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MICROSATELLITE LETTERS

Development and characterization of polymorphic microsatellite marker for an endangered freshwater fish in Korea, *Gobiobotia brevibarba*

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Abstract We developed and characterized 11 polymorphic microsatellite loci in *Gobiobotia brevibarba* (Cypriniformes: Gobioninae), an endangered freshwater fish in Korea. The number of observed alleles per locus ranged from 3 to 20 within 20 individuals, the observed and expected heterozygosities per locus ranged from 0.150 to 0.950 and 0.145 to 0.958, respectively. Nine of the microsatellite loci were applicable to other endangered Korean fishes in the Gobioninae genera. The developed microsatellite loci will be useful in the investigation of genetic diversity and population genetic structure, which will enhance future conservation strategies.

Keywords Cross-species amplification · Endangered species · *Gobiobotia brevibarba* · Gobioninae · Microsatellites

Gobiobotia brevibarba (Gobioninae; Cyprinidae) is endemic to Korea, and resides under the riverbed gravel in the rapids of the Imjin, Han and Geum Rivers. However, this species faces a high risk of extinction caused by anthropogenic activities such as river channel development, gravel mining, damming and so forth. To preserve *G. brevibarba*, the Korean Ministry of Environment has designated it as a category II endangered species, and efforts are being made to increase the population size using artificial means. Genetic variability is important for population persistence, and determination of population structure is an essential component in successful management programs such as distinguishing the management units and reintroduction (Frankham et al. 2004). Therefore, the development of efficient DNA markers is essential.

A total of twenty individuals of *G. brevibarba* used in the present study were collected from the Han River, Korea, in 2010. Genomic DNA was extracted from the pectoral fins using TNES-urea buffer with the phenol–chloroform extraction method. The quantity of DNA was determined using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific Inc., Germany). The quality of DNA was loaded on a Fragment Analyzer^{TM} (Advanced Analytical Technologies Inc., USA).

A partial genomic library enriched with GT repeats was generated using a slight modification of the procedure described by Hamilton et al. (1999). Fragments ranging from 200 to 600 bp were isolated, ligated into the *Xba* I-digested pUC18 vector (Amersham Pharmacia Biotech Inc., USA) and used to transform XL1-Blue MRF' strain cells (Stratagene Inc., USA). Positive clones with repeats were identified by colony PCR using $(GT)_{10}$ and M13 primers; approximately 400 colonies with inserts were screened. The PCR products were analyzed on 1.5 % agarose gels, and the clones that produced multiple bands or smearing patterns were considered to contain a microsatellite. DNAs from the positive colonies were purified using a QIAprep Spin Miniprep Kit (Qiagen Inc., CA). All positive colonies were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc., USA) and an ABI 3730*xl* DNA Analyzer (Applied Biosystems). Primers were designed from the sequences flanking the microsatellite motifs using PRIMER 3 software (Rozen and Skaletsky 2000). Designed primers contained a dye-labeled universal primer system (Schuelke 2000) with an M13-tagged tail (5′-

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Table 1 Details of 11 microsatellite loci isolated from *G. brevibarba*

Locus	Repeat motif	Primer sequences (5′ → 3′)	Allele size range (bp)	T_a	n	k	H_o	H_e	GenBank accession no.
GBms157	$(CA)_6$ …$(CA)_{16}$	F:GGAGTTATGAACGATAGAGCAGAC	153–175	58	20	10	0.800	0.874	JX179136
		R:GAGCCTCATCAGCGACAACG							
GBms258	$(CA)_{13}$	F:GCAACATGTGTGTCATATCGGC	247–287	58	20	12	0.700	0.771	JX179137
		R:TTGTGTTTTTAGACACGAGATCAACA							
GBms278	$(CA)_{14}$	F:CGGCTGGGACTTCCAGTAATC	120–192	58	20	20	0.900	0.958	JX179138
		R:CGTTCGGATTTGAACCAGACA							
GBms304	$(CA)_{14}$	F:TGACAATGGCATTTGCTCCT	131–167	58	20	16	1.000	0.928	JX179139
		R:CAATGGAAAGTCCCCATGAA							
GBms316	$(CA)_{14}$	F:ACTGATATTGGCCAGGCGGT	220–232	58	20	5	0.500	0.494	JX179140
		R:CATTGTCTGCTCTTTATGCTGAC							
GBms381	$(CA)_{21}$	F:GCTTCGGGTCAGGGATAAAG	205–253	58	20	15	0.950	0.921	JX179141
		R:GGTTTCAGCTCAAGGGGAAG							
GBms392	$(CA)_{15}$	F:CAGATCCAGGTACGCCTCAGTC	155–191	58	20	14	0.950	0.921	JX179142
		R:TTTCCTGCAGGGTGAAGTCG							
GBms419	$(CA)_{11}$	F:TTGAGCAGGACCAGCCCTTT	197–201	58	20	3	0.150	0.145	JX179143
		R:GATTGCCCTGAGCGTAACCC							
GBms479	$(CA)_{12}$	F:ACAGAACTGATCGTCCGCAG	159–181	58	20	10	0.800	0.772	JX179144
		R:AGTGAGCTGTGTGCCTGTCC							
GBms427	$(CA)_{18}$	F:CAGCAGGTCTTGACCGCATT	284–298	58	20	7	0.800	0.792	JX179145
		R:AGAAGGGAGGGCCAGTCACA							
GBms481	$(CA)_{13}$	F:TGCTGAGCGAGAGAAGCAAT	122–146	58	20	7	0.700	0.771	JX179146
		R:CCCAGACTAACACCTCATTTTTATG							

