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Characterization of novel microsatellite markers derived from Korean rose bitterling (*Rhodeus uyekii*) genomic library

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ABSTRACT. Korean rose bitterling (*Rhodeus uyekii*) is a freshwater fish endemic to Korea. Natural populations of this species have experienced severe declines as a result of habitat fragmentation and water pollution. To conserve and restore *R. uyekii*, the genetic diversity of this species needs to be assessed at the population level. Eighteen novel polymorphic microsatellite loci for *R. uyekii* were developed using an enriched partial genomic library. Polymorphisms at these loci were studied in 150 individuals collected from three populations. The number of alleles at each locus ranged from 3 to 47 (mean = 17.1). Within the populations, the observed heterozygosity ranged from 0.032 to 1.000, expected heterozygosity from 0.082 to 0.967, and polymorphism information content from 0.078 to 0.950. Six loci showed significant deviation from Hardy-Weinberg equilibrium after Bonferroni's correction, and no

significant linkage disequilibrium was detected between most locus pairs, except in three cases. These highly informative microsatellite markers should be useful for genetic population structure analyses of *R. uyekii*.

Key words: *Rhodeus uyekii*; Korean rose bitterling; Microsatellite loci; Genetic diversity

INTRODUCTION

Bitterlings belong to the subfamily Acheilognathinae, which includes approximately 40 species and subspecies. They are distributed in temperate regions of Europe and Asia, including Korea, Japan, Taiwan, and China (Banarescu, 1990). Korean bitterlings are classified into 2 genera and 14 species, including 9 endemic species (Kim et al., 2005). The Korean rose bitterling (*Rhodeus uyekii*) is a common freshwater fish endemic to Korea, and it inhabits tributaries of the Nagdong, North Han, and Tamjin Rivers. Recently, this species was named as a candidate for development as an ornamental fish because of its small size and beautiful coloration (Kang et al., 2005). Natural populations of this species have recently experienced severe declines as a result of overfishing, habitat fragmentation, and water pollution.

Conservation projects are currently under way to promote increases in population size and distribution. However, knowledge of the population genetic structure for this species is essential to develop effective plans, and suitable DNA markers are needed for the evaluation of population genetic diversity. Because microsatellite markers have high numbers of polymorphisms, codominant inheritance, a genome-wide distribution, and high reproducibility, they are the most popular and powerful molecular markers in population genetics research (Liu and Cordes, 2004). In the present study, we developed the first set of microsatellite makers for *R. uyekii* and assessed polymorphisms at these loci in 150 individuals collected from three different populations.

MATERIAL AND METHODS

Genomic DNA was extracted from muscle tissue of *R. uyekii* using the TNES-urea buffer method (Asahida et al., 1996). A partial genomic library enriched for GT repeats was constructed using a slightly modified version of the procedures described by Hamilton et al. (1999). The DNA was then digested with *Alu*I, *Rsa*I, and *Hae*III (New England Biolabs, Ipswich, MA, USA), and DNA fragments ranging from 300 to 1000 bp were isolated and ligated to SNX/SNX rev linker sequences. The linker-ligated DNA was amplified by polymerase chain reaction (PCR) using SNX as the primer, and the products were hybridized to biotinylated $(GT)_{10}$ probes attached to streptavidin-coated magnetic beads (Promega, Madison, WI, USA). The enriched fragments were amplified again; the products were digested with *Nhe*I and ligated into the *Xba*I-digested pUC18 vector (Pharmacia LKB Biotechnology Inc., Gaithersburg, MD, USA), and the resulting constructs were transformed into competent *Escherichia coli* DH5α cells. Positive clones with repeats were identified by PCR with $(GT)_{10}$ and M13 primers. A negative control with no template was included in each set of reactions. The PCR products were analyzed on 1.5% agarose gels, and those clones producing two or more bands were considered to contain a microsatellite locus. Plasmid DNA from the positive clones was purified using AcroPrep 96-well filter plates (Pall Corp., Port Washington, NY, USA). All

positive colonies were bidirectionally sequenced using M13 forward or reverse primer with a BigDye Terminator Cycle Sequencing Ready Reaction kit and an ABI 3130*xl* automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Primers were designed on the basis of the sequences flanking the microsatellite motifs using the Oligo 5.0 software (National Biosciences Inc., Plymouth, MN, USA). The PCR conditions were initially optimized with the DNA samples originally used for microsatellite isolation to establish whether a product of the desired size was amplified by changing the annealing temperature, primers, and $MgCl_2$ concentration. Thirty-two microsatellite loci were genotyped to test the level of genetic polymorphisms using 150 individuals from three natural populations in Cheongpyeong Stream (N = 59; CS), Yangcheon Stream (N = 60; YS), and Tamjin Stream (N = 31; TS) in Korea.

