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Isolation and Characterization of 21 Microsatellite Loci in *Cardiocrinum giganteum* var. *yunnanense* (Liliaceae), an Important Economic Plant in China

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Abstract: Twenty-one microsatellite markers from the genome of *Cardiocrinum giganteum* var. *yunnanense*, an important economic plant in China, were developed with a fast isolation protocol by amplified fragment length polymorphism of sequences containing repeats (FIASCO). Polymorphism within each locus was assessed in 24 wild individuals from Gaoligong Mountains in western Yunnan Province, China. The number of alleles per locus ranged from 2 to 4 with a mean of 2.9. The expected and observed levels of heterozygosity ranged from 0.042 to 0.726 and from 0.000 to 1.000, with averages of 0.44 and 0.31, respectively. These polymorphic microsatellite markers should prove useful in population genetics studies and assessments of genetic variation to develop conservation and management strategies for this species.

Keywords: *Cardiocrinum giganteum* var. *yunnanense*; microsatellite markers; polymorphism; population genetics; Liliaceae

1. Introduction

The herbaceous perennial genus *Cardiocrinum* (Endlicher) Lindley is a member of the lily family (Liliaceae) and contains three species and one variety, namely *Cardiocrinum cathayanum* (E. H. Wilson) Stearn, *C. cordatum* (Thunb.) Makino, *C. giganteum* (Wall.) Makino, and *C. giganteum* var. *yunnanense* (Leichtlin ex Elwes) Stearn [1]. All the species are characterized by ovate-cordate leaves with reticulate veins, and terminal racemes with many large flowers [2].

Cardiocrinum giganteum var. *yunnanense* is a perennial herb and mainly distributed in Gansu, Guangdong, Guangxi, Guizhou, Henan, Hubei, Hunan, Shaanxi, Sichuan, and Yunnan provinces of China and adjacent regions of Myanmar [3]. It grows in forests at altitudes ranging from 1200 to 3600 m elevation [3]. With its attractive flowers (Figure 1), this species has attracted the attention of botanists and horticulturalists, who have taken an interest in the commercial development of this species as an ornamental resource [4]. It is an important economic plant in China. The seeds are used as a replacement for *Aristolochia* fruits to treat cough [5], and bulbs are used as a starch staple by the local people in Guangxi and Yunnan [6]. Its economic attributes have made *Cardiocrinum giganteum* var. *yunnanense* at risk of overexploitation of natural populations. Moreover, its habitat has been badly degraded and fragmented due to heavy logging and forest destruction in past decades, which has reduced the distribution of this species to a fragmented range with small populations [7].

Figure 1. Inflorescence of *Cardiocrinum giganteum* var. *yunnanense*, showing the attractive flowers.



To provide effective conservation and management strategies for this important economic plant, it is necessary to understand the spatial genetic structure, genetic diversity, and levels of gene flow

within and among its populations. However, in the genus *Cardiocrinum*, except 13 microsatellite loci were developed from the genome of *C. cordatum* [8], no nuclear microsatellite primers or other types of markers have been reported for *C. giganteum* var. *yunnanense*. Microsatellites show numerous advantages over other fingerprinting methods such as RAPD, ALFP, and ISSR because they are locus-specific, codominant, highly reproducible, and usually highly polymorphic [9]. Hence, we have developed and characterized 21 microsatellite markers for *Cardiocrinum giganteum* var. *yunnanense*, which will facilitate further investigations on the genetic diversity, population structure, and gene flow of this species.

2. Results and Discussion

In total, 273 positive clones were sequenced. A total of 223 (82%) sequences were found to contain simple sequence repeats (SSRs); 114 of these with appropriate microsatellite and sufficient flanking regions were selected to design locus-specific primers. Polymorphisms of all 114 microsatellite loci were assessed in 24 wild individuals. Of these primers, 30 successfully amplified the target regions, and 21 of them displayed polymorphisms and 9 showed monomorphism (Table 1). The number of alleles per locus ranged from 2 to 4, with a mean of 2.9. The expected (H_E) and observed (H_O) heterozygosities ranged from 0.042 to 0.726 and from 0.000 to 1.000, with average of 0.44 and 0.31, respectively (Table 2). Among 21 microsatellite markers, 16 loci showed significant deviation from Hardy-Weinberg equilibrium (HWE) ($P < 0.01$) (Table 2), probably due to heterozygote deficiency or the limitation of sample size, also because of the presence of null alleles. Twenty-eight loci pairwise (13.3%) showed significant genotypic linkage disequilibrium (LD) between pairs of loci ($P < 0.001$).

Table 1. Characteristics of 30 microsatellite loci in *Cardiocrinum giganteum* var. *yunnanense*.

