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Microsatellite loci to assess genetic variation in *Tor putitora*

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Summary

Seven polymorphic microsatellite DNA loci were identified in golden mahseer, *Tor putitora*, through cross-species amplification. Thirty-two primers developed for three cyprinid fishes were tested in the study. The genetic variation detected at each microsatellite locus in *T. putitora* specimens (n = 107), collected from three different rivers and one lake was assessed. The allele frequencies deviated significantly from that expected under Hardy–Weinberg equilibrium. The mean observed heterozygosity values ranged from 0.29 to 0.40. Significant genotype heterogeneity indicated that the samples were not drawn from the same gene pool. The results indicate that the identified microsatellite loci exhibit promise for use in fine scale population structure analyses of *T. putitora*.

Introduction

Microsatellites are short tandem repeat motifs with high levels of allelic polymorphism and co-dominant inheritance, useful for direct assessment of pattern and distribution of genetic variability at the intraspecific level (O'Connell and Wright, 1997; Nalon et al., 2000). Variability detected at microsatellite loci has several applications, e.g. in brood stock management for genetic improvement of farmed species (Wolfus et al., 1997), propagation-assisted rehabilitation for conservation of endangered species (Brown et al., 2000), and monitoring genetic bottlenecks in natural populations (Reilly et al., 1999). The flanking sequences of microsatellites within related taxa are highly conserved (Scribner et al., 1996). The potential of these markers is enhanced when primers designed for one species amplify homologous loci in other species (Zardoya et al., 1996).

Tor putitora, the golden mahseer (Family: Cyprinidae, subfamily: Cyprininae), is a component of the biodiversity-rich Himalayan ecosystem. It inhabits cold-water streams and is distributed from Bangladesh, Nepal, and India to Pakistan and Afghanistan (Jayaram, 1999; Froese and Pauly, 2003). *Tor putitora* is important as a principal game as well as food fish and is reported to grow up to 54 kg (Froese and Pauly, 2003). Fish abundance has been declining in certain natural distribution ranges, stated to be the result of overexploitation and habitat alterations (Chonder, 1999). To save this important resource, effective conservation and propagation-assisted rehabilitation strategies are necessary. However, this may not be feasible unless data is available for *T. putitora* on stock structure and genetic variation throughout its distribution range. Identification of polymorphic markers with consistent scorable alleles is a crucial step to generate population genetics data (Ferguson et al., 1995). There is no recorded information on any class of genetic markers or stock structure in *T. putitora*. This is true as well for all species belonging to the genus *Tor*, whereby genetic research has been limited to karyological studies (Khuda-Bukhsh, 1982; Lakra, 1996; Kushwaha et al., 2001). The present study examines cross-species amplification of primers, developed for three cyprinids, in *T. putitora*. The objective was to identify polymorphic microsatellite loci and evaluate suitability of the identified loci in population structure analysis of *T. putitora*.

Materials and methods

Sampling sites and sample collection

Tor putitora specimens were obtained through commercial catches from three rivers, the Satluj (Bhakra, n = 24), Beas (Pong, n = 28), Yamuna (Yamunanagar, n = 27) and one lake, Lake Bhimtal (Bhimtal, n = 28). The riverine locations were chosen to cover geographically isolated populations of *T. putitora*. The rivers Beas and Satluj are part of the Indus River system whereas the Yamuna belongs to the Ganges River. The Beas and Satluj join downstream beyond the distribution range of *T. putitora* (ECAFE, 1966). Lake Bhimtal harbours *T. putitora*, which was transplanted from the River Kosi, an associate river of the Ganges. The samples were obtained from July 1999 to August 2001. Body weight in the Satluj River ranged from 100 to 5000 g, Beas from 700 to 2500 g, Yamuna from 75 to 1100 g, and in Lake Bhimtal from 600 to 3000 g. Collection was performed at actual fishing sites to obtain fresh and the best samples. The blood samples, collected through caudal puncture, were fixed in 95% ethanol (1 : 5) and stored at 4°C until used.

