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Microsatellite markers for *Dayaoshania cotinifolia* (Gesneriaceae), a critically endangered perennial herb[1]

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- *Premise of the study:* Microsatellite primers were developed for the critically endangered species *Dayaoshania cotinifolia* (Gesneriaceae) to evaluate population genetic diversity and detect population history.
- *Methods and Results:* In our study, 15 primer sets were developed using an enriched genomic library. These are the first microsatellite loci developed for this genus. Genetic diversity was measured using 40 individuals. The number of alleles per locus ranged from one to six, and the incidence of observed heterozygosities was 0.365 and 0.410 in two populations.
- *Conclusions:* The described markers will be useful in future population genetics studies of this critically endangered species.

Key words: *Dayaoshania cotinifolia*; microsatellite marker; population genetics.

Dayaoshania cotinifolia W. T. Wang is the sole representative of the monospecific genus *Dayaoshania* (Gesneriaceae) (Wang, 1983). It is a perennial herb endemic to southern China, where it has a very restricted distribution in the Dayaoshan Mountains. *Dayaoshania cotinifolia* is an older species in the Gesneriaceae family; thus, it is valuable in expanding our understanding of the evolutionary history of this family (Liu, 2003). In addition, it is a valuable wildflower resource (Li and Wang, 2004). Because of increasing anthropogenic disturbances, the number and size of *D. cotinifolia* populations have drastically decreased in past decades. At present, only two populations are known to exist, and a mere 1000 individuals have been found (Wang et al., 2008). It was listed among the "first class protected key wild plants of China" in 1999 and has been registered as a critically endangered species on the China Species Red List (Wang and Xie, 2004). Due to its endangered status, it is necessary to conduct a population genetics study of *D. cotinifolia* to improve approaches for its protection.

It is well known that simple sequence repeat (SSR) markers are powerful tools for population genetics investigations of wild species. Microsatellite markers are unique in their abundant and random distribution throughout the eukaryotic genome. Here we present the first report of the development and characterization of 10 microsatellite loci in *D. cotinifolia*. These data will facilitate the study of the genetic diversity and population history of this endangered species.

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METHODS AND RESULTS

Genomic DNA (30 µg) was extracted from silica gel–dried leaves using the CTAB method (Doyle, 1991) and digested with *Rsa*I and *XMn*I enzymes. The digested DNA was linked to forward (5′-GTTTAAGGCCTAGCTAGCAGAATC-3′) and reverse (5′-GATTCTGCTAGCTAGGCCTTAAACAAAA-3′) adapters using 2 U of T4 DNA ligase. After incubation for 14 h at 18°C, the fragments were separated using 2% agarose gel electrophoresis. DNA fragments with lengths between 500 and 800 base pairs (bp) were recovered using the QIAquick Gel Extraction kit (QIAGEN, Shanghai, China). Individual fragments were hybridized with three kinds of biotin-labeled probes (New England Biolabs, Beijing, China): $(AC)_8$, $(AG)_{18}$, and $(ATG)_{12}$. Fragments were then recovered using streptavidin-coated magnetic beads. After cloning and solid culturing, we screened the positive clones by PCR amplification using M13F and M13R primers. Finally, 81 positive clones were selected and sequenced on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, California, USA); 64 of these clones (about 79%) contained SSRs.

PCR primers were designed for 38 sequences using the program Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA). These primers were tested for polymorphism in 10 *D. cotinifolia* individuals, which were drawn from two populations. PCR was performed in 15 µL of a solution containing approximately 75 ng of genomic DNA, 10 µM of each primer, and 1× PCR Mix (Tiangen Biotech, Beijing, China). Microsatellites were amplified under the following conditions: 5 min initial denaturation at 95°C, 36 cycles of 30 s at 94°C, 30 s at 48–64°C (Table 1), 1 min at 72°C, and a final extension at 72°C for 10 min. The products were checked on 2.0% agarose gels. Fifteen pairs of primers showed clear bands (one or two bands per individual) (Table 1). Finally, 10 primer pairs (Table 2) were selected, and the forward or reverse primer was labeled with one of the fluorescent dyes (FAM or HEX) to detect polymorphism.

Individual loci were assessed in 40 plant samples collected from two populations, Dumuqiao ($N = 20$) and Qingnianqu ($N = 20$). Voucher specimens of *D. cotinifolia* populations could not be included because collecting permits did not authorize collection of voucher specimens. In each population, the number of alleles per locus (N_a), the observed and expected heterozygosity (H_o and H_e), and the deviation from Hardy–Weinberg equilibrium (HWE) were analyzed using the software package Arlequin ver. 3.1 (Excoffier et al., 2005). Ten primers were successfully amplified for all samples drawn from the two populations. All primers produced one or two bands for each individual, which was consistent with expectations for a diploid species. The number of alleles per locus ranged from one to six, with a mean of 2.2 in the Dumuqiao population and 2.5

TABLE 1. Characteristics of 15 microsatellite loci in *Dayaoshania cotinifolia*. Information on each primer pair includes forward and reverse sequences, repeat motif, size range of the original fragment (bp), annealing temperature (T_a), and GenBank accession number.

