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TECHNICAL NOTE

Characterization of novel microsatellite loci in rare minnow (*Gobiocypris rarus*) and amplification in closely related species in Gobioninae

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Abstract Rare minnow (*Gobiocypris rarus*) is an endangered small fish endemic to upper reach of the Yangtze River. From a (GT)*n* enriched genomic library, 32 microsatellites were isolated and characterized. Nineteen of these loci were polymorphic in a test population with alleles ranging from 2–7, and observed and expected heterozygosities from zero to 0.8438, and 0.2679 to 0.8264, respectively. In the cross-species amplifications, 13 out of 19 polymorphic loci were found to be also polymorphic in at least one of the 7 closely related species of the subfamily Gobioninae. These polymorphic microsatellite loci should provide sufficient level of genetic diversity to evaluate the fine-scale population structure in rare minnow and its closely related species for the conservation purpose.

Keywords *Gobiocypris rarus* · Microsatellite · Genetic diversity · Cross-species amplification · Conservation genetics

The rare minnow (*Gobiocypris rarus*) is a small freshwater fish species endemic to upper reach of the Yangtze River, an area limited to some rivulets in Sichuan, Southwest China. *G. rarus* belongs to subfamily Danioninae of Cyprinidae based on morphological traits (Ye and Fu 1983), or subfamily Gobioninae based on recent molecular phylogenetics (He et al. 2004). Because of its narrow distribution and limited stocks, together with the use of pesticides, rare minnow populations have declined in recent decades and it became an endangered species (Yue and Chen 1998). Some hydroelectric projects have been constructing in the Yangtze or its tributary rivers, such as the Three Gorges Dam, Xiluodu Dam and Pubugou Dam etc., which will significantly raise the water level of the upper Yangtze River and thus the habitats of the rare minnow would be affected directly or indirectly. A conservation program for endemic fishes in the upper Yangtze River has been proposed in China recently. Population genetic studies are necessary for this and future endeavors towards protection and sustainable utilization of fish resources in the Yangtze River. However, due to the lack of polymorphic DNA markers, to date studies on population genetics of *G. rarus* has been rare (Wang et al. 2000). Because of their high level of polymorphism and co-dominant inheritance in Mendelian fashion, microsatellites have been widely used as DNA markers in the studies of population structure and conservation genetics in threatened species. In this paper, the isolation and characterization of novel microsatellites from the genomic library of *G. rarus* were reported, which will be important to the genetic studies of the species and/or closely related species.

Adult rare minnow were sampled from a rivulet in Hanyuan, Sichuan Province. Genomic DNA for constructing microsatellite-enriched library was extracted using traditional phenol-chloroform protocol

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with RNase treatment. DNA samples from a test panel (32 individuals) were extracted using high salt protocol (http://sciencepark.mdanderson.org/mbcore/protocols.html) with the following modifications: about 100 mg fin tissues of each individual were chopped up and mixed with 400 μl TNES buffer (10 mM Tris, pH7.5, 400 mM NaCl, 100 mM EDTA, 0.6 % SDS) and 5 μl of Proteinase-K (20 mg/ml). The mixture was incubated in water at 55°C until the tissues were digested completely. Then 120 μl 6 M NaCl were added to each digested sample. After shaken for 5 min vigorously, samples were spun down at 13,800 g for 25 min at 4°C. The supernatants were transferred to a fresh tube. A double volume of cold 100% ethanol were added to each sample, mixed well, and precipitated DNA were collected using micropipette tip and transferred to another tube. The DNA were washed with 70% ethanol for two times, dried at room temperature, and finally resuspended in 200 μl sterile TE (10 mM Tris–HCl, 1.0 mM EDTA, pH8.0).