PCR amplification and electrophoresis

Total genomic DNA was extracted from the blood samples following the procedure of Ruzzante et al. (1996). Polymerase chain reaction (PCR) amplifications were performed (MJ Research thermal cycler PTC-200, MA, USA) in a final volume of 25 μl, containing 25–50 ng of genomic DNA, 1X PCR buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl; 0.01% gelatin), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of each primer and 1.5 units of *Taq* DNA polymerase. Amplification conditions were 94°C for 5 min followed by 25 cycles at 94°C for 30 s, T_a for 30 s and 72°C for 1 min, with a final extension of 72°C for 4 min. After amplification, 8 μl of PCR products were electrophoresed on non-denaturing polyacrylamide (19 : 1 acrylamide : bisacrylamide) gels (10 × 10.5 cm; Amersham Biosciences Ltd, NJ, USA). Electrophoresis was done with 1X TBE buffer for 5 h at 10 v cm^{-1} at 4°C. The gel concentration was optimized according to allele size for better

resolution. The gels were silver stained (Silver Staining Kit; Amersham Biosciences) to visualize microsatellite loci and their allelic patterns. The alleles were designated according to PCR product size and calculated relative to a molecular marker (pBR322 DNA/*Msp*I digest) with Image master 1D Elite v3.01 (Amersham Biosciences).

Screening of primers and genetic diversity analysis

Microsatellite primers from *Cyprinus carpio*, *Barbus barbus* and *Catla catla* (Table 1) were tested for amplification of homologous loci. The three species are termed as resource species in the study. This cross-species amplification experiment was carried out with eight specimens of *T. putitora*. The optimum annealing temperature, to achieve a scoreable band pattern, was determined through experimental standardization for each primer pair. The primers yielding scoreable amplified products were again evaluated with a larger sample size (107 individuals from three rivers and one lake) to evaluate their suitability in quantification of genetic divergence in *T. putitora*. Data was analysed using the software GENETIX 4.02 (Belkhir et al., 1997) to obtain allele frequencies, mean number of alleles per locus, and expected (H_e) and observed (H_o) heterozygosity values. Tests for conformity to Hardy–Weinberg expectations were performed by the Markov chain method with parameters dememorization = 1000, batches = 100 and iteration = 100 (GENEPOP ver. 3.3, 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 ver. 3.3, genotype differentiation test). This test was performed on genotype tables and possible non-independence of alleles within genotypes will not affect test validity (Raymond and Rousset, 1995b; Goudet et al., 1996). The standard Bonferroni correction applied to significant levels was made for the simultaneous tests (Lessios, 1992).

Results and discussion

Of the 32 heterologous primer pairs tested, 13 (40.63%) primers provided successful amplification of homologous loci in *T. putitora* (Table 1). It is evident (Table 2) that the

Table 1
Primers of microsatellite loci tested for cross-species amplification in *Tor putitora*

Species	Number of primer pairs tested	Locus	GenBank accession number	Reference	Successful primer pair amplified in *T. putitora* [n (%)]
Catla catla	5	*CcatA7, A12, C3 G1, G2*	AF045378 to AF045382	Naish and Skibinski (1998)	–
Cyprinus carpio	21	*MFW1, 2, 5, 6, 7, 9, 11, 14, 15, 16, 17, 18, 19, 23, 24, 26, 28, 29, 30, 31, 32*	–	Croojimans et al. (1997)	9 (43)
Barbus barbus	6	*Barb 54, 59, 65, 79, 37, 62*	–	Chenuil et al. (1999)	4 (67)
Total tested	32				13 (40.6)

Table 2
Characteristics of amplified microsatellite loci in details of *Tor putitora*

