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Development and characterization of polymorphic microsatellite markers in *Dysosma pleiantha* (Berberidaceae)

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- *Premise of the study:* The development of compound microsatellite markers was conducted in *Dysosma pleiantha* to investigate genetic diversity and population genetic structure of this threatened medicinal plant.
- *Methods and Results:* Using the compound microsatellite marker technique, 14 microsatellite markers that were successfully amplified showed polymorphism when tested on 38 individuals from three populations in eastern China. Overall, the number of alleles per locus ranged from 2 to 14, with an average of 7.71 alleles per locus.
- *Conclusions:* These results indicate that these microsatellite markers are adequate for detecting and characterizing population genetic structure and genetic diversity in *Dysosma pleiantha*.

Key words: *Dysosma pleiantha*; genetic diversity; microsatellite markers; threatened medicinal plant.

The genus *Dysosma* Woodson (Berberidaceae) consists of seven herbaceous perennial species, all of which are endemic to China. *Dysosma pleiantha* (Hance) Woodson, an important threatened medicinal plant (TMP) species, is restricted in distribution to southeastern China and the island of Taiwan (Ying et al., 1993). In recent years, the species has been subject to a rapid demographic decline. As to its overall conservation status, *D. pleiantha* has been ranked as "threatened" on the China Species Red List (Wang and Xie, 2004). In a previous study, ISSR markers were used to assess clonal and genetic diversity, as well as population genetic structure for *D. pleiantha* (Zong et al., 2008). However, the dominant nature of ISSR markers can lead to an underestimation of the recessive allele frequency in a population, causing a bias in the estimates of genetic diversity and genetic differentiation (Nybom, 2004). Therefore, more polymorphic codominant markers are required to be developed. Microsatellites (simple sequence repeats, SSRs), due to the advantages of high variability, codominance, and ubiquity in eukaryotic genomes, have become a useful molecular marker in population genetic analyses (Walter and Epperson, 2001), which have not been well developed in *D. pleiantha*. Here, we identified 14 polymorphic compound microsatellite markers from the genome of *D. pleiantha* using a recently developed isolation technique (Lian et al., 2006) to further investigate spatial genetic structure, interpopulation diversity, mating system, and gene flow of *D. pleiantha*, which will provide additional insight and help to develop strategies for the conservation and management of this medicinally important, severely endangered, endemic species.

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METHODS AND RESULTS

An improved technique for isolation of codominant compound microsatellite markers (Lian et al., 2006) was used to develop SSR markers for *D. pleiantha*. An adaptor-ligated DNA library was constructed according to the protocol of Lian et al. (2001). Briefly, total genomic DNA extracted from silica-gel-dried leaf material was digested with a blunt-end restriction enzyme, *Ssp*I (Takara, Dalian, Liaoning, China), and the restricted fragments were ligated to an unequal-length adaptor, using the DNA Ligation Kit Version 2.0 (Takara). Then, fragments flanked by a microsatellite at one end were amplified from the *Ssp*I DNA library using the compound SSR primer $(AC)_6(AG)_5$ or $(TC)_6(AC)_5$ and an adaptor primer AP_2 (5′-CTATAGGGCACGCGTGGT-3′). Fragments amplified from the *Ssp*I DNA library were purified and ligated into a pGEM-T vector (Promega, USA) and transformed into JM109 competent cells (Takara). Three hundred and thirty-one clones were analyzed using M13 primers to amplify the complete microsatellite-containing insert. A total of 160 positive clones were obtained and sequenced on an ABI Prism 3730 automated DNA sequencer (Applied Biosystems, Foster City, California, USA). One hundred twenty-one sequences were found to contain $(AC)_6(AG)_n$ or $(TC)_6(AC)_n$ compound SSR motifs. Only 59 sequences containing $(AC)_6$(AG)n or $(TC)_6(AC)_n$ compound SSR sequences at one end were suitable for designing primers, and a specific primer (IP1) was designed using PRIMER version 5.0 (Clarke and Gorley, 2001) from these sequences flanking the compound SSR. The primer pairs of IP1 and compound SSR primer were used as a compound SSR marker (Table 1). Polymerase chain reactions (PCR) were performed in 10-µl reaction volumes containing 60 ng/µl of template DNA, 0.25 U of *Taq* polymerase (Takara), 1 µl of 10× PCR buffer, 0.5 µl of $MgCl_2$ (2.5 mM), 1 µl of dNTPs (2.5 mM each), 0.5 µl of each primer (10 µM) and 0.05 µl bovine serum albumin (BSA) (Takara). PCR amplification conditions were as follows: initial denaturation at 95°C for 5 min; 35 cycles of 30 s of denaturation at 95°C, 45 s of annealing at the optimized annealing temperature (Table 1), 90 s of elongation at 72°C; and final extension at 72°C for 10 min. PCR products were resolved on a 6% polyacrylamide denaturing gel using a 50-bp ladder (Takara) as the reference and visualized by silver staining. After excluding those that did not amplify or yielded nonspecific amplification products, 14 primer pairs were chosen to test for polymorphism. The compound primers were labeled with a fluorescent dye (5′HEX or 5′6-FAM). PCR amplification followed the above. Fragment analysis was

