations, * $P < 0.05$).

amplification of microsatellite loci in cyprinids (Zheng et al., 1995). Successful amplification in other Indian major carps has already been achieved using primers from closely related species (Lal et al., 2004).

The presence of null alleles could be one of the possible factors for observed heterozygote deficiency at locus *R*3*. Null alleles are not represented in PCR amplification due to mutation at primer binding site that in turn contribute to the homozygote excess (Paetkau and Strobeck, 1995). The various estimates provided strong evidence that the two sample sets were not drawn from the same randomly mating gene pool. Analysis of larger sample sizes from more geographic locations will provide fine scale assessment of population genetic structure of *L. calbasu* and also more insight into the observed homozygote excess.

In conclusion, nine polymorphic microsatellite loci were shown to be promising markers to determine genetic variation in *L. calbasu* natural populations, enabling suitable measures to be taken.

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