Isolation and characterization of novel microsatellite loci in the wrinkled frog

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ABSTRACT. The wrinkled frog, *Rana rugosa*, is a species complex that inhabits plains and mountains near freshwater bodies throughout East Asia, encompassing China, Korea, Japan, and the Russian Primorye region. Although extensive efforts are required to estimate cryptic diversity in the *R. rugosa* complex, no specifically designed microsatellite loci are available. Here, novel and polymorphic microsatellites were isolated based on the construction of a microsatellite-enriched library and characterized using *R. rugosa* specimens collected on the Korean Peninsula. A total of 72 primer sets were designed from approximately 400 positive clones, and 22 were validated as being reliably amplified and polymorphic. Overall, high genetic variability was observed (mean number of alleles per locus = 22.23; mean observed and expected heterozygosities = 0.770 and 0.816, respectively) from a total of 60 individuals sampled from two geographically isolated localities. In the two sites analyzed, an extremely low level of relatedness was inferred from the estimation of pairwise relatedness, and no evidence of a genetic bottleneck was detected. The two sites showed a

high level of genetic differentiation, suggesting a clear signature of isolation following colonization. With high statistical power in parentage and sibship exclusion, these microsatellite loci will be suitable for the identification of cryptic diversity and population structure as well as the recognition of individuals in social interaction and captive breeding practice.

Key words: Wrinkled frogs; *Rana rugosa*; *Rana emeljanovi*; Microsatellites; Ranidae

INTRODUCTION

Since the late 1980s, declines in amphibian populations have occurred more frequently across the globe (Crawford et al., 2010; Hayes et al., 2010), and approximately one-third of all recorded species are presently threatened with extinction (Stuart et al., 2004). Despite this unprecedented worldwide crisis, the discovery of new amphibian species has increased explosively, as a result of the rigorous application of molecular tools in recent years (Hanken, 1999; Wynn and Heyer, 2001; Gower et al., 2005; Lemmon et al., 2007), indicating that the level of amphibian diversity has thus far been greatly underestimated (Vieites et al., 2009; Nair et al., 2012). Indeed, efforts to recognize hidden lineages or species could be regarded as a critical foundation for the development of long-term management programs in amphibian species, such as delineating conservation priorities and setting protection/recovery strategies.

The wrinkled frog, *Rana rugosa*, is a type of true frog that generally inhabits plains and mountains near freshwater bodies, and provides a model system for investigating cryptic diversity within taxa previously regarded as a single species. This species is widely distributed across East Asia encompassing China, Korea, Japan, and the Russian Primorye region (Máeda and Matsui, 1999; Yang et al., 2000). Uncertainty still remains concerning its taxonomic status. *R. rugosa* is a species complex that likely represents at least three different species (Frost et al., 2006; Sekiya et al., 2012); *R. emeljanovi* in east China and the Korean Peninsula, *R. rugosa* in Japan, and *R. tientaiensis* in central China. In addition, recent studies based on molecular and cytogenetic tools clearly showed that five distinct lineages (species candidate) of *R. rugosa* exist in Japan (Miura, 2007; Sekiya et al., 2012). Recently, *R. rugosa* populations from Sado Island in Japan were found to be phenotypically distinct and postzygotically isolated from other *R. rugosa* populations, and were described as a new species, which was named *R. susurra* (Sekiya et al., 2012).

It is imperative that extensive efforts are made to identify cryptic diversity in the *R. rugosa* complex across East Asia, especially given the rising likelihood of habitat degradation and extirpation in many areas (Stuart et al., 2004). Although microsatellites may provide an appropriate solution to evaluate population structure and to find cryptic lineages, no specifically designed microsatellite loci are currently available. Here, 22 novel and polymorphic microsatellites were isolated and characterized using a specimen of *R. rugosa* (*R. emeljanovi*) collected from the Korean Peninsula.

MATERIAL AND METHODS

Sample collection

Sampling and tissue collection were carried out under the national legislation of South Korea. A total of 60 individuals were sampled during 2012 and 2013 from two localities (Yangsan

and Jincheon) on the Korean Peninsula. Yangsan is on the southeastern side of the peninsula (N 36°92'81.90'', E 127°40'40.25''), while Jincheon is located in the center (N 35°31'19.99'', E 129°06'86.52''). Those two localities are separated by complicated landscape structures and an approximate distance of more than 200 km (straight line distance).