Locus	Primer sequence (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	GenBank Accession No.
JT1	F: CCTGAAGATTAAGAACA	$(TA)_8(TG)_9$	254	51	JF830807
	R: TACCTGAAAGTATGGAA				
JT3	F: CAAACAGCAATCCAGCA	$(AG)_{14}$	424–446	60	HQ916335
	R: ATTCCCCCAAATCAGCG				
JT12	F: GCAGAAATGACTATAACAAGG	$(TG)_9$	128	60	HQ916336
	R: TAACAGCACAAGACACAGGAG				
JT14	F: CCAGTCCAACCAGTGAT	$(TC)_{21}$	279–288	52	HQ916337
	R: AGGGGTCTGAAGAAAAT				
JT15	F: GTGGCTCTATTCAGATGGC	$(TC)_{25}$	137	55	JF830808
	R: TTCTTCAACAATGTGGGAT				
JT16	F: ACTGTGTTGGCTGATTC	$(TG)_{29}$	331	50	JF830809
	R: TGTTATGGTGAGGTTGG				
JT17	F: GGAGCCTGGACACCTATGATAC	$(TG)_{11}$	365–367	58	HQ916338
	R: ACTGTGGGAGGAACTTTGATGA				
JT21	F: GCAACACTATCTTCACTCA	$(TG)_{12}(AG)_9$	335–343	53	HQ916339
	R: CTTTTTATACGATCATCTC				
JT25	F: TGCCATCGCATTTATTG	$(TA)_5(TG)_{18}$	323	52	JF830810
	R: CGGTTGAGCAGCTTTTT				
JT26	F: GGAACTATCACAAGGAG	$(CA)_{19}(GA)_6$	379–403	48	HQ916340
	R: TAAGGTTTTATCGCATT				
JT28	F: AGTATGACTGACCGACC	$(TG)_{11}$	242	50	HQ916341
	R: CAACTGAAAACCAAGAG				
JT29	F: TGGCCTAGTCAAGTCCT	$(TG)_{11}(AG)_4$	319–322	53	HQ916342
	R: CCAGTTTCTTTCCTCCT				
JT34	F: CTTCAACACTGCCTTAG	$(GAT)_{10}$	183–186	52	HQ916343
	R: ATACATACCATCAACAAC				
JT36	F: AATCCTACTAATCGCAC	$(GAT)_8$	320–326	50	HQ916344
	R: CACCAATACTTCTCCAAC				
JT37	F: CCGCACCTCACAGTCAC	$(GT)_9(GA)_4$	298	56	JF830806
	R: TCAATCAGTCAGCCAACC				

TABLE 2. Results of initial primer screening in *Dayaoshania cotinifolia*. For each primer pair, the number of alleles (N_a) and average observed (H_o) and expected (H_e) heterozygosity are reported. Sample size within each population (N) is indicated in parentheses.

Locus	N_a	H_o	H_e
Dumuqiao (N = 20)			
JT3	5	0.400	0.355
JT12	1	0.000	0.000
JT14	2	0.550	0.409
JT17	2	0.500	0.385
JT21	1	0.000	0.000
JT26	5	0.300**	0.673
JT28	1	0.000	0.000
JT29	2	0.900**	0.508
JT34	2	1.000**	0.513
JT36	1	0.000	0.000
Average	2.2	0.365	0.284
Qingnianqu (N = 20)			
JT3	6	0.500	0.590
JT12	1	0.000	0.000
JT14	2	0.500	0.385
JT17	2	0.600	0.431
JT21	2	0.150	0.142
JT26	4	0.400*	0.650
JT28	1	0.000	0.000
JT29	4	0.950**	0.671
JT34	2	1.000**	0.513
JT36	1	0.000	0.000
Average	2.5	0.410	0.338

Deviations from Hardy–Weinberg: *P < 0.01, **P < 0.001.

in the Qingnianqu population (Table 2). The markers JT12, JT28, and JT36 were shown to be monomorphic in both populations; the marker JT21 was monomorphic only in the Dumuqiao population. The microsatellite loci JT3 and JT26 were the most polymorphic, with four to six alleles in both populations. The estimated average heterozygosity value of the microsatellite locus was relatively low; values of 0.365 (H_o) and 0.284 (H_e) were found in the Dumuqiao population and 0.410 (H_o) and 0.338 (H_e) in the Qingnianqu population. Low genetic diversity in *D. cotinifolia* could be related to events in its population history, such as a bottleneck effect. Three loci (JT26, JT29, and JT34) showed significant deviation from HWE (P < 0.05) in both populations. These data suggest good genetic potential for conservation in situ of *D. cotinifolia*. These are the first microsatellite loci described for this genus.

CONCLUSIONS

The novel microsatellite markers developed in this work are suitable for future population genetics studies of *D. cotinifolia*. Such studies could include those focused on genetic diversity, mating systems, and population history. These data will be helpful in developing conservation strategies to aid this critically endangered species.

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