Amplification was performed in a 10-µL reaction mixture containing 10 ng genomic DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 3 pmol of each primer, 0.5 X Band Doctor, and 0.5 U f-Taq DNA polymerase (Solgent Co. Ltd., Daejeon, Korea). The forward primers were end-labeled commercially with the dyes 6-FAM, NED, or HEX (Applied Biosystems). The reactions were conducted using a PTC-200 thermocycler (MJ Research Inc., Waltham, MA, USA) with an initial denaturation at 95°C for 15 min, followed by 35 cycles of 30 s at 95°C, 30 s at a primer-specific annealing temperature (Table 1), and 30 s at 72°C, with a final 30 min extension at 72°C. The lengths of the products were determined with an ABI 3130*xl* Genetic Analyzer (Applied Biosystems) using the GeneScan-400HD (ROX) size standard (Applied Biosystems).

The number of alleles, polymorphism information content (PIC), and observed and expected heterozygosity at each locus were calculated using Cervus 3.03 (Marshall et al., 1998). Deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were estimated using Genepop 4.0 (Raymond and Rousset, 1995), and adjusted P values for both analyses were obtained using a sequential Bonferroni test for multiple comparisons (Rice, 1989). We also estimated inbreeding coefficient (F_{IS}) values (Weir and Cockerham, 1984), which can identify departures from HWE within a population. The presence of null alleles was examined using Microchecker 2.2.3 (Van Oosterhout et al., 2004).

RESULTS AND DISCUSSION

In total, 400 white colonies with inserts were randomly selected and screened for repeats using PCR, yielding 250 (62.5%) true positive clones. These were sequenced, producing a total of 218 (54.5%) sequences containing simple sequence repeats, of which 163 (40.8%) were eliminated because they possessed no flanking sequences. Fifty-five (13.8%) sequences containing microsatellites were obtained and primers were designed to amplify microsatellite-containing regions of the genome. Only 32 of the 55 primer pairs tested successfully amplified the target region, and the remaining pairs either failed to amplify or produced nonspecific bands. We labeled 32 primer pairs with fluorescent dyes. When the PCR products were genotyped, 11 were not scorable due to excessive stutter and 3 were monomorphic. Ultimately, we chose 18 microsatellite markers and examined the genetic diversity of 150 individuals collected from three populations. The number of alleles, repeat motif, product size range, optimized annealing temperature, and GenBank accession number for each of the 18 microsatellite loci are presented in Table 1. In total, 308 alleles were detected at the 18 loci analyzed in 150 individuals. The number of alleles per locus varied from 3 to 47, with a mean of 17.1 in the total population. Within populations, the observed heterozygosity ranged from 0.032 to

1.000, expected heterozygosity from 0.082 to 0.967, and PIC from 0.078 to 0.950 (Table 2). Although it varied among loci, the mean observed heterozygosity was lower than the mean expected heterozygosity in all three populations. Significant deviations from HWE after sequential Bonferroni correction ($P < 0.0028$) were detected at six loci in at least one population [Ru48 (CS), Ru129 (CP and TS), Ru249 (CS), Ru276 (TS), Ru573 (TS), and Ru792 (TS)] (Table 2). A significant heterozygote deficiency was detected at these loci. An analysis with Microchecker indicated the possible occurrence of null alleles at five loci, except Ru792, but no evidence of allelic dropout was found for any of the loci (Bonferroni's correction). These five loci showed a highly significant positive F_{IS} (i.e., heterozygote deficiency) together with a high estimated null allele frequency, strongly suggesting a causative relationship. In addition, no significant linkage disequilibrium between loci was detected for most of the locus pairs after Bonferroni's correction ($P < 0.0028$), except in three cases (Ru48-Ru235, Ru48-Ru280, and Ru235-Ru280) (Table 2). These microsatellite markers will be useful for studying the population genetic structure and establishing effective conservation strategies for *R. uyekii*. We are currently analyzing the fine-scale structural variation in Korean *R. uyekii* populations.