Microsatellite isolation

For microsatellite isolation, a microsatellite-enriched library was constructed based on previously employed protocols (Suk et al., 2009; Park et al., 2014). Genomic DNA was extracted from the first toe tissues of five individuals (from Yangsan) using the Wizard Genomic DNA purification kit (Promega, Fitchburg, WI, USA) based on the manufacturer protocol, and was subsequently digested with *Mbo*I (Enzynomics, Daejeon, South Korea). DNA fragments of 500-1000 bp in size were isolated by gel elution and then ligated to a double-stranded adaptor (EP; forward: 5'-CCC CCA CCT CCT GCC CAT CAT AAA AA TC-3'; reverse: 5'-phosphate - GAT CGA TTT TTT ATG ATG GGC AGG AGG TGG GGG-3') using T4 DNA ligase (Enzynomics). The ligated fragments were recovered by polymerase chain reaction (PCR; annealing at 65°C) with the primer EP3 (5'-CCC CCA CCT CCT GCC ATC AT-3'). The PCR product was purified and hybridized to biotinylated $(GT)_{12}$ and $(CT)_{12}$ probes (65°C for 12 h). The hybridized fragments were isolated using streptavidin-coated magnetic beads (Promega) and recovered by PCR using the EP3 primer. The PCR products were ligated into a pGEM-T Easy Vector (Promega). The ligated vector was transformed into DH5α cells (Enzynomics) and spread onto LB-ampicillin agar plates containing IPTG and X-gal. About 400 positive clones were chosen and sequenced at NICEM (Seoul National University, Seoul, South Korea) with M13 (-40) forward and reverse universal primers.

Validation and genotyping

Flanking regions of the sequences were used to design microsatellite PCR primer sets. Each forward primer of the validated sets was fluorescently labeled with FAM, HEX, and TET (Cosmo Genetech, Seoul, South Korea; Table 1). PCR amplifications were carried out for each primer set on a GenePro thermocycler (BIOER, Hangzhou, China). Thermal cycling consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles comprising 94°C for 30 s, 54°-58°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. The fluorescently labeled PCR products were genotyped on an ABI 3730xl Genetic Analyzer (NICEM) and scored using GeneMapper 3.7 (Applied Biosystems, Foster City, CA, USA). Those polymorphic markers were deposited in GenBank under the accession Nos. KR259851-KR259873.

Statistical analysis

Characteristics of the 22 polymorphic loci, the number of alleles, expected (H_E) and observed (H_O) heterozygosity, and inbreeding coefficient (F_{IS}) were estimated using Genepop ver. 4.2 (Raymond and Rousset, 1995) and Arlequin 3.5 (Excoffier and Lischer, 2010). Allelic richness was estimated using FSTAT ver. 2.9.3.2 (Goudet, 2001). Departures of genotypic proportions from those predicted under Hardy-Weinberg equilibrium (HWE) were tested for each locus-site combination using Genepop with the exact test following Markov chain parameters with 1000 batches and 10,000 iterations per batch (Guo and Thompson, 1992). The Fisher exact test of

Table 1. Characterization of 22 polymorphic microsatellite loci for *Rana rugosa* and a summary of genetic diversity indices, being comprised of locus name, primer sequence, GenBank accession No. (Acc. No.), repeat motif (R-Motif), number of alleles per locus (N_A), allele size range (bp), observed (H_O) and expected (H_E) heterozygosities, inbreeding coefficient (F_{IS}), and pairwise-F_{ST} between two sites.

