Characterization of 19 Microsatellite Loci in the Clonal Monkshood Aconitum kusnezoffii (Ranunculaceae)

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PRIMER NOTE

CHARACTERIZATION OF 19 MICROSATELLITE LOCI IN THE CLONAL MONKSHOOD *ACONITUM KUSNEZOFFII* (RANUNCULACEAE)[1]

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- *Premise of the study:* Microsatellite loci were isolated and characterized from *Aconitum kusnezoffii* (Ranunculaceae) to estimate male and female reproductive success and evaluate the effects of clonal growth on sexual reproduction.
- *Methods and Results:* A genomic enrichment approach was used to develop microsatellite markers. In three investigated *A. kusnezoffii* populations, a total of 19 microsatellite loci were successfully amplified, and 13 of these loci were polymorphic. Most of the primer pairs designed for the identified loci also amplified corresponding microsatellite loci in *A. barbatum* var. *puberulum* and *A. alboviolaceum*.
- *Conclusions:* The identified microsatellite loci will be useful for quantifying male and female fitness in *A. kusnezoffii* and evaluating the effects of clonal growth on sexual reproduction.

Key words: *Aconitum kusnezoffii*; clonal growth; genomic enrichment cloning; microsatellite; Ranunculaceae; reproductive success.

Clonal growth typically results in plants concurrently exhibiting sexual and asexual reproduction. This type of clonal growth is thought to have mixed effects on sexual fitness (Vallejo-Marín et al., 2010; Liao and Harder, 2014; Barrett, 2015; Van Drunen et al., 2015). *Aconitum kusnezoffii* Rchb. (Ranunculaceae), a hermaphroditic and self-compatible perennial herb, grows clonally via root tubers to form a clumped clonal architecture and therefore provides an ideal system for evaluating the effects of clonality on plant sexual reproduction (Liao et al., 2009; Hu et al., 2015). Moreover, *A. kusnezoffii* and many Delphinieae species have attracted a great deal of research attention that has focused on the molecular bases and evolution of floral zygomorphy, perianths, and nectar spurs (Jabbour and Renner, 2012).

To gain a better understanding of the potential effects of clonality on sexual reproduction, male and female fitness with respect to sexual reproduction should be thoroughly evaluated. The most common approach for evaluating male and female reproductive success is to conduct paternity analyses in isolated populations based on molecular markers (e.g., this approach has been used for *Juglans mandshurica* Maxim. [Bai et al., 2007]). Due to their high degree of polymorphisms and codominance, microsatellites have been widely used in various plants to estimate mating systems and quantify male and female reproductive success. Here, we characterize the first set of 13 polymorphic microsatellite loci in three populations of *A. kusnezoffii*. We also test cross-amplification in two related species, *A. barbatum* Pers. var. *puberulum* Ledeb. and *A. alboviolaceum* Kom., because these three *Aconitum* L. species are sympatric in western Beijing and exhibit different floral colors. This set of microsatellite markers will facilitate further studies that estimate reproductive success and evaluate the effects of clonality on sexual reproduction.

METHODS AND RESULTS

Aconitum kusnezoffii reproduces clonally through root tubers, resulting in a clumped architecture. Therefore, each clone is separately distributed in populations and can be easily identified. We randomly sampled more than 24 clones with a distance of more than 3 m among sampled clones from each of the three *A. kusnezoffii* populations (Appendix 1) and collected one leaf from each clone for the molecular experiments.

We applied a genomic enrichment approach to identify microsatellite loci from the genome of *A. kusnezoffii*, using a protocol based on procedures described by Zane et al. (2002). Genomic DNA of nine individuals was extracted from dried leaves using a Plant Genomic Purification Kit (TIANGEN, Beijing, China). Approximately 250 ng of genomic DNA was digested with 5 units of *Mse*I (New England Biolabs, Ipswich, Massachusetts, USA), and the digested fragments were ligated with *Mse*I adapters and amplified with adapter-specific primers. Purified PCR products were hybridized with probes consisting of 15 repetitive sequences of AT, AC, AG, CAG, GAC, and GATA (Sangon Biotech, Shanghai, China). Streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA) were used to capture fragments hybridized to the probes. Enriched microsatellite fragments were sequenced by DNA cloning. A total of 356 sequences ranging from 300 to 800 bp in length were obtained; 127 primer pairs for these sequences were designed using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA). We chose primers with lengths of 15–30 bp and similar annealing temperatures between forward and reverse primers. The annealing temperatures for all the isolated microsatellite loci ranged from 47°C to 60°C.

