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Characterization of 23 microsatellite markers and development of multiplex PCRs for the Amur viper *Gloydius saxatilis* (Viperidae) and cross-species amplification in other *Gloydius* species

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ABSTRACT. The Amur viper (family Viperidae), *Gloydius saxatilis* (Emelianov, 1937) is found in Russia, China, and the Korean Peninsula, and suffers from considerable poaching pressure driven by financial incentives. Its unique distribution has attracted attention as an indicator species for climate change. Herein, we report the isolation and characterization of 23 novel microsatellite loci in *G. saxatilis* using next-generation sequencing technology consisting of six multiplex panels. We collected 42 muscle samples from *G. saxatilis* that had either been road-killed or captured at various natural sites in South Korea. Twenty-two samples were collected from Gangwon-do, four from Gyeonggi-do, nine from Gyeongsang-do, five from Jella-do, and two from Chungcheong-do. The observed and expected heterozygosities of the loci varied from 0.447 to 0.937 and 0.436 to 0.815, respectively. The number of alleles per locus ranged from four to 15. All loci were in Hardy–Weinberg equilibrium, and no linkage disequilibrium was detected among any pairs of the loci. Examination of cross-species amplification showed that 20 loci were transferable to two other *Gloydius* snakes (*G. ussuriensis* and *G. brevicaudus*) and helped in identifying each

species and hybrid. The six multiplex panels of 23 polymorphic microsatellite loci will play an important role in future population genetic studies and in the conservation and management of the *Gloydius* species.

Key words: *Gloydius saxatilis*; Microsatellite marker; Cross-species amplification; Population genetics

INTRODUCTION

The Amur viper (*Gloydius saxatilis*), Ussuri pit viper (*Gloydius ussuriensis*), and short-tailed pit viper (*Gloydius brevicaudus*) are pit vipers belonging to the genus *Gloydius* (family Viperidae) that are distributed throughout the Korean Peninsula (David and Vogel, 2015; Do et al., 2016). *Gloydius saxatilis* is the largest of the three *Gloydius* species in South Korea and has a preference for high-altitude mountainous regions and shows a clumped distribution (Do et al., 2017a and 2017b). The population of *G. saxatilis* is declining due to poaching driven by high prices in the illegal wildlife market (NIBR, 2020). In 2005, *G. saxatilis* was legally designated as an endangered species in South Korea, and is currently reported as protected. Recently, *Gloydius saxatilis* has become ecologically important as an indicator species of climate change (Do et al., 2022).

Only a limited number of genetic studies on *G. saxatilis* have been conducted to date. Xu et al. (2016) and Lee et al. (2021) reported the complete mitochondrial genome sequence of this species. Yan et al. (2012) evaluated phylogenetic relationships within the Asian pit viper genus *Gloydius* based on mitochondrial and nuclear DNA sequences, whereafter Lee et al. (2022) revealed the phylogenetic relationships between the three *Gloydius* species in South Korea. Although previous studies have contributed to the understanding of phylogenetic relationships at the species level, population-level research has not yet been conducted to elucidate the level of genetic diversity and population structure of *G. saxatilis*, which could aid in establishing management plans.

In the present study, we aimed to develop a microsatellite genetic marker for population genetics. Ma et al. (2013) developed eight microsatellite loci for *G. brevicaudus*. In addition, Wang et al. (2014) isolated ten more microsatellite primers for *G. ussuriensis*. However we do not know whether the newly developed loci from *G. saxatilis* can be used across the *Gloydius* species. In this study, we tested their cross-amplification utility.

MATERIAL AND METHODS

Sample collection and DNA extraction

We collected 42 muscle samples from *G. saxatilis* that had either been road-killed or captured at various natural sites in South Korea from 2005 to 2021. Twenty-two samples were collected from Gangwon-do, four from Gyeonggi-do, nine from Gyeongsang-do, five from Jella-do, and two from Chungcheong-do. The collected samples were deposited at the National Institute of Biological Resources (NIBR: https://www.nibr.go.kr) in Incheon, South Korea, before DNA isolation. We investigated this study after approval from NIBR animal ethics committee (NIBR IACUC 20220001). Genomic DNA was extracted from muscle tissue using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. All the samples used in this study were collected with relevant legal permissions.