Enriched partial genomic library for repeat motif (GT)*n* in *G. rarus* was constructed essentially following the FIASCO (fast isolation by AFLP of sequences containing repeats) protocol (Zane et al. 2002) with slight modifications. Briefly, 100 ng DNA of an adult fish was digested with 5U *Mse*I (NEB) at 37°C for 2 h. Then 10 μl of the digested fragments was ligated to *Mse*I adaptor (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) using 3U T4 DNA ligase (Takara) in a volume of 30 μl. Diluted (1:10) digestion-ligation mixtures were amplified with adaptor-specific primers *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′) in a 25 μl reaction containing 5 μl diluted digestion-ligation DNA via PCR using a program of 94°C 30 s, 53°C 1 min, 72°C 1 min for 17 cycles. After denaturation at 95°C for 5 min, 25 μl amplified DNA fragments were hybridized with 0.1 μM 5′-biotinylated $(GT)_{13}$ oligonucleotide probe in a 100 μl volume of hybridization buffer (4.2 × SSC, 0.07% SDS) at 60°C for 1 h. Three hundred microlitres streptavidin paramagnetic particles (Promega) were used to selectively capture the probes and microsatellites. The mixtures were incubated at room temperature for 30 min with constant gentle agitation. The beads-probe-DNA complex was separated by Magnetic Separation Stand (Promega). Three low stringency washes in 400 μl TEN1000 (10 mM Tris–HCl, 1 mM EDTA, 1 M NaCl, pH7.5) were performed at room temperature followed by three high stringency washes in 400 μl buffer (0.2 × SSC, 0.1% SDS) at 55°C. Two additional washes were performed using 400 μl 1 × SSC to eliminate remnant SDS. Targeted DNA fragments were separated from the beads-probe complex by incubating for 5 min at 95°C in 50 μl TE (pH8.0).

Released DNA fragments were amplified using *Mse*I-N primers for 17 cycles under the conditions described above. After purified using PCR Products Extraction Kit (Omega), the enriched fragments were ligated into pMD18-T vector (Takara) and transformed into DH5α *E. coli* competent cells (Invitrogen). Clones with positive inserts were confirmed by PCR amplification using *Mse*I-N primers. Forty positive clones were sequenced using BigDye termination (Applied Biosystems) with the products being resolved on an ABI3730 sequencer, and 39 out of 40 clones (97.5%) contained microsatellites (>8 times for dinucleotide repeats). Primers were designed for 32 sequences flanking the repeat regions of interest using online software PRIMER3.

Eight unrelated individuals were primarily used to test the amplification feasibility of the newly designed primers and polymorphism of the respective microsatellite loci. PCR amplifications were carried out in 12.5 μL volumes containing 1 × PCR buffer, 10–50 ng genomic DNA, 0.2 μM for each primer, 120 μM dNTPs and 0.5U *Taq* DNA polymerase (Biostar) on a PTC-100 thermocycler (MJ Research). The amplifications were programmed using following conditions: 94°C for 5 min, then 35 cycles at 94°C for 30 s, proper temperature (Table 1) for 30 s, 72°C for 40 s, and a final extension at 72°C for 10 min. Amplified products were separated on 6% denaturing polyacrylamide gels using silver staining. pBR322 DNA/*Msp* I molecular weight marker (SABC Biotech.) was used as size standard to identify alleles. Once the polymorphism was confirmed for a given locus, 32 individuals were genotyped to determine the heterozygosity. The observed and expected heterozygosities (H_O and H_E) were calculated using Arlequin software (Schneider et al. 2000), and tests for deviation from the Hardy–Weinberg equilibrium (HWE) were performed using Genepop (Raymond and Rousset 1995). Genepop program was also used to test for genotypic linkage disequilibrium (LD), to calculate *f*, an estimator of *F*is (Weir and Cockerham 1984), and to determine heterozygote deficiencies per locus by calculating and comparing the observed and expected heterozygosities for deviations from HWE. The software MICRO-CHECKER (Van Oosterhout et al. 2004) was employed to infer the most probable technical cause of HWE departures, including null alleles, mis-scoring due to stuttering, and allelic dropout due to short allele dominance. All results were adjusted for multiple simultaneous comparisons using a sequential Bonferroni correction (Rice 1989).

