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Development of microsatellite markers to assess the genetic diversity of the red-tongue viper, *Gloydius ussuriensis* (Reptilia: Viperidae) on the Korean Peninsula

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The red-tongue viper (*Gloydius ussuriensis*) is one of only three species of the genus *Gloydius* found in South Korea. *Gloydius ussuriensis* has a narrow activity radius and is distributed nationwide, and this species was reported to have the largest population among the Korean species in genus *Gloydius*. Preliminary results of a phylogenetic analysis using part of the mitochondrial DNA indicated that domestic *G. ussuriensis* is not comprised of monophyletic groups, and morphological analysis showed differences between domestic populations. In this study, we developed 17 microsatellites for the analysis of *G. ussuriensis* genetic diversity based on these characteristics. These microsatellites were developed using six multiplex panels, which could be employed to validate 80 *G. ussuriensis* specimens from different geographical regions in South Korea. The average number of alleles per locus was 12.2 and ranged from 4 to 25 alleles; the observed heterozygosity ranged from 0.238 to 0.950 and the expected heterozygosity ranged from 0.213 to 0.933. As a result of assessing four inland populations, a high level of genetic diversity was confirmed. These newly developed markers will be useful for further studies on the population structure and evolutionary history of the *G. ussuriensis*.

Keywords: *Gloydius ussuriensis*, microsatellite marker, multiplex panel, population structure, red-tongue viper

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Introduction

The red-tongue viper (*Gloydius ussuriensis*) belonging to the family Viperidae, is a venomous snake distributed among the Russian Far East, northeastern China, and the mainland of South Korea and its associated islands (Do and Yoo, 2014; Oh and Banjade, 2021). In total, 19 known species currently belong to the genus *Gloydius*, and there are three species in South Korea, *G. brevicaudus*, *G. saxatilis*, and *G. ussuriensis* (Shim *et al*., 1998; Xu *et al*., 2012). *Gloydius ussuriensis* is the smallest among the three species reported in South Korea with a body length of 25–50 cm, but it forms the largest population and mainly inhabits valleys, paddy fields, and wetlands (David and Vogel, 2015; Do *et al*., 2016). Nevertheless, the threat of population decline due to poaching is high, and the population is steadily declining, like that of other *Gloydius* species (Do *et al*., 2016; NIBR, 2020a). Although molecular phylogenetic studies have not been carried out on species inhabiting Korea, a preliminary analysis of the phylogenetic tree using mtDNA did indicate that phylogenetic species do not form monophyletic groups, and the morphological analysis results also confirmed differences between domestic populations (NIBR, 2020b).

To date, there have been studies using *G. shedaoensis* and *Boiga irregularis* as examples of genetic diversity in snakes, using microsatellite markers, and both studies on island populations showed low genetic diversity (Wang *et al*., 2015; Kierepka *et al*., 2019). In China, *G. ussurien-*

sis is designated and protected as an endangered species owing to its narrow distribution range, and microsatellite markers have been reported for the investigation of genetic diversity and to characterize the genetic structure of the species (Wang *et al*., 2014). We tested the developed 10 markers (Wang *et al*., 2014) on Korean specimens of *G. ussuriensis*. Three out of 10 loci were successfully and clearly amplified. Regarding to subsequent population studies of Korean *Gloydius* species, we determined that more than 10 microsatellites need to be developed. In this study, we successfully mined and characterized 17 new microsatellite markers with six multiplex sets from *G. ussuriensis* genome data, and the genetic diversity of 80 individuals collected inland, in South Korea, was tested.