Genomic library construction, next generation sequencing, and microsatellite identification

One of the 42 samples was used to construct a genomic library for NGS. A voucher specimen (voucher no. NIBR0000625204) was collected from Samcheok-si, Gangwon-do, South Korea (37.109096 N 129.180022 E). An average library insert size of 550 bp (Truseq Nano DNA Prep Kit) was constructed, and NGS was performed on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) at DNA Link Inc. (Seoul, South Korea). Approximately 50 Gb of DNA sequence data were generated. De novo assembly was performed using CLC Genomics Workbench v.10.0.0.1 (CLCBio, Cambridge, MA, USA). Singleton reads that were not assembled were used for microsatellite identification in the case of tandem repeats of 2–4 bp, with a minimum of four repeats. Primers targeting the flanking regions of candidate microsatellite loci were designed using Primer v3 (Rozen and Skaletsky, 2000). A total of 233 candidate microsatellite loci were selected for the initial screening of polymorphisms in three individuals of *G. saxatilis*.

PCR validation in simplex PCR

Simplex polymerase chain reaction (PCR) was performed on three randomly selected individuals to confirm whether the newly designed primer pairs were properly amplified. The simplex PCR reactions were performed using AccuPower® PCR Master Mix (Bioneer, Daejeon, South Korea) in a 20 μL reaction volume containing 10 μL 2X PCR Master Mix, 0.04 μM forward primer tagged with M13 (5-GGATAACAATTTCACACAGG-3) at their 5' ends, 0.2 μM reverse primer, 0.2 μM each fluorescent dye, ~20 ng genomic DNA, and 7 μL RNase-free water. The thermal cycling profile was as follows: 95 °C for 5 min, 10 cycles of 95 °C for 1 min, 60 °C to 50 °C for 1 min (decreasing by 1 °C per cycle), 72 °C for 1 min, and 72 °C for 5 min. The annealing temperature for the last 25 cycles was 50 °C, with denaturation. All PCR products were run on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) with Hi-Di formamide (Applied Biosystems, Foster City, USA) and size standard GeneScan-LIZ 500 (Applied Biosystems, Foster City, USA). This process provided basic information, including the shape of the peaks, allele size range, and level of polymorphism of each locus, which helped compose the loci for multiplex PCR.

Multiplex PCR amplification

Using simplex PCRs, we screened 23 microsatellite loci which were used to design six multiplex panels. The candidate forward primers were ordered from Applied Biosystems (Life Technologies) with the 5' fluorescent dye 6-FAM (M13), VIC (Hill:5-TGACCGGCAGCAAAATTG-3), NED (T3:5-AATTAACCCTCACTAAAGGG-3), and PET (neomycin:5-AGGTGAGATGACAGGAGATC-3). PCR chemistry and primer volumes were optimized for each panel using the Multiplex PCR Master Mix (Qiagen, Valencia, CA, USA) in a 50 μL reaction volume containing 25 μL 2X Multiplex PCR Master Mix (including HotStarTaq Plus DNA Polymerase and Multiplex PCR Buffer with 3 mM $MgCl_2$), 0.05–0.4 mM primer (Table 1), ~20 ng genomic DNA, and 19 μL RNase-free water. The touchdown PCR parameters were as follows: 95 °C for 15 min; 10 cycles of 95 °C for 30 s, 60 °C for 90 s (decreasing by 1 °C per cycle), and 72 °C for 60 s; 25 cycles of 95 °C for 30 s, 50 °C for 90 s, and 72 °C for 60 s; and 60 °C for 30 min. Each panel was sized using an ABI 3730xl DNA Analyzer and a GeneScan-LIZ 500 size standard. The samples were genotyped as described above. The 23 microsatellite loci were deposited in National Center for Biotechnology Information (NCBI; Table 1).4

Table 1. Characteristics of 23 newly developed microsatellite loci in *Gloydius saxatilis* in the six PCR multiplexes.