Table 1. Primer sequences and characteristics of 18 polymorphic microsatellite loci in *Rhodeus uyekii*.

Locus	Repeat motif	Primer sequence (5'-3')	Ta (˚C)	Size range (bp)*	GenBank accession No.
Ru35	$(AC)_{14}$	F: CACCGCATGCTTCTTTAATATCCAG R: ATGTAAAGTCCGCGTGCTTGTG	55	160-194	KF049471
Ru48	$(GT)_{13}$	F: ACATACGTGCCGATCTTCTTA R: TGTGTTGATCAGGTGTTCTCA	55	109-199	KF049472
Ru62	$(GT)_{31}$	F: CTCTCACGCTTTCTCCATTC R: GTAATAGAGTTTTGATTCCCAACC	58	203-351	KF049473
Ru127	$(GAG)_{9}$	F: ATTCACACTCCTGCTTCCTGAC R: TATGATAGCAAAGAGAACGAAGGG	60	207-216	KF049474
Ru129	$(GT)_{11}$	F: GAGGAAAACAGAAAGCGATTCATC R: GACCTCCAGCCCTCATTCAAT	55	241-289	KF049475
Ru132	$(AC)_{15}GC(AC)_{8}$	F: CCTGTGCAATTGAGTTTGACAC R: TGTTTCCTTTCTCGACCTGTTTAC	60	117-149	KF049476
Ru235	$(GA)_{10}GG(GA)_{8}$	F: GAAGTAAGCCTCCCACAAGAACTG R: GCAGCAGGGCACTGATTTATCT	60	119-217	KF049477
Ru249	$(GT)_{3}(GAGT)_{3}(GT)_{5}$	F: TTGACCGTATGTTGACCTCCTG R: TTACAGCCAGAAGACCAGCATTTA	60	54-236	KF049478
Ru276	$(GT)_{20}$	F: ACACGCCTCACCATCATATCACAG R: CAAACGCTTCACGCCATCAG	55	250-274	KF049479
Ru280	$(AC)_{8}$	F: CTTGTCCTCTGCATCTCTTTCTCA R: ACAAATGACACATGCTTCCACTG	58	60-72	KF049480
Ru382	$(AC)_{10}$	F: TTTCCCATTATACTTCTTACAGCA R: TATACCTCCCTTTTTATGTCACTG	58	128-138	KF049481
Ru396	$(GT)_{11}$	F: TGATTCTTCTCTCGGCTGGTTG R: GAGGTTTACTGATTCAGCGATGGA	60	111-153	KF049482
Ru414	$(GT)_{12}$	F: TCCATCTGTCCTCCAACACAAAAT R: CCAACTCCACCTTCCCCTGTAA	55	225-263	KF049483
Ru461	$(CT)_{10}$	F: ACTGAACTCCCTACTAAGATGATT R: GGGAGAAAGTGAGAGGTAAAG	58	109-141	KF049484
Ru573	$(GT)_{20}$	F: TTATGGTTCAGATGTGGCATTATT R: TGTATGTGACCTCCAGTGCTG	58	146-248	KF049485
Ru657	$(AC)_{15}$	F: TGCTGACACCTGCTGGACACA R: GCTCCATTTCTGCAGGAACATTCA	55	155-189	KF049486
Ru721	$(GT)_{17}AT(GT)_{8}$	F: ATCAATGAATGAAACAAGTGCTGT R: TTAGACCATCTGCGAAACTACAAA	60	82-240	KF049487
Ru792	$(AC)_{20}$	F: TCACATGGCACTTCCTCCGTGTTG R: CCTGGTTTCCTGTCGGCTCCTGTC	60	152-186	KF049488

*Size range of alleles genotyped by 150 individuals pooled from three populations. F = forward primer sequences; R = reverse primer sequences; Ta = annealing temperature.