Materials and Methods

Samples, DNA isolation and next-generation sequencing (NGS)

The *G. ussuriensis* specimens were collected from across South Korea and deposited at the National Institute of Biological Resources (NIBR: https://www.nibr.go.kr) in Incheon, South Korea. Among them, VZMLGR000 0000283, collected from Buan-gun, Jeollabuk-do (35°39′ 22.4″N, 126°34′25.9″E), was used for NGS analysis. For microsatellite primer characterization and genetic diversity estimation, we used 80 samples collected from Chungchengnam-do (CN, n = 20), Gyeongsangnam-do (GN, n = 20), Jeollanam-do (JN, n = 20), and Gangwon State (GW, n = 20). For each individual, scale and muscle tissue samples (approximately 0.2 cm of tail) were taken and preserved in 98% ethanol, and the sampling location, with latitude and longitude, was recorded. All sampled snakes were released immediately after sampling, and bleeding was stopped using an alcohol swab. Genomic DNA was extracted from 10−20 mg of tissue using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The library for NGS was constructed using the Truseq Nano DNA prep Kit (Illumina, San Diego, California, USA) and with an average insert size of 550 bp. NGS was performed on the Illumina platform, NovaSeq6000 with 150 bp paired-end reads by DNA Link Inc. (Seoul, South Korea).

Primer design, screening and touchdown PCR

The microsatellite loci were detected and primers for each single sequence repeat (SSR) sequence were designed with the program QDD version3.1.2 (Meglecz *et al*., 2014). After securing each SSR marker from the NGS data obtained from the two samples, the loci commonly mined from the two individuals were obtained through filtering. SSRs were defined as having di-, tri-, and tetranucleotide motifs with a minimum of four tandem repeats. Primers were designed according to the optimum melting temperature range of 56−61°C and a product size ranging from 150 to 300 bp. For the initial screening of polymorphisms, simplex PCR was performed using AccuPower® PCR Master Mix (Bioneer, Daejeon, South Korea) in a 20 μL reaction volume containing 10 μL 2× PCR Master Mix, 0.04 μM forward primer (tagged at the 5′ end with M13 (5′-GGATAACAATTTCACACAGG-3′), Hill (5′-TGACCGGCAGCAAAATTG-3′), T3 (5′-AATTAACCC TCACTAAAGGG-3′), or neomycin (5′-AGGTGAGATG ACAGGAGATC-3′), 0.2 μM reverse primer, 0.2 μM fluorescent dye (6-FAM for M13, VIC for Hill, NED for T3 and PET for neomycin), approximately 20 ng genomic DNA, and 7 μL RNase-free water. For multiplex PCR, PCR chemistry and primer volumes were optimized for each panel using the Multiplex PCR Master Mix (Qiagen) in a 50 μL reaction volume containing 25 μL 2× Multiplex PCR Master Mix (including HotStarTaq Plus DNA Polymerase and Multiplex PCR Buffer with 3 mM $MgCl_2$), 0.05−0.4 mM primers (Table 1), approximately 20 ng genomic DNA, and 19 μL RNase-free water. The parameters for touchdown PCR were as follows: 95°C for 5 min; 10 cycles of 95°C for 1 min, 60°C to 50°C for 1 min (decreasing at a rate of 1°C per cycle), and 72°C for 1 min; 25 cycles of 95°C for 30 s, 50°C for 1 min, and 72°C for 1 min; and 72°C for 5 min.

Genotyping and data analysis

PCR products for all primer sets were multiplexed and added to a mixture of Hi-Di formamide and GeneScan-LIZ500 Size Standard (both from Applied Biosystems, Foster City, CA, USA) and run on an ABI 3730xl DNA Analyzer (Applied Biosystems). Allele sizes were verified and scored using Geneious Pro v.8.1.9 (Biomatters, Auckland, New Zealand; Kearse *et al*., 2012). GenAlEx version 6.1 (Peakall and Smouse, 2006) was used for allele data processing, including the expected heterozygosity (H_E), observed heterozygosity (H_O), fixation index, number of alleles, and effective number of alleles. Genepop v.4.2 (Rousset, 2008) was used to calculate deviation from the Hardy-Weinberg equilibrium at each locus and linkage disequilibrium between locus pairs.