In total, 19 of 32 loci were polymorphic with the number of alleles per locus ranging from 2 to 7, and

Table 1 Characterization of 19 novel microsatellites in Gobiocypris rarus. *T*a: annealing temperature; N: number of alleles; H_O: observed heterozygosity; H_E: expected heterozygosity; * indicated significant deviation from HWE after Bonferroni correction ($P < 0.00263$); § indicated $P < 0.01$

Locus (GenBank accession no.)	Repeat motif	Primer sequence (5′–3′)	*T*a (°C)	Size range (bp)	N	H_O/H_E	*P*-value	F_{IS} (W&C)
Gra01 DQ490140	$(CA)_{10}CG(CA)_4$	F:TGGATCTAATGTCTCCCCATTT R:GCACAGCAATGAAAGCATGA	63	180–238	5	0.2581 0.7065	0.0000*	0.639§
Gra02 DQ490141	$(CA)_{17}$	F:GGTTCTGGGAGATTCTTTGGA R:GCGGTTCTCTTCAAATGAGC	63	160–201	4	0.2667 0.6599	0.0000*	0.593§
Gra03 DQ490142	$(CA)_9$	F:CGCAGTAAAGGGGTGACACT R:CGAATCATGCCCTCAATTTT	63	160–190	7	0.7186 0.7832	0.1208	0.084
Gra04 DQ490143	$(GT)_{14}$	F:TTGACCTCTCACCCTGCTTT R:CACGGCTTCTTTCTTCTTGC	55	201–238	3	0.4839 0.6029	0.2254	0.162
Gra05 DQ490144	$(AC)_4AA(AC)_{20}$	F:AGCCAATGAAGCCTACCAAC R:AGGATGAGTAGACCGTCAGACA	48	170–210	4	0.5625 0.6974	0.0011*	0.194§
Gra06 DQ490145	$(GT)_{10}$	F:ATTTTGCGGGGTTATGACAG R:TGGTTTTCCGACAGTGTTCA	55	190–201	2	0.5313 0.5382	1.0000	–0.048
Gra08 DQ490146	$(GT)_{12}$	F:GCCCTGACAATTTGATTGGT R:GCTGGGCTAACATATGTGCTG	52	180–242	6	0.8438 0.7733	0.0305	–0.093
Gra15 DQ490147	$(CA)_5TA(AC)_{17}$	F:CGCCCTGTTGTGTTACCTTT R:TGGCCCATCAAGCATACATA	50	230–270	4	0.2188 0.6032	0.0000*	0.623§
Gra16 DQ490148	$(AC)_{11}$	F:GGTTAGGACCAGTGGCAAAA R:TTAATGCAGCTCCCCCTAGA	50	220–270	5	0.5667 0.6881	0.0093	0.170
Gra17 DQ490149	$(TG)_3TT(TG)_8$	F:CTCATGCTTCCATTGTGATAGG R:GGAATCAGGGTCAAAAGCAG	54	249–280	4	0.4063 0.6448	0.0035	0.370§
Gra19 DQ490150	$(TG)_{11}$	F:AAAGCCCATCCAGTCATCTG R:AGCTTGTCCCAGCAGACAGT	55	130–201	6	0.2500 0.6880	0.0000*	0.631§
Gra20 DQ490151	$(CA)_{10}$	F:TTGTGAGAGGCTTCATGTGC R:GAAGGGGTCAGCAGGATACA	55	150–182	6	0.7813 0.7093	0.7686	–0.103
Gra21 DQ490152	$(CA)_{10}$	F:TCCTTCGTAAAGCCTCTCTGA R:CCAGAGGCAATAATCATTTGAA	55	180–201	4	0.7000 0.6927	0.1554	–0.049
Gra22 DQ490153	$(AC)_{12}$	F:AACACATGGCAGATGTCCAA R:CAGCATGTTTCCTGTGATGG	48	217–238	2	0.0000 0.2986	0.0000*	1.000§
Gra25 DQ490154	$(TG)_9$	F:CTGGAGGGTCGGGACTTTAT R:GCAGCAGAACTGAACCCACT	55	160–201	4	0.7188 0.6766	0.3747	–0.089
Gra26 DQ490155	$(AG)_{24}$	F:GCCTGTGATCACCTCTAGCA R:TCAGCACCACTCTGTTCCAC	45	217–242	6	0.6875 0.8264	0.0020*	0.170§
Gra27 DQ490156	$(TG)_8TA(TG)_8$	F:GAAAAGCCAAAATCCACGTC R:TGCAAAATGGTGTAGCGAGA	54	238–280	4	0.7097 0.6119	0.5870	–0.201
Gra30 DQ490157	$(AC)_{11}$	F:TTAGCACACGCAAAGGAATG R:CAATGCATCTGTCACATCCTG	55	180–190	2	0.1250 0.2679	0.0125	0.537
Gra31 DQ490158	$(TC)_{18}$	F:TGGAAGGAAAAGTGGGTGAG R:CACATGAATTGAAGGCTGGA	50	230–260	6	0.7188 0.8026	0.0178	0.106