Locus	Primer sequence (5'→3')	Flu	Acc. No.	R-Motif	N_A	bp	H_O	H_E	F_{IS}	F_{ST}
Gr001	F-TGAGCTACTCCTCCCATCTTGA R-GGCAGGGGTGACATGTATGA	HEX	KR259851	$(GT)_{19}$	26	142-208	0.932	0.941	0.009	0.016
Gr002	F-TCGGCTGTGTGAAGAAAGGT R-ACCACTTTTATCCACAGAGACACA	TET	KR259852	$(GT)_{25}$	19	130-180	0.751	0.797	0.053	0.142
Gr004	F-ATCCTGCTTCCTTTCACCGC R-TGGATTAAACAAGAAGCACAGCA	TET	KR259853	$(GA)_{13}\text{-}(GA)_{17}$	7	100-174	0.334	0.272	-0.230	0.171
Gr006	F-GTGATGCCGTTTAGATGAAGTT R-CACCTCTTAAAGTGTGTGATTGAT	HEX	KR259854	$(CA)_{29}$	24	108-187	0.779	0.870	0.097	0.131
Gr008	F-TTCATATCATCGCCCTCCCC R-CTCCCACCCTCCATTCAAGG	TET	KR259855	$(GT)_{26}$	17	214-283	0.870	0.842	-0.030	0.071
Gr015	F-TGAGAGTCAATCACAGCCTGT R-CGTGCAGGTTTGTTTCCGAG	6-FAM	KR259856	$(GT)_{35}\text{-}(GT)_4\text{-}(GT)_3$	34	111-215	0.822	0.884	0.127	0.061
Gr017	F-TCCTTTTCTATCCTTGATTCCCC R-GCTCCATAACGCCGGAAAAT	6-FAM	KR259857	$(CA)_{23}$	28	173-254	0.884	0.934	0.056	0.035
Gr023	F-TTGTCCATTGTGACGGGTGA R-CAGAAGTATGCCCACACAGT	TET	KR259858	$(GT)_{23}$	21	156-249	0.829	0.840	0.014	0.006
Gr024	F-TGTCTGGTGAGTGAGGAGTG R-TGACGGTTCCATGTCCTTCC	HEX	KR259859	$(GT)_{22}$	21	221-278	0.750	0.865	0.133	0.103
Gr029	F-CCCTTCTACTCGCTATTCAGGT R-AGCAGCTCAGAATAAGGCGA	TET	KR259860	$(GT)_{31}$	30	179-271	0.918	0.897	-0.027	0.065
Gr030	F-GCCTGGACCACAAAACTAGC R-GTGTAAAGGCAGGTGTCCCA	TET	KR259861	$(TG)_{24}(TA)_5$	16	133-191	0.492	0.597	0.169	0.357
Gr032	F-CAGGTAATGATTCGCTGAGGC R-TCCATAAATACCTGAAACCAGTGT	6-FAM	KR259863	$(TG)_{27}(TAGA)_5(GA)_2$	24	116-317	0.850	0.934	0.090	0.040
Gr033	F-TGGCAGTTACATAGTTGAAGACA R-ATTGACCGCTGCAATATCGC	HEX	KR259864	$(TG)_{43}(TA)_4$	42	95-281	0.927	0.943	0.017	0.039
Gr034	F-GCTGGAACTCAACTTTCTGTGTC R-CAGTAGCTCTCACAGACAAAGC	HEX	KR259865	$(TCTA)_{15}\text{-}(TCTA)_{10}$	26	144-271	0.872	0.925	0.052	0.038
Gr035	F-AGCTCTCCTCTGTCCCTCTG R-GGCTGGATAAAAGAGAGGGATTG	TET	KR259866	$(TG)_{29}\text{-}(TG)_5$	18	125-199	0.692	0.657	-0.056	0.312
Gr037	F-CCCGGTTGGTAAGTGGTTT R-TCTACTGGGCCCTTTTAAAACA	HEX	KR259867	$(GT)_{22}$	18	152-186	0.851	0.893	0.046	0.057
Gr038	F-CCCCATAGAGACATGCCGAT R-CCCTGAAATCTGAGAGGCCA	6-FAM	KR259868	$(GT)_{37}$	19	92-204	0.638	0.765	0.166	0.033
Gr043	F-CTGTACAGTCGCATGTGATTG R-AATGGTAATCGTTCGCTCCT	6-FAM	KR259869	$(AT)_3(GT)_{12}\text{-}(GT)_9$	18	192-274	0.822	0.884	0.089	0.038
Gr051	F-AGGCTTCTCTGAATGGGCA R-GGTCTTCCCTGAACATAATGGAC	6-FAM	KR259870	$(GT)_{24}(CT)_8$	25	112-194	0.810	0.818	0.009	0.016
Gr053	F-CCTGTGGTGAAGGAGAGGAG R-TTGTTGTGGAGAGGGGTCTG	6-FAM	KR259871	$(GT)_{27}$	23	172-259	0.637	0.721	0.123	0.259
Gr054	F-TCCACATGCACTGCTCTTCT R-AGTAGTGCCCCTCCATAGCA	HEX	KR259872	$(TG)_{24}$	14	100-174	0.715	0.744	0.038	0.234
Gr055	F-AGGTATGGAAAGGTCATGCTGT R-AGCTCCTTGACTGCCAACAA	HEX	KR259873	$(GT)_{32}\text{-}(GT)_2$	19	137-191	0.764	0.874	0.123	0.068

linkage disequilibrium between all pairs of loci disclosed presumed gametic disequilibria for all paired loci in every population under the Markov chain parameters in Genepop. Critical significance values were corrected for multiple tests by the sequential Bonferroni correction (Rice, 1989). Bottleneck ver. 1.2.02 (Piry et al., 1999) was used to examine the possibility of a recent reduction in population size based on a significant excess of H_E under mutation-drift equilibrium relative to HWE with three different models of microsatellite mutation, infinite allele model (IAM), stepwise mutation model (SMM) and two-phased model (TPM), with the proportion of SMM = 70% and IAM = 30. Genetic divergence between two analyzed sites was assessed by calculating the pairwise-F_{ST} and -R_{ST} in Genepop. Relatedness for each pair of individuals within sites was determined in ML-Relate (Kalinowski et al., 2006).