Table 2. Genetic characterization of 18 polymorphic microsatellite loci in three natural populations of *Rhodeus uyekii*.

	N_T	CS (N = 59)						YS (N = 60)						TS (N = 31)					
		N_A	H_O	H_E	PIC	P	F_{IS}	N_A	H_O	H_E	PIC	P	F_{IS}	N_A	H_O	H_E	PIC	P	F_{IS}
Ru35	12	5	0.627	0.600	0.530	0.097	-0.046	3	0.267	0.254	0.234	0.531	-0.052	9	0.935	0.839	0.804	0.245	-0.118
Ru48	9	6	0.407	0.720	0.667	0.000*	0.437	5	0.500	0.617	0.534	0.079	0.191	6	0.484	0.635	0.550	0.222	0.241
Ru62	47	22	0.966	0.912	0.897	0.219	-0.060	11	0.750	0.753	0.716	0.704	0.004	34	0.871	0.967	0.950	0.006	0.101
Ru127	3	2	0.051	0.082	0.078	0.085	0.381	2	0.517	0.454	0.349	0.387	-0.140	3	0.032	0.095	0.091	0.017	0.663
Ru129	0	9	0.610	0.796	0.760	0.002*	0.235	3	0.533	0.557	0.451	0.517	0.043	7	0.548	0.777	0.730	0.001*	0.298
Ru132	17	14	0.898	0.893	0.874	0.011	-0.006	8	0.300	0.309	0.290	0.524	0.031	6	0.290	0.266	0.251	1.000	-0.093
Ru235	28	19	0.898	0.875	0.857	0.529	-0.027	12	0.767	0.737	0.693	0.012	-0.040	12	0.774	0.747	0.698	0.216	-0.037
Ru249	25	17	0.661	0.862	0.839	0.002*	0.234	4	0.450	0.417	0.373	0.249	-0.080	6	0.484	0.540	0.504	0.162	0.106
Ru276	10	8	0.610	0.657	0.597	0.200	0.071	3	0.633	0.649	0.569	0.717	0.025	3	0.032	0.464	0.395	0.000*	0.932
Ru280	5	2	0.203	0.184	0.166	1.000	-0.105	4	0.267	0.296	0.276	0.003	0.099	4	0.258	0.290	0.269	0.081	0.111
Ru382	6	2	0.085	0.082	0.078	1.000	-0.036	2	0.150	0.140	0.129	1.000	-0.073	6	0.581	0.701	0.631	0.066	0.174
Ru396	14	11	0.797	0.790	0.758	0.464	-0.008	2	0.283	0.245	0.214	0.587	-0.157	5	0.323	0.368	0.344	0.043	0.124
Ru414	16	12	0.831	0.854	0.828	0.015	0.028	5	0.550	0.526	0.441	0.651	-0.046	9	0.548	0.586	0.555	0.065	0.066
Ru461	10	7	0.542	0.581	0.531	0.051	0.068	5	0.250	0.258	0.245	0.076	0.032	3	0.452	0.466	0.367	0.475	0.031
Ru573	33	23	0.983	0.938	0.926	0.012	-0.049	12	0.733	0.749	0.707	0.672	0.021	16	0.452	0.882	0.855	0.000*	0.492
Ru657	14	13	0.763	0.820	0.789	0.003	0.070	5	0.450	0.525	0.417	0.112	0.145	3	0.065	0.124	0.118	0.097	0.485
Ru721	37	15	0.831	0.856	0.835	0.364	0.030	15	0.817	0.824	0.794	0.721	0.009	28	1.000	0.963	0.945	0.470	-0.039
Ru792	12	10	0.847	0.761	0.718	0.073	-0.115	5	0.283	0.259	0.248	1.000	-0.094	4	0.516	0.665	0.585	0.001*	0.226
Mean	17.1	10.9	0.645	0.681	0.652			5.9	0.472	0.476	0.427			9.1	0.480	0.576	0.536		

N = number of individuals tested; N_T = total number of alleles per locus scored by 150 individuals; N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; PIC = polymorphism information content; P = probability of deviation from Hardy-Weinberg equilibrium (HWE); F_{IS} = inbreeding coefficient. *Significant deviations from HWE after sequential Bonferroni's correction ($P < 0.0028$).

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