Locus	Panel	Dye label	Primer sequence (5'-3')	repeat motif	size range	*Ta*(℃)	Accession No
AS260	A	6-FAM	F:GATCCACCCATCCAACCATT R:ACCTGATTCATGGTCTAAACTGCT	$(AGAT)_9$	132-168	60-50	MZ344791
AS112	A	6-FAM	F:CCTGATGAGACTGCAGGTAAA R:CTTCAACAGCAGGTCCAAGC	$(ACAT)_8$	296-324	60-50	MZ344770
AS220	A	VIC	F:GGGTCTTCAAACTTGGCCCT R:TGTGACTTCTAACCGCACAA	$(ATCC)_9$	232-268	60-50	MZ344783
AS206	A	PET	F:TCCTCACATGAGATGGCTGG R:TGAATTCTATCGTGTCAGGAAAGA	$(ATCC)_{12}$	266-310	60-50	MZ344780
AS258	B	6-FAM	F:AATGACTCTGAGCCACCCAG R:GGAGCACTTCTGCCTCAGTT	$(AATG)_8$	150-178	60-50	MZ344790
AS132	B	VIC	F:GCCCAATGGAAGGAACTGCT R:TCCCTCCTTCTATCCTCCACT	$(AAAG)_{15}$	174-230	60-50	MZ344772
AS212	B	VIC	F:TCGAAATACAGTTTGGTGCGA R:ACGGTCTAACAGAGCCCAGA	$(AAAG)_{11}$	249-289	60-50	MZ344782
AS239	B	NED	F:CAGCCACAGAAGCTACACGA R:TTTGTAATGGTGTTTCAGTCTTGT	$(AAAG)_9$	212-244	60-50	MZ344786
AS265	C	6-FAM	F:CAGCTCCCTGCACATGAGTT R:GCATCTGGCTAATATCCACAGT	$(ATCC)_{10}$	141-181	60-50	MZ344792
AS244	C	NED	F:TCCTGACTCAGAGCACTCAGA R:AGCGTGGTCCAACATTAGCA	$(ATCC)_{12}$	186-240	60-50	MZ344787
AS189	C	NED	F:ACTCCTTCAGTGCTATGCAGG R:ATGTATGCATGTATTTGACCAAGGA	$(AAAG)_{15}$	281-337	60-50	MZ344776
AS125	C	PET	F:TGTACACAGTGACTATTGACCC R:TGGCATTCAGAACATTGCATCT	$(AAAG)_{12}$	231-287	60-50	MZ344771
AS226	D	VIC	F:TGGCCTTGCAGTCTTCTTGA R:GGTTCTAGGAGAGGTGTGCA	$(AAAG)_{10}$	236-272	60-50	MZ344784
AS246	D	NED	F:TGCACTTAGCTACAGTGGCA R:CTGCAATTGTTTCCAAGGATGACT	$(AAAC)_4$	216-293	60-50	MZ344788
AS184	D	NED	F:GATGAGGACCCAGATTGTTGG R:TCAGGATTTGGAAGCATTGAGC	$(AAAC)_8$	312-340	60-50	MZ344775
AS204	D	PET	F:GGGTCCAAAGGCAGATGACAT R:GCTCTCGGCAGGTGAATTCT	$(ACAG)_{11}$	269-313	60-50	MZ344778
AS229	E	NED	F:TCCGCCCAGCCTCAGATTAT R:ACAAGTGGTGCCCTAATGACA	$(AAAG)_{11}$	204-252	60-50	MZ344785
AS182	E	NED	F:AGCTTTGCTTGATGACTGGT R:TTCTACTTTAGGTGTACTGCTTAGT	$(AAAG)_{14}$	303-359	60-50	MZ344774
AS205	E	PET	F:TCCTATGTGGCACTTCTGGC R:CCCATGCAGCTTCTCAACCA	$(ATCC)_9$	267-299	60-50	MZ344779
AS139	F	6-FAM	F:GGACGGCTATGATTTCCGCT R:TCAGGAGGCAAGCAATTCTGA	$(ATCC)_{11}$	148-192	60-50	MZ344773
AS192	F	6-FAM	F:ATGGCGAGTGTGCTGATAGG R:GAACCAGGAGTCCACGGAAG	$(AAAG)_{15}$	298-366	60-50	MZ344777
AS247	F	NED	F:GCACAGAACTGGCTTAGATGC R:TTAGTGAATGCTCTGCTTTGAAAT	$(AAAT)_{11}$	187-239	60-50	MZ344789
AS209	F	PET	F:TACAGTGCCTGCTGTCCAAG R:ATGCTCTGGCTTGCCTGATT	$(AAAG)_{14}$	226-286	60-50	MZ344781

Ta is Annealing temperature. Accesstion number is GenBank accesstion number.