T_a optimized annealing temperature, n number of individual genotyped, k number of alleles, H_o observed heterozygosity, H_e expected heterozygosity

GTTGTAAAACGACGGCCAGT-3′) added to the 5′ end of the forward primer. Approximately 50 ng of template DNA was used in a reaction volume of 20 µl containing 0.5 µM of each primer and 1.0 µM fluorescent (6-FAM, NED, PET, VIC)-labeled M13 primer with *AccuPower*® PCR Premix (Bioneer Inc., Korea). The thermal cycler protocol consisted of an initial denaturation of 10 min at 94 °C; followed by 35 cycles of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 45 s; and a final extension of 72 °C for 5 min. Microsatellite polymorphisms were screened using an ABI 3730*xl* DNA Analyzer (Applied Biosystems) and alleles were designated according to the PCR product size relative to a standard size (GeneScan™ 500 LIZ; Applied Biosystems). Allele frequency, observed heterozygosity (H_o) and expected heterozygosity (H_e) were calculated by CERVUS version 3.0.3 (Marshall et al. 1998). All loci were tested for fitness to the Hardy–Weinberg equilibrium (HWE), and all pairwise combinations of loci were tested for linkage disequilibrium (LD) by GENEPOP on the web (Rousset 2008).

The number of alleles, repeat motifs, allele size, annealing temperatures, and observed and expected heterozygosities in 11 microsatellite loci are presented in Table 1. No significant deviations ($P > 0.05$) from the HWE were observed, and the LD between GBms258 and GBms419 was statistically significant ($P < 0.05$); however, none of the loci displayed significant LD after the Bonferroni adjustment. The number of alleles per locus ranged from 3 to 20 (with an average of 10.8), and the observed and expected heterozygosities ranged from 0.150 to 0.950 (with an average of 0.750) and 0.145 to 0.958 (with an average of 0.759), respectively. Cross-species amplification was successful in five other endangered fish species belonging to the Gobioninae subfamily in Korea. GBms381 was amplified in all individuals from five other species, showing moderate to polymorphism. In addition, we developed nine microsatellite markers (excluding GBms157, GBms258) that were applicable to other endangered Korean fishes in the Gobioninae genera (see Table 2). Therefore, the microsatellites reported herein are promising tools for identifying the genetic structure of fish populations, which will enhance future conservation strategies. Moreover, these markers provide valuable tools for population genetic studies in other endangered fish species belonging to the Gobioninae subfamily in Korea.

Table 2 Cross-species amplification of developed microsatellite loci for other endangered Gobioninae species in Korea

Locus	*Pseudopungtungia*						*Gobiobotia*						*Micophysogobio*		
	nigra ($N = 6$)			*tenuicorpa* ($N = 3$)			*naktongensis* ($N = 9$)			*macrocephala* ($N = 6$)			*koreensis* ($N = 6$)		
	n	*k*	Size range (bp)	*n*	*k*	Size range (bp)	*n*	*k*	Size range (bp)	*n*	*k*	Size range (bp)	*n*	*k*	Size range (bp)
GBms157	0	–		0	–		9	15	174–326	6	12	174–286	4	8	198–344
GBms258	0	–		0	–		0	–		6	2	252–254	0	–	
GBms278	0	–		0	–		9	9	114–166	6	7	132–152	6	8	122–248
GBms304	6	1	108	3	1	110	4	2	102–112	6	3	110–114	6	2	112–156
GBms316	6	5	230–246	0	–		0	–		0	–		6	1	248
GBms381	6	6	166–188	3	6	262–272	9	8	170–186	6	8	230–292	6	5	270–346
GBms392	6	6	166–188	3	4	170–200	9	15	170–248	1	2	170–236	2	1	192
GBms419	6	1	190	3	1	190	9	3	196–204	6	4	198–206	6	1	196
GBms427	1	2	164–182	0	–		8	6	262–318	0	–		2	3	218–344
GBms479	3	1	214	3	4	192–292	9	12	278–368	3	2	282–286	5	2	254–276
GBms481	6	2	128–166	3	6	136–166	9	6	110–130	6	2	114–116	4	7	150–228

N number of individuals, *n* number of successfully genotyped individuals, *k* number of alleles, – no or nonspecific amplification

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