Resource species					*Tor putitora*	
Species	Locus	Primer sequence (5′ → 3′)	Repeat motif	T_a (°C)	T_a (°C)	Number of alleles
Cyprinus carpio	*MFW1*	GTCCAGACTGTTCATCAGGAG GAGGTGTACACTGAGTCACGC	CA	55	57	M
	MFW 2	CACACCGGGCTACTGCAGAG GTGCAGTGCAGGCAGTTTGC	CA	55	59	1
	MFW7	TACTTTGCTCAGGACGGATGC ATCACCTGCACATGGCCACTC	CA	55	59	1
	MFW11	GCATTTGCCTTGATGGTTGTG TCGTCTGGTTTAGAGTGCTGC	CA	55	57	5
	MFW 15	CTCCTGTTTTGTTTTGTGAAA GTTCACAAGGTCATTTCCAGC	CA	55	55	M
	MFW 17	CAACTACAGAGAAATTTCATC GAAATGGTACATGACCTCAAG	CA	55	55	5
	MFW19	GAATCCTCCATCATGCAAAC CAAACTCCACATTGTGCC	CA	55	55	M
	MFW 24	CTCCAGATTGCACATTATAG TACACACACGCCAGAGCCTTT	CA	55	51	1
	MFW26	CCCTGAGATAGAAACCACTG CACCATGCTTGGATGCAAAAG	CA	55	51	5
Barbus barbus	*Barb37*	AAATACGCTCTCCTCATTAC GTACAAAAGCAAAAATAAATTA	ATTT	50	50	3
	Barb54	GTTGTTTTGATTCACACTGAG TACCATCTGCTGCTGCTTC	CA	58	47	M
	Barb59	CTGTATCCATCACATAGGCT CATGATTTAATAGAACACACAC	GATA	56	47	11
	Barb62	GGCACAAAAATGGATTCATATC GTACACGAGCATATGGACAA	ATTT	58	50	2(*Barb62-1*) 3(*Barb62-2*)

M-Multiple Bands.

optimum annealing temperature (T_a °C) observed in *T. putitora* differed from that reported in the resource species for the respective primer pair. Primer pairs *MFW1*, *15*, *19* and *Barb54* revealed two, or more than two, bands per individual. This could be the expression of duplicate loci. Duplication of certain loci is known to occur among polyploids (McQuown et al., 2002). Because of a high chromosome number (n = 100), *T. putitora* was considered to be a natural polyploid fish (Khuda-Bukhsh, 1982; Lakra, 1996). The four loci (*MFW1*, *15*, *19* and *Barb54*) were not considered for genotyping. It is interesting that primer pair *Barb62* amplified two distinct loci, in contrast to the other seven primer pair that consistently amplified single locus. Primers *MFW2* and *7* produced a monomorphic band in all individuals tested. Based on the results, it can be stated that certain sequences flanking the microsatellite regions of the genome might be conserved among the cyprinids. The inference is consistent with earlier reports, suggesting the possibility of using primers interspecifically among cyprinids (Zheng et al., 1995; Mohindra et al., 2001; Lal et al., 2004).

In the present study, seven polymorphic microsatellite loci (*MFW11, 17, 26, Barb37, Barb62-1, Barb62-2,* and *Barb59*) exhibiting 2–11 alleles, could be successfully identified for *T. putitora*. The parameters of genetic variation at each locus and over all loci differed among the four sample sets (Table 3). The observed heterozygosity values over all loci ranged from 0.28 (Satluj) to 0.39 (Yamuna). Mean number of alleles per locus ranged from 3.57 (Beas) to 4.42 (Yamuna). The probability test provided the evidence that the observed allele frequencies significantly ($P < 0.05$) deviated from that expected under the Hardy–Weinberg equilibrium. Deviation was observed in all four sample sets at one or more loci (Table 3), possibly due to the small sample size that did not have true representation of population allele frequencies. The other possibility of more serious concern is that the assumptions underlying the Hardy–Weinberg equilibrium, relevant to natural population of *T. putitora*, are violated (Ferguson et al., 1995). One of the reasons could be reduction in the effective breeding population size in *T. putitora* as a result of overexploitation, restricted migrations and habitat alterations, etc.