Genotypic data analysis

Allele sizes were verified and scored using Geneious Pro v.8.1.9 (Biomatters, Auckland, New Zealand; Kearse et al., 2012). MICRO-CHECKER v2.2.3 (Van Oosterhout et al., 2004) was used to check for the presence of null alleles, large dropouts, or genotyping errors. GenAlEx v 6.1 (Peakall and Smouse, 2006) was employed for allele data processing, which included expected heterozygosity (H_E), observed heterozygosity (H_O), fixation index (F_{IS}), and the number of alleles (Na). Genepop v.4.2 on the web (Rousset, 2008) was used for each locus and pair of loci to detect deviation from Hardy–Weinberg equilibrium (HWE) and genotypic linkage disequilibrium (LD), respectively. The Bayesian software, STRUCTURE v2.3.4 (Falush et al., 2003), was used for species identification and hybrid detection. The admixture model and correlated allele frequencies were selected. Five independent simulations for each value of *K*, ranging from 1 to 5 were carried out. Markov chain Monte Carlo parameters were set to 100,000 iterations, and the length of the burn-in was 10,000 iterations.

Cross-species amplification in other *Gloydius* snakes

We tested the cross-species amplification of the six multiplex panels using the same amplification conditions as mentioned above. We collected 26 muscle samples from *G. ussuriensis* (n = 12) and *G. brevicaudus* (n = 14) that had either been road-killed or captured at various natural sites in South Korea. Samples were collected from Gangwon-do, Gyeonggi-do, Jella-do, Incheon and Chungcheong-do. The two other species in the *Gloydius* genus were used for this examination; *G. ussuriensis* (n = 12; NIBR specimen: NIBRGR0000644241–NIBRGR0000644252) and *G. brevicaudus* (n = 14; NIBR specimen: NIBRGR0000644253–NIBRGR0000644265, NIBRGR0000644268).

RESULTS AND DISCUSSION

The NovaSeq 6000 run generated 667,525,848 (2×333,762,924) reads for *G. saxatilis* and identified 125,359 reads containing microsatellite motifs. In the 125,359 identified microsatellites of *G. saxatilis* were 81,224 di-, 18,885 tri-, 21,946 tetra-, 2,908 penta-, and 396 hexanucleotides. We screened di-, tri-, and tetra-nucleotide repeats with minimal complexity in the repeat regions, which allowed for the selection of 233 potential primer pairs for microsatellites. Singleplex PCRs with 233 microsatellite loci were initially performed, of which 88 loci were identified as polymorphic and were consistently amplified. After optimization of the multiplex PCRs, the 23 microsatellite markers consisted of six multiplex panels (Table 1).

Six multiplex panels were successfully genotyped in 42 individuals of *G. saxatilis*. The number of alleles ranged from four (AS246) to 15 (AS132, AS189, and AS192). The observed heterozygosity ranged from 0.447 (AS246) to 0.937 (AS132), with an average of 0.768; and expected heterozygosity ranged from 0.436 (AS246) to 0.815 (AS189), with an average of 0.734 (Table 2). There was no observed deviation from HWE, and no LD was detected between the pairs of loci.

Table 2. Characteristics of 23 polymorphic microsatellite loci for *Gloydius saxatilis* tested on 42 individuals.