TABLE 1. Characteristics of 14 compound microsatellite loci developed for *Dysosma pleiantha*. Shown for each locus are the locus name, the forward (F) and reverse (R) primer sequence, the optimized annealing temperature (*Ta*), allele size ranges, the total number of alleles per locus (*Na*), and the GenBank accession number. Size ranges and the total number of alleles include all values detected within three *Dysosma pleiantha* populations used in this study (see Table 2).

Locus	Repeat	Primer sequence (5′–3′)	Size range (bp)	*Ta* (°C)	*Na*	GenBank Accession No.
Dp1	$(AC)_6(AG)_{15}$	F: ACACACACACAGAGAGAGAG	190	55	10	HQ871674
		R: GACTATGTTTGGTTTCAGGGA	172–198			
Dp2	$(AC)_6(AG)_5$	F: ACACACACACAGAGAGAGAG	281	54	9	HQ871675
		R: AGAAGACAGGGAGCACAACA	279–311			
Dp3	$(AC)_6(AG)_7GGGGAT(AG)_8$	F: ACACACACACAGAGAGAGAG	163	55	14	HQ871676
		R: TCAGCCACCAATAGGACTT	127–187			
Dp4	$(AC)_6(AG)_5$	F: ACACACACACAGAGAGAGAG	176	55	2	HQ871677
		R: TTCCAACAGGGGATAACAAT	176–194			
Dp5	$(AC)_6(AG)_7$	F: ACACACACACAGAGAGAGAG	202	55	7	HQ871687
		R: GGGCTGGTATCCATTTTC	198–242			
Dp6	$(AC)_6(AG)_5$	F: ACACACACACAGAGAGAGAG	149	54	4	HQ871679
		R: ACAAAGGAAACGAACCTAACA	147–161			
Dp7	$(AC)_6(AG)_8$	F: ACACACACACAGAGAGAGAG	189	55	7	HQ871680
		R: TTAGGGCACTTCCTGATTGT	185–209			
Dp8	$(AC)_6(AG)_8$	F: ACACACACACAGAGAGAGAG	192	55	7	HQ871681
		R: CATTTACCATTCACCTTCCAT	190–222			
Dp9	$(AC)_6(AG)_{11}$	F: ACACACACACAGAGAGAGAG	135	55	10	HQ871683
		R: AAACAGCATCACATCTTCCTC	127–161			
Dp10	$(AC)_6(AG)_{12}$	F: ACACACACACAGAGAGAGAG	261	57	12	HQ871684
		R: GTGCGGATTTGTCTACTGATG	249–303			
Dp11	$(AC)_6(AG)_9$	F: ACACACACACAGAGAGAGAG	313	55	5	HQ871685
		R: GATGCGTATCCAACTAACAGG	311–353			
Dp12	$(AC)_6(AG)_{11}$	F: ACACACACACAGAGAGAGAG	183	55	4	HQ871686
		R: ATTGGCTTTACCCGATAGAA	179–213			
Dp13	$(TC)_6(AC)_{21}$	F: TCTCTCTCTCTCACACACACAC	181	55	12	HQ871678
		R: ACATGCGTATGACTACCACTTC	163–199			
Dp14	$(TC)_6(AC)_{19}$	F: TCTCTCTCTCTCACACACACAC	125	55	5	HQ871682
		R: TCGGAGGAAACTTCTCACC	115–147			

performed on a MegaBACE 1000 autosequencer (GE Healthcare Biosciences), and the data were scored and compiled using Genetic Profiler version 2.2 (GE Healthcare Biosciences).

Thirty-eight individuals of *D. pleiantha* collected from Tianmu Mountain (30°19′04″N, 119°26′50″E), Daiyun Mountain (25°38′34″N, 118°11′19″E) and Wuyanling Mountain (27°40′29″N, 119°40′37″E) were used to test the polymorphism of the microsatellite primers. Voucher specimens for the sampled populations are stored at the Herbarium of Nanchang University (JXU) (see Appendix 1). The number of alleles (*Na*), observed (H_o) and expected (H_E) heterozygosities, linkage disequilibrium (LD), and deviations from Hardy–Weinberg equilibrium (HWE) were analyzed using GENEPOP version 4.0.7 (Rousset, 2008). CERVUS version 3.0.3 (Kalinowski et al., 2007) was employed to calculate the value of polymorphic information content (PIC).