In view of the observed non-conformity to Hardy–Weinberg expectations, the genetic heterogeneity was tested on the tables based on genotype rather than allele frequencies (Raymond and Rousset, 1995b; Goudet et al., 1996). Significant heterogeneity ($P < 0.05$) in genotype proportions was observed at six of seven loci (Table 3). After the Bonferroni correction was made to the probability levels, four loci still exhibited significant heterogeneity. Combined probabilities over all loci were < 0.0001 (Table 3). Global tests for pairwise comparisons indicate that all six possible tests had significant heterogeneity ($P < 0.02$, between Beas and Satluj; $P < 0.0001$, other pairs tested). However, analyses of larger sample sizes from different

Table 3
Parameters of genetic variability for each microsatellite locus in *Tor putitora* samples from three rivers (S, Satluj; B, Beas; Y, Yamuna) and one lake (BH, Bhimtal)

Locus	River/ lake	Size range (bp)	Number of alleles at each locus	H_o	H_e	HW (P-value)[1]	Genotype homogeneity (P-value)[2]
MFW11	S	169–183	3	0.090	0.087	1.0000	< 0.0001*
	B	171–183	3	0.087	0.298	0.0002*	
	Y	169–181	4	0.500	0.411	1.0000	
	BH	169–183	5	0.629	0.634	0.0122**	
MFW17	S	213–235	4	0.363	0.649	0.0026**	0.0063**
	B	213–233	4	0.409	0.614	0.0441**	
	Y	213–235	5	0.421	0.659	0.0176**	
	BH	213–233	3	0.320	0.640	0.0001*	
MFW26	S	114–140	5	0.478	0.715	0.0013*	< 0.0001*
	B	114–140	5	0.538	0.750	0.0092**	
	Y	114–148	6	0.428	0.630	0.0035**	
	BH	114–140	5	0.392	0.697	0.0009*	
Barb62–1	S	117–121	2	0.000	0.090	0.0244**	0.2921
	B	121	1	0.000	0.000	–	
	Y	117–121	2	0.071	0.132	1.0000	
	BH	117–121	2	0.125	0.186	0.2057	
Barb62–2	S	94–98	2	0.478	0.439	1.0000	< 0.0001*
	B	94–98	2	0.538	0.473	1.0000	
	Y	94–110	3	0.000	0.000	0.0000*	
	BH	98	1	0.036	0.196	–	
Barb37	S	197–213	3	0.272	0.372	0.1404	0.0472**
	B	205–213	2	0.040	0.112	0.0602	
	Y	197–213	3	0.375	0.440	0.5309	
	BH	197–213	3	0.185	0.170	1.0000	
Barb59	S	98–138	7	0.333	0.634	0.0000*	< 0.0001*
	B	98–150	9	0.777	0.806	0.0599	
	Y	98–146	10	0.900	0.844	0.7976	
	BH	98–134	8	0.807	0.850	0.2657	
M	S	–	3.714	0.288	0.427	–	< 0.0001*
B	–	3.571	0.339	0.437			
Y	–	4.428	0.395	0.482			
BH	–	3.714	0.351	0.454			

Given are the Hardy–Weinberg (HW) observed (H_o) and expected (H_e) heterozygosity values with associated probability, and the probability of genotype homogeneity between samples. Significant probability values are marked.
$(P)^1$ *$P < 0.0017$ and ** $P < 0.05$ (Bonferroni correction applied, 28 simultaneous tests).
$(P)^2$ *$P < 0.0071$ and ** $P < 0.05$ (Bonferroni correction applied, seven simultaneous tests).

geographical locations are required before the conclusive assessment of genetic variation and population structure of *T. putitora* can be made.

Conclusion

The present study identifies seven polymorphic microsatellite loci. The genetic variation detected with these seven loci exhibits considerable promise for use as a marker in stock structure analysis. This will also provide potential tools for assessing the genetic bottlenecks occurring in natural populations of *T. putitora*.

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