RESULTS AND DISCUSSION

About 400 positive clones were selected for sequencing. A total of 72 microsatellite PCR primer sets were designed using the Primer 3 program (ver. 0.4.0; http://bioinfo.ut.ee/primer3-0.4.0/) and screened using 12 individuals from Yangsan. Primer sets were discarded if they failed to amplify consistently and were monomorphic. From 72 primer sets, 22 were validated and were reliably amplified and clearly polymorphic in the 12 individuals.

From a total of 60 individuals sampled from two geographically isolated sites (Yangsan and Jincheon), high genetic variability was observed at the 22 loci examined (Table 1). The average number of alleles per locus was 22.23, ranging from 7 (*Gr004*) to 42 (*Gr033*). The average H_O and H_E were 0.770 and 0.816, respectively, ranging from 0.334 (*Gr004*) to 0.932 (*Gr001*), and 0.272 (*Gr004*) to 0.943 (*Gr033*), respectively. A single locus (*Gr053*) showed a significant deviation in genotype proportion from HWE after Bonferroni correction (N = 22, α = 0.0023) owing to a significant deficiency in the observed number of heterozygotes. At the population level, a significant heterozygosity deficiency was found for *Gr043* and *Gr053* from Yangsan and for *Gr006* from Jincheon. Such deviations might arise from a failure of amplification of one allele or because of an allele size falling outside of the normal calling range. The Fisher exact test revealed significant disequilibrium for only 10 of the possible pairs of the 22 loci (231 pairs) after sequential Bonferroni correction (N = 44, α = 0.0011), indicating that there was no likelihood of linkage between loci or population subdivision within sampling sites.

The two sampling sites showed similar levels of genetic variation (allelic richness; Table 2). No signature of a genetic bottleneck was detected, since no significant excess of H_E under mutation-drift equilibrium relative to that observed under HWE was detected based on the Wilcoxon test (Table 2). In addition, the Bottleneck program did not reveal any signature of a population decline with the test of shift in allele class distribution mode (normal L-shaped distribution). In future studies with widespread sampling, the *M*-ratio and critical *M* (Garza and Williamson, 2001) can also be calculated and compared with the results of the Bottleneck program to find historical signatures of population decline during colonization and genetic divergence among populations.

Table 2. Genetic diversity parameters for two sites of *Rana rugosa* sampling, Yangsan and Jincheon, being comprised of total number of individuals analyzed (N), mean number of alleles per locus (N_A), mean number of allelic richness (A_R), the probability results of Wilcoxon tests under three different models (IAM, SMM, and TPM) for Bottleneck and frequency of family relationship (ML-Relate; Freq. fam.).

Site	N	N_A	A_R	Bottleneck			Freq. fam.
				IAM	SMM	TPM	
Yangsan	31	15.27	11.57	0.339	0.979	0.999	0.028
Jincheon	29	14.00	10.48	0.980	0.999	1.000	0.015

IAM = infinite allele model; SMM = stepwise mutation model; TPM = two-phased model.

The two sites analyzed in the present study showed significant genetic differentiation between each other (pairwise-F_{ST} = 0.110, P < 0.001; pairwise-R_{ST} = 0.124, P < 0.001). Such detectable genetic differentiation between two sites could relate to a long history of geographic isolation. In addition to geographic distance, those two sites might have been isolated from each other by complex mountain ranges and by the signature of geological uplifts (Kyung, 2010), which probably limited the level of gene flow between them. A more comprehensive resolution of population

structure and dispersal could be obtained by extensive sampling throughout most of its distribution range (i.e., the Korean Peninsula and east China) and genotyping the loci characterized here.

Upon maximum likelihood calculation of relatedness within sites by ML-Relate, an extremely low level of family relatedness was found at both sites (Table 2), indicating that the microsatellites used here are highly informative for parentage analysis. With a high statistical power in parentage and sibship exclusion, these microsatellite loci will be suitable for the identification of individuals in studies of reproductive success and mating systems and in captive breeding practice as well as in the evaluation of cryptic diversity, population genetic structure, and metapopulation dynamics. Further study will include tests of cross-amplification across different species (or lineages) in the *Rugosa* complex as well as in Ranidae.

Conflicts of interest

The authors declare no conflict of interest.

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