No	Locus	N	Na	H_O	H_E	F_{IS}	P-value
1	AS260	42	9	0.833	0.713	-0.168	0.661
2	AS112	42	8	0.751	0.706	-0.063	0.552
3	AS220	42	9	0.756	0.710	-0.065	0.602
4	AS206	42	12	0.798	0.784	-0.018	0.423
5	AS258	42	8	0.711	0.743	0.043	0.380
6	AS132	42	15	0.937	0.798	-0.175	0.664
7	AS212	42	11	0.805	0.726	-0.108	0.468
8	AS239	42	9	0.891	0.748	-0.192	0.723
9	AS265	42	10	0.826	0.718	-0.151	0.633
10	AS244	42	12	0.851	0.726	-0.172	0.740
11	AS189	42	15	0.856	0.815	-0.051	0.558
12	AS125	42	12	0.811	0.747	-0.086	0.502
13	AS226	42	10	0.561	0.742	0.244	0.269
14	AS246	42	4	0.447	0.436	-0.025	0.443
15	AS184	42	8	0.587	0.719	0.183	0.31
16	AS204	42	11	0.671	0.690	0.027	0.258
17	AS229	42	11	0.908	0.803	-0.131	0.633
18	AS182	42	14	0.924	0.766	-0.206	0.806
19	AS205	42	9	0.680	0.760	0.105	0.272
20	AS139	42	11	0.819	0.731	-0.121	0.540
21	AS192	42	15	0.673	0.737	0.088	0.574
22	AS247	42	11	0.789	0.757	-0.042	0.596
23	AS209	42	14	0.773	0.797	0.03	0.490

F_{IS} is inbreeding coefficient. H_E is expected heterozygosity. H_O is observed heterozygosity. N is number of individuals genotyped at each locus. Na is number of alleles. P value for Hardy-Weinberg equilibrium test.

Cross-species amplification tests indicated that 20 of the 23 microsatellite loci were successfully amplified and scored in the two other *Gloydius* snakes under the aforementioned PCR conditions (Table 3). Three loci (AS220, AS132, and AS205) were excluded owing to either poor amplification or unexpected size range within the *G. ussuriensis* and *G. brevicaudus* samples. The twenty loci were polymorphic in both species except for the AS206 locus, which was monomorphic in *G. brevicaudus* (Table 3). In our study, both *G. ussuriensis*, and *G. brevicaudus* showed a relatively high genetic diversity (2-12 alleles per locus and 1-12 alleles per locus, respectively), which was similar with the results of Wang et al. (4-13 alleles per locus for *G. ussuriensis*) and Ma et al. (7-16 alleles per locus for *G. brevicaudus*), indicating high representative power of our primer sets. STRUCTURE analysis using 20 microsatellites demonstrated no evidence of hybridization among individuals of the three species (data not shown), and was sufficient to distinguish between these three species. Recently, a possible hybrid between *G. brevicaudus* and *G. intermedius* in the wild was observed based on morphological characteristics (Do et al., 2019). The newly developed microsatellites in this study are helpful in clarifying hybridization and contact zones.

Table 3. Cross-species transferability of 23 microsatellite loci identified in *G. brevicadus and G. ussuriensis*

Locus	*Gloydius brevicaudus* (n=14)	*Gloydius ussuriensis*(n=12)
AS260	8 [160-188]	7 [148-176]
AS112	3 [307-315]	5 [299-319]
AS220	-	-
AS206	1 [222]	6 [218-246]
AS258	3 [158-166]	5 [150-178]
AS132	-	-
AS212	3 [206-218]	4 [214-234]
AS239	7 [223-255]	6 [207-231]
AS265	6 [161-181]	7 [161-185]
AS244	8 [200-232]	5 [168-248]
AS189	10 [304-340]	7 [288-332]
AS125	12 [263-315]	12 [219-291]
AS226	8 [248-280]	7 [236-272]
AS246	2 [196-200]	2 [216-220]
AS184	7 [312-340]	3 [312-328]
AS204	5 [289-305]	8 [277-305]
AS229	9 [248-312]	11 [256-348]
AS182	8 [303-339]	8 [303-355]
AS205	-	-
AS139	5 [176-204]	11 [172-260]
AS192	8 [286-326]	7 [326-362]
AS247	6 [187-211]	7 [199-247]
AS209	6 [242-262]	7 [270-314]

*Number of alleles and size ranges (in base pairs, shown in brackets) of the PCR product are indicated.

CONCLUSIONS

We isolated and characterized 23 new polymorphic microsatellite loci with tetra-nucleotide repeat motifs in *G. saxatilis* using next-generation sequencing (NGS) technology.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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