Results and Discussion

Illumina sequencing yielded more than 50 Gb of data comprising 332,854,173 sequence reads from VZMLGR 0000000283 to use for sequence assembly, from which 152,868 contigs containing 6,357 microsatellite motifs were obtained. Among them, 150 primer pairs consisting of di-, tri-, and tetranucleotide motifs were selected in

Table 1. Primer sequences for the 17 *Gloydius ussuriensis* microsatellite loci and optimized six multiplex panels.

Panel name	Locus	Motif	Allele range (bp)	FL	Primer sequence (5′-3′)
Panel A	GU130	$(AATG)_6$	169–222	PET	F: TGGTTGGGCTTTGAAGGAAGA R: AAGTGGCAGCTTGGGTTGAT
	GU137	$(AGAT)_{12}$	214–358	NED	F: ACCTCAGTGATATCCCAGTGT R: GCAAATGCCTGAAATGAGCT
	GU116	$(AACG)_5$	298–354	6FAM	F: TGAAACAGGCCAGCCATAGA R: CAAGCTGCATAACACCTGGC
Panel B	GU110	$(AAAG)_{19}$	148–232	PET	F: AGCACCTGCAATGATGAGAA R: GGAACAAAGCAATTCCAACCA
	GU129	$(AATG)_{15}$	228–288	VIC	F: TGGGTTTGGCTTAACTGTGAA R: CCCTGGGTTAGAGCATAGGC
Panel C	GU001	$(AC)_8$	155–181	6FAM	F: AAACACTCCATTGACTCTACAGA R: CCACTGGACTAGGAGGTGCA
	GU119	$(AAGG)_{18}$	187–271	NED	F: AAAGCTTCCCACTGTCAACA R: TTGTCAGTCAGAAGCCAGGC
	GU147	$(ATCC)_{11}$	260–316	PET	F: TCCCTCCCTCCTACCTCAAA R: CCCACAGTTGTTGTTCAGCC
Panel D	GU150	$(ATCC)_{13}$	134–200	6FAM	F: TGTGCAGACAGTTCCAGGTC R: AACCCAAATGTGCACTTGCC
	GU127	$(AATC)_{11}$	212–284	NED	F: AGCTTTGCTCTCTTGGATGCT R: GCTAACATTGCCTGCAGTGC
	GU078	$(AGC)_5$	301–313	PET	F: AAACACACAACCTGCAACCC R: TGTCATACCTCAGTGCTTGGA
	GU126	$(AATC)_8$	298–342	6FAM	F: AAGGACTCCGCTTGGAAAGG R: TTCCCACAGTGTCAGCCATC
Panel E	GU115	$(AAAT)_{19}$	134–254	PET	F: ACAATGGGACTGGCCTATGC R: AGATTGCTAGATGTAAATGGAAGGT
	GU072	$(ACC)_8$	226–244	NED	F: TACTGGATTCAGACGCGAGC R: GAGGGCAGACTGAAGACTGG
Panel F	GU105	$(AAAC)_6$	184–204	PET	F: AGATTGAGCTGGCCCTCATG R: GAGCTGATAGATGTGTGGCTGA
	GU122	$(AAGG)_{15}$	194–286	NED	F: AATTGGCTTCCCTACTGGCG R: GGCCAGCATTATTCAAGGGC
	GU133	$(AATG)_{13}$	286–330	6FAM	F: TGTGTGAATGCTCGGTCCTC R: GGCAATCTCATCGGGACCAA

FL, fluorescent label

simple PCR using samples from three *G. ussuriensis* specimens. We selected 17 novel microsatellites that were ultimately used to generate six multiplex panels (Table 1). The six multiplex panels confirmed to be capable of identifying 2–4 markers per panel were used.

All 17 markers were in agreement with Hardy-Weinberg

Table 2. Characterization and accession numbers of the microsatellite markers for *Gloydius ussuriensis*.