All 14 primer pairs displayed polymorphism for *D. pleiantha*. The mean number of alleles per locus (*Na*) was 6.5 (range: 2–10), 6.5 (range: 2–10), and 7.0 (range: 2–11) for populations in Tianmu Mountain, Daiyun Mountain, and Wuyanling Mountain, respectively (Table 2). On average, the observed heterozygosities (H_o) were 0.687 (range: 0.538–0.846), 0.696 (range: 0.500–0.917), and 0.681 (range: 0.462–0.923), respectively (Table 2). The expected heterozygosities (H_E) were 0.810 (range: 0.471–0.923), 0.811 (range: 0.522–0.902), and 0.816 (range: 0.492–0.926), respectively (Table 2). The PIC's were 0.745 (range: 0.350–0.877), 0.742 (range: 0.375–0.849), and 0.752 (range: 0.361–0.880),

TABLE 2. Results of initial primer screening in three populations of *Dysosma pleiantha*. Shown are locus name, the number of alleles per locus (*Na*), mean values of observed (H_o) and expected (H_E) heterozygosity, and polymorphism information content (PIC). The sample size for each population is shown in parentheses.

	Population TM(13)				Population DY(12)				Population WYL(13)			
Locus	*Na*	H_o	H_E	PIC	*Na*	H_o	H_E	PIC	*Na*	H_o	H_E	PIC
Dp1**	8	0.692	0.883	0.831	8	0.667	0.873	0.817	9	0.692	0.849	0.798
Dp2 n.s	7	0.846	0.877	0.823	8	0.917	0.902	0.849	9	0.538	0.877	0.824
Dp3***	10	0.692	0.898	0.849	9	0.833	0.902	0.849	11	0.769	0.926	0.880
Dp4 n.s	2	0.538	0.471	0.350	2	0.500	0.522	0.375	2	0.462	0.492	0.361
Dp5**	7	0.692	0.846	0.788	7	0.667	0.848	0.787	7	0.692	0.834	0.775
Dp6 n.s	4	0.615	0.751	0.673	4	0.750	0.728	0.645	4	0.769	0.748	0.669
Dp7**	6	0.615	0.837	0.776	7	0.833	0.855	0.797	7	0.615	0.843	0.786
Dp8**	7	0.692	0.871	0.816	6	0.583	0.804	0.740	7	0.692	0.862	0.805
Dp9***	7	0.692	0.849	0.792	9	0.667	0.899	0.846	9	0.615	0.877	0.824
Dp10**	10	0.846	0.923	0.877	10	0.583	0.891	0.838	10	0.769	0.889	0.839
Dp11*	5	0.692	0.778	0.711	5	0.583	0.786	0.716	5	0.692	0.815	0.750
Dp12*	4	0.538	0.717	0.635	4	0.667	0.757	0.674	4	0.692	0.711	0.633
Dp13***	10	0.769	0.895	0.846	7	0.750	0.833	0.770	9	0.615	0.892	0.842
Dp14 n.s	4	0.692	0.748	0.666	5	0.750	0.754	0.679	5	0.923	0.809	0.743

Notes: *, ** and ***, significant departures from Hardy–Weinberg equilibrium at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively. n.s. = not significant.

respectively (Table 2). Significant heterozygote deficiencies ($P < 0.05$) were detected in 10 loci (Table 2), which may result from the excess of homozygotes. Significant linkage disequilibrium (LD) was not detected between any pair of loci. Microsatellite loci were all identified and their respective sequences were deposited in GenBank (accession nos. HQ871674–HQ871687). Details about the 14 microsatellite loci and their variability across the 38 individuals were summarized in Table 1. These 14 polymorphic loci should be useful for conducting population genetic studies of *D. pleiantha*.

CONCLUSIONS

Our pilot data suggest that these microsatellite markers are adequate for detecting and characterizing population genetic structure and genetic diversity in *D. pleiantha*. We are currently using these microsatellite primers together with chloroplast DNA markers to assess patterns of geographical molecular variation in *D. pleiantha* at the population level and across the species' ranges in southeastern China. Furthermore, we anticipate that the polymorphic microsatellite loci will be helpful in future studies of genetic variation and population ecology in other six congenerics.

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APPENDIX 1. Information on representative voucher specimens deposited at the Herbarium of Nanchang University (JXU).

Taxon*—*Voucher specimens, Locality in southeast China.

Dysosma pleiantha (Hance) Woodson–*B.C. Guan 100429–100430*, Mt. Tianmu, Zhejiang; *B.C. Guan 100509–090510*, Mt. Wuyanling, Zhejiang; *B.C. Guan 100612–100613*, Mt. Daiyun, Fuzhou.