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TABLE 1. Characteristics of 19 microsatellite loci identified in *Aconitum kusnezoffii*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)[a]	GenBank accession no.
Ak1	F: GGACAAGTAATCGCCGTGGA	$(TC)_{12}$	469–477	TD60–45	KU302084
	R: CTAGTGTGATGTGGAGCTTA				
Ak2	F: TGTGTTGGTCCTTACATGTG	$(GT)_{10}$	390–420	57	KU302085
	R: GTTGCACTCATGCACAGACA				
Ak3	F: CTTATTCAAGGCGGCACTTC	$(CA)_8$	280–340	53	KU302086
	R: TCTAGGGTCGGCCCTGAATC				
Ak4	F: TTCGGTGTTGCAAGCAAGGT	$(CA)_8$	140–160	50	KU302087
	R: GCAGCTGATGTCATCTGAGT				
Ak5	F: TTGGCACACTGAGCTACAAC	$(GT)_{17}$	400–450	50	KU302088
	R: CCGTGATCTCTAGCATAGTC				
Ak6	F: CTTGTTATTATGATTGGTGATGGGAT	$(TA)_7(TG)_{13}$	210–250	49	KU302089
	R: CTATCATCACTTGCCGTACTTTTCAG				
Ak7	F: AATCAAAGTCTACAGCGG	$(CA)_6$	240–260	TD60–45	KU302090
	R: GAATAGGATGCGTCAGTT				
Ak8	F: CTTCTCACCATCACTGCCAC	$(GAG)_3(GAA)_4(GAT)_3$	281–289	51	KU302091
	R: GATGTCCATCATGTTCCCTC				
Ak9	F: TCACGCATTACTACTAGGCACAGG	$(AC)_{11}$	164–190	50	KU302092
	R: GTGACGTGTACAAAGAAACGACGG				
Ak10	F: TGAAATTCCCTGAAATGCAAGAT	$(GA)_5$	318–328	49	KU302093
	R: TGGCAAGGGTTGTGAGTTGG				
Ak11	F: TGTTATTTGGTAGCCCAAAGCTG	$(CA)_8$	270–330	52	KU302094
	R: ATGAAACCTCCACGATCACGAC				
Ak12	F: AATACGTCGTCGTCACGCAAAC	$(AC)_5$	340–360	49	KU302095
	R: TCTGTTCCTGGGCTTCTCATCTC				
Ak13	F: AATACGTCGTCGTCACGCAAAC	$(AC)_5$	420–480	49	KU302096
	R: TCTGTTCCTGGGCTTCTCATCTC				
Ak14	F: CTCTGCTCAGTCCCTTCCCTCTT	$(TC)_6$	171	49	KU302097
	R: CCTCAAATCTCAAACCGAACAAA				
Ak15	F: AGGCGCATGTTTAGATGGACAA	$(AG)_{10}$	356	49	KU302098
	R: CCCAGCAAGCACCACCGT				
Ak16	F: AAGCCTTCATCCTTCACCACC	$(GA)_6$	323	51	KU302099
	R: TCGCTCCAAACGCCGTCATA				
Ak17	F: GTTGTAGACTCGGTGGGAGCATTG	$(TC)_8$	212	50	KU302100
	R: GGGTGGATTCGGTTATAGAGGGTG				
Ak18	F: AAGCACGCTGGATCTTGACTTTG	$(CT)_{15}$	247	49	KU302101
	R: GGGTCTGGAGGGTGAGGTTTG				
Ak19	F: CCTACCCGGCCTCCTTTCTTC	$(CA)_5$	309	50	KU302102
	R: CCATCGGTTTCTCACCTGAATCTTG				

Note: T_a = annealing temperature.
[a]A touchdown (TD) protocol was applied. Annealing started at the highest temperature and decreased by 0.5°C each PCR cycle.

PCR amplification with these primer pairs was conducted as follows, using Veriti thermal cyclers (Applied Biosystems, Grand Island, New York, USA). Each PCR amplification included approximately 50 ng genomic DNA, 2.5 mM Mg^{2+}, 0.5 mM forward and reverse primers, 0.2 mM dNTPs, and 1 unit *Taq* polymerase (TaKaRa Biotechnology Co., Dalian, Liaoning, China) in a final volume of 20 µL. Gradient PCRs were performed as follows: 95°C for 5 min; 30 cycles of 95°C for 30 s, 49–57°C for 45 s, and 72°C for 45 s; and a final extension at 72°C for 7 min. The PCR products of another eight individuals were run on 1.5% agarose

TABLE 2. Genetic diversity in three *Aconitum kusnezoffii* populations[a] based on the 13 newly developed polymorphic microsatellite loci.