Locus	*A*	A_R	H_O	H_E	*P* value	F	GenBank
GU001	12	1.628	0.650	0.662	0.121	0.018	MZ935648
GU072	5	0.362	0.725	0.664	0.843	−0.092	MZ935651
GU078	4	0.189	0.463	0.460	0.757	−0.006	MZ935652
GU105	4	0.437	0.238	0.213	1.000	−0.114	MZ935654
GU110	15	1.214	0.875	0.892	0.813	0.019	MZ935655
GU115	25	2.711	0.950	0.933	0.297	−0.018	MZ935656
GU116	8	0.848	0.513	0.528	0.757	0.029	MZ935657
GU119	18	1.676	0.888	0.924	0.172	0.039	MZ935658
GU122	16	1.765	0.763	0.888	0.162	0.141	MZ935659
GU126	8	0.164	0.825	0.850	0.229	0.029	MZ935660
GU127	15	1.478	0.738	0.857	0.083	0.139	MZ935661
GU129	13	1.143	0.763	0.861	0.132	0.114	MZ935662
GU130	12	0.930	0.813	0.878	0.581	0.074	MZ935663
GU133	11	0.867	0.788	0.844	0.089	0.066	MZ935664
GU137	16	1.660	0.850	0.881	0.697	0.035	MZ935665
GU147	10	0.550	0.788	0.849	0.714	0.072	MZ935666
GU150	15	1.372	0.838	0.894	0.362	0.064	MZ935667

A, total number of alleles; A_R, allelic richness; H_O, observed heterozygosity; H_E, expected heterozygosity; *P* value, probability values of test for Hard-Weinberg equilibrium; F, fixation index; GenBank, NCBI GenBank accession number.

equilibrium even without a significance level correction for multiple comparisons (after Bonferroni correction, $P = 0.05/17 = 0.0029$; Table 2) and all analyzed polymorphic loci have shown no evidence of linkage disequilibrium. The polymorphisms of the markers comprised 4–25 alleles per locus. The H_O and H_E ranged from 0.238 to 0.950 (average 0.733) and from 0.213 to 0.933 (average 0.769), respectively (Table 2).

The four populations (n = 80) from inland South Korea had similar levels of genetic variation, for which the H_O and H_E ranged from 0.715 to 0.750 and from 0.726 to 0.769, respectively (Table 3). The average number of alleles ranged from 8.647 to 9.235, and the fixation index ranged from −0.004 to 0.026 (Table 3). When looking at the results of previous studies, the genetic diversity of snakes was confirmed in two studies limited to the island, which reported average H_O and H_E values of 0.32 and 0.37 in *G. shedaoensis* (Wang *et al*., 2015) and 0.570 and 0.677 in *B. irregularis*, respectively (Kierepka *et al*., 2019). Therefore, compared to other snakes (*G. shedaoensis* and *B. irregularis*), the genetic diversity of South Korean *G. ussuriensis* was relatively high.

Here, we report the successful application of six multiplex panels for 17 newly developed microsatellite markers. These microsatellite markers will contribute to confirming the genetic diversity of the domestic *G. ussuriensis* population and could be used to determine whether populations in geographically isolated areas form different genetic structures. Moreover, the ability to genotype various domestic and foreign populations of *G. ussuriensis* will also be useful for future studies on origins and evolutionary history.

Table 3. Genetic diversity estimates of four populations on the Korean Peninsula.

Population	N	N_A	N_E	H_O	H_E	F
CN	20	9.059	6.046	0.750	0.761	0.007
GN	20	9.235	6.041	0.726	0.744	0.026
JN	20	9.176	6.354	0.741	0.769	0.022
GW	20	8.647	5.444	0.715	0.726	−0.004
Mean	20	9.029	5.971	0.733	0.750	0.013

N, number of samples; N_A, number of alleles; N_E, number of effective alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F, fixation index; CN, Chungcheongnam-do; GN, Gyeongsangnam-do; JN, Jeollanam-do; GW, Gangwon State

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