Table 2 Cross-species amplification of polymorphic microsatellite loci from Gobiocypris rarus in seven species of Gobioninae. T_a: annealing temperature; Che: Coreius heterodon; Cgu: Coreius guichenoti; Sda: Saurogobio dabryi; Ppa: Pseudorasbora parva; Rve: Rhinogobio ventralis; Rcy: Rhinogobio cylindricus; Rty: Rhinogobio typus; P: Polymorphic; M: Monomorphic; NA: No amplification product; numbers in brackets are alleles numbers

Locus	T_a(°C)	*Che*	*Cgu*	*Sda*	*Ppa*	*Rve*	*Rcy*	*Rty*
Gra01	55	P(3)	M	P(3)	NA	M	NA	NA
Gra02	55	P(4)	M	P(3)	P(4)	NA	NA	NA
Gra03	50	NA	NA	NA	NA	M	NA	NA
Gra04	50	P(2)	P(2)	NA	P(3)	M	M	M
Gra05	55	M	P(2)	NA	M	M	M	M
Gra06	50	M	M	M	P(4)	M	M	M
Gra08	50–55	NA	NA	NA	NA	NA	NA	NA
Gra15	50	M	M	NA	P(4)	M	NA	NA
Gra16	50	NA	NA	NA	NA	NA	NA	NA
Gra17	50	NA	NA	NA	NA	NA	NA	NA
Gra19	50	NA	P(3)	P(2)	P(3)	M	M	M
Gra20	50	M	M	P(2)	M	M	M	M
Gra21	50	M	M	NA	NA	NA	NA	NA
Gra22	45	M	M	NA	NA	NA	M	M
Gra25	50	P(2)	P(2)	P(2)	NA	M	M	M
Gra26	45	P(5)	P(4)	P(2)	P(4)	P(2)	M	P(2)
Gra27	50	P(3)	P(2)	P(2)	P(2)	P(2)	M	M
Gra30	50	M	P(2)	NA	P(2)	NA	M	NA
Gra31	45	M	M	M	M	M	M	M

expected heterozygosity (H_E) from 0.2679 to 0.8264, respectively (Table 1). The remaining 13 loci were monomorphic or could not amplify any scorable products. Seven loci deviated from the HWE in the sampled population after Bonferroni correction (adjusted P value = 0.00263) with significant heterozygote deficits (Table 1), and the remaining 12 loci were in HWE. Null alleles were found in 7 loci (Gra01, 02, 15, 17, 19, 22 and 30) detected with MICRO-CHECKER utility ($P < 0.05$), but no evidences for stuttering and allelic dropout were found in all loci ($P > 0.05$). All pairwise tests for linkage disequilibrium among loci were non-significant after applying sequential Bonferroni correction ($P > 0.004$).

Nineteen polymorphic loci were further investigated for cross-species amplifications in other 7 species (*Coreius heterodon*; *Coreius guichenoti*; *Saurogobio dabryi*; *Pseudorasbora parva*; *Rhinogobio ventralis*; *Rhinogobio cylindricus*; *Rhinogobio typus*) belonging to subfamily Gobioninae using the same PCR programs as used for amplification in *G. rarus* expected for some changes in annealing temperatures (Table 2). In 7 species, primers of the 3 loci (Gra08, Gra16 and Gra17) failed to amplify any PCR products, and other 3 loci (Gra03, Gra22 and Gra31) were either monomorphic or failed to amplify. The rest of the microsatellites (13 loci) were found to be polymorphic in at least one of the 7 species (Table 2).

These polymorphic microsatellites derived from a (GT)n enriched genomic library are the first batch to be published in rare minnow. Isolations and characterization of these microsatellites, including the tests of trans-species amplifications, have provided PCR-based dominant molecular markers with sufficient level of genetic diversity to evaluate the fine-scale population structure in rare minnow. These results will facilitate future genetic studies in *G. rarus* and its closely related species in the upper Yangtze River, which is important for the establishment of a conservation zone in the future.

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