	DLS (n = 33)			NLY (n = 24)			WMG (n = 24)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
Ak1	4	0.222	0.330	4	0.125	0.498	4	0.044	0.125
Ak2	6	0.444	0.667	5	0.500	0.639	4	0.417	0.554
Ak3	3	0.261	0.584	4	0.286	0.505	4	0.609	0.660
Ak4	2	0.483	0.373	2	0.500	0.383	2	0.583	0.422
Ak5	2	0.433	0.481	2	0.417	0.422	2	0.500	0.507
Ak6	8	0.235	0.802	7	0.125	0.813	8	0.333	0.784
Ak7	3	0.035	0.341	2	0.250	0.223	2	0.083	0.082
Ak8	2	0.750	0.476	2	0.583	0.479	2	0.333	0.337
Ak9	2	0.107	0.223	3	0.083	0.231	3	0.381	0.508
Ak10	2	0.897	0.508	2	0.478	0.476	2	0.522	0.510
Ak11	2	0.368	0.462	2	0.353	0.513	3	0.191	0.441
Ak12	2	0.800	0.488	2	0.792	0.489	2	0.667	0.479
Ak13	3	0.625	0.446	3	0.542	0.414	4	0.667	0.520

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.
[a]Voucher and locality information are provided in Appendix 1.

TABLE 3. Genetic properties of the microsatellite loci developed for *Aconitum kusnezoffii* in single populations[a] of *A. barbatum* var. *puberulum* and *A. alboviolaceum.*

	A. barbatum var. *puberulum* (n = 24)		*A. alboviolaceum* (n = 24)	
Locus	*A*	Allele size range (bp)	*A*	Allele size range (bp)
Ak2	2	320–340	—	—
Ak3	2	350–390	1	379
Ak4	—	—	2	340–390
Ak6	2	160–220	—	—
Ak7	1	282	2	280–310
Ak9	2	345–370	2	345–370
Ak10	—	—	2	300–320
Ak11	4	360–380	2	200–210
Ak12	—	—	2	300–320

Note: — = not amplified; *A* = number of alleles; n = number of individuals sampled.

[a]Voucher and locality information are provided in Appendix 1.

gels. Nineteen primer pairs revealed unambiguously observable fragments in the expected size range. Forward primers for the 19 successfully amplified loci were labeled with FAM and used for amplifications with the same protocol. The loci Ak1 and Ak7 were amplified using the following touchdown protocol: 95°C for 5 min; 30 cycles of 95°C for 30 s, 60°C with a decrease of 0.5°C/cycle for 45 s, and 72°C for 45 s; and a final extension at 72°C for 7 min (Table 1). The labeled PCR products were analyzed on an Applied Biosystems 3730 Genetic Analyzer with a GeneScan 500 LIZ Size Standard (Applied Biosystems), and the genotyping was scored using GeneMapper version 3.2 (Applied Biosystems).

All 19 successfully amplified microsatellite loci were used to estimate the genetic diversities in three populations of *A. kusnezoffii*. GENEPOP version 4.2 (Rousset, 2008) was used to calculate the number of alleles per locus and the observed and expected heterozygosity. Among the 19 loci, there were six monomorphic loci and 13 polymorphic loci in all three populations. The number of alleles per locus ranged from two to eight, with allele sizes ranging from 140 to 477 bp. Observed heterozygosity was 0.491 ± 0.251 (mean ± SD), ranging from 0.035–0.897 in the DLS population; 0.387 ± 0.208, ranging from 0.083–0.792 in the NLY population; and 0.410 ± 0.209, ranging from 0.044–0.667 in the WMG population. The expected heterozygosities were 0.475 ± 0.150, 0.468 ± 0.153, and 0.456 ± 0.191 in the DLS, NLY, and WMG populations, respectively (Table 2). Cross-amplifications demonstrated that the PCR products showed fragments of the expected size in six and seven microsatellite loci in *A. barbatum* var. *puberulum* and *A. alboviolaceum*, respectively (Table 3). Five of the six amplified loci in *A. barbatum* var. *puberulum* were polymorphic, with two to four alleles per locus, whereas six of the seven amplified loci in *A. alboviolaceum* were polymorphic, with two alleles per locus.

CONCLUSIONS

A total of 57 alleles were identified for 13 polymorphic loci in three *A. kusnezoffii* populations. This set of microsatellite loci will be a valuable tool for future studies on estimating reproductive success and evaluating the effects of clonality on sexual reproduction.

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APPENDIX 1. Locality and voucher information for species sampled in this study. Voucher specimens were deposited in the herbarium of Beijing Normal University (BNU), Beijing, China.

Species	Population[a]	Geographic coordinates	n	Voucher no.
Aconitum kusnezoffii Rchb.	DLS	39°57′37″N, 115°27′30″E	33	DLSAcok120808
	NLY	39°58′5″N, 115°25′48″E	24	NLYAcok130603
	WMG	39°58′7″N, 115°25′41″E	24	WMGAcok130604
Aconitum barbatum Pers. var. *puberulum* Ledeb.	NG	39°57′49″N, 115°26′11″E	24	NGAcob120813
Aconitum alboviolaceum Kom.	DLS	39°57′37″N, 115°27′30″E	24	DLSAcoa150801

Note: n = number of individuals sampled.

[a]All populations are located near Xiaolongmen National Forest Park, Beijing, China.