Locus	Primer sequences (5'-3')	Repeat motif	Size range (bp)	T_a (°C)	GenBank Accession No.
CGY003 *	F: TATGGAGGGTTCTATTGCC R: GGTTCAGAGTTCATTGGAT	(AG) ₉	214–223	52	JQ340036
CGY005	F: AAAGGAGGGGAAGGCATAGT R: CTAAGAGACCGCTCCTCAT	(AG) ₅ AT(AG) ₅	257	55	JQ340037
CGY006 *	F: TGGTATTGTCAGAATCTCAT R: AGGTTGTTGGGTGGAGTAGT	(TG) ₁₀ (AG) ₁₃	169–182	50	JQ340038
CGY007 *	F: TGTGTGAGTTTGAGCATAAT R: CGGATACTCAGTGAACCTAC	(AG) ₁₅	130–145	52	JQ340039
CGY011 *	F: ACAATAACCCCAGTAGACC R: TGGGTGCTCATCAAGTGTC	(AG) ₁₄	164–168	55	JQ340040
CGY012 *	F: CGAACTGAACATTGAGAAGA R: ATTACACTCTGACAACACCG	(AG) ₁₅	119–127	50	JQ340041
CGY015	F: TCTCAAGTAAATCCAACAAAT R: AAGGTATTGGAATGGCGAT	(AG) ₁₆	156	50	JQ340042
CGY028 *	F: ATGAGAAAGAGAGATGAAAG R: TAAAGTGTGTGTAGGTGGAG	(AG) ₁₃	147–153	48	JQ340043

Table 1. Cont.

CGY029 *	F: TTCATTATCATCTCGGACAC R: AGAAGGTCAACCGAACACAT	(TG) ₇ (AG) ₁₄	157–165	48	JQ340044
CGY031 *	F: ACTCCTCTACCCCTTTCACCA R: CATGATATTTATACTGAGGTTCT	(AG) ₉	163–173	48	JQ340045
CGY035	F: AACAAAAGAAAAGCAGTAGAA R: TATGATAGAGCAAAAAGAGGG	(TC) ₁₁	184	48	JQ340046
CGY036 *	F: TATCGCCTTCTTACACTTA R: TGAGCCGATTCTTACATTTT	(AG) ₁₈	178–182	50	JQ340047
CGY037	F: TCCAAGAGAGAAAGCATCAA R: ATGGCAGAATCACAATAAGT	(TC) ₉	144	50	JQ340048
CGY043 *	F: TTTCAGCCACCCTCACTATT R: CTCCTATTTTACAAGACGC	(AG) ₈	180–186	50	JQ340049
CGY053 *	F: TGCCAGAAAAGAATCACAA R: ATGACCCTTCCTAATTCG	(AG) ₁₁	144–152	50	JQ340050
CGY054 *	F: ACCCAAATAAAGTAACAGACCA R: TGCCCCATCACATCCCCACC	(TC) ₁₆	197–207	57	JQ340051
CGY058 *	F: GTAGTTTCCTTCATCGCCTT R: CCACACAGGGAGGCATCTTT	(TC) ₁₅	243–251	57	JQ340052
CGY064 *	F: TATTTCTTATTCTTCACCTC R: AAAACCAATAAAAATCCCTC	(AG) ₁₄	121–127	47	JQ340053
CGY065 *	F: CCGTTGGGATTATGAGTATT R: CAGCATAGAGCATAGCCCTT	(TC) ₁₉	167–175	50	JQ340054
CGY066 *	F: TGGAGAGATTTCAGGTTTATA R: GAGACCATAACATCACTAAATCA	(AG) ₁₄	218–230	52	JQ340055
CGY067	F: GTGACCTTAGGAGTATATTAGC R: CGGAAATGGCTACTAACTAAGA	(AG) ₁₀	237	55	JQ340056
CGY072	F: AGATGAAGGAGTAGGGACAA R: CAAACTCCCCTCACCATTTC	(TC) ₈	305	55	JQ340057
CGY073 *	F: GTCTCCCTCCTTCTCAAAAT R: CTTCTTGCCCCCACTAACTT	(TC) ₇	250–258	55	JQ340058
CGY075 *	F: GCCATAGAGACATAGGGAGG R: ATGAAACCTGACCTAAGCAC	(AG) ₁₉	213–221	55	JQ340059
CGY083 *	F: CCTACTCATTTTTCAACTTTC R: GCCCATTCCTCAACCACTATT	(TG) ₁₃	290–298	52	JQ340060
CGY091 *	F: TGGACACATTTTTGGCTAAG R: CGACGATTAGGGCAAAGGTA	(AC) ₅ AT(AC) ₁₁	120–132	50	JQ340061
CGY099	F: TCATTCCACTCCACCATAAA R: ATACCTAACCATCTTCCAAT	(AC) ₁₂	119	50	JQ340062
CGY105	F: CCCAAAAATAATCATCAAGC R: CACCTACCCTGCTTTGTTCA	(AC) ₇	152	52	JQ340063
CGY110 *	F: ATAGTGTAGCAGTGAAGCGA R: TGTGGTTGGTTTCTCATTGC	(TG) ₇	115–119	57	JQ340064
CGY111	F: TGACACCCCCATACTTAGAC R: TCTCATCACTCTATCTCATT	(TG) ₇	115	50	JQ340065

T_a : PCR annealing temperature; * displayed polymorphisms.

Table 2. Result of 21 polymorphic microsatellite loci screening in 24 wild individuals of *Cardiocrinum giganteum* var. *yunnanense*.

Locus	N_A	H_E	H_O	F_{is}		Locus	N_A	H_E	H_O	F_{is}	
				W&C	R&H					W&C	R&H
CGY003 *	2	0.042	0.042	—	—	CGY054 *	2	0.042	0.042	—	—
CGY006 *	3	0.434	0.042	0.906	0.493	CGY058 *	3	0.543	0.917	−0.715	−0.411
CGY007	4	0.611	0.875	−0.446	−0.243	CGY064 *	2	0.550	0.083	0.851	0.486
CGY011 *	4	0.621	0.292	0.536	0.370	CGY065 *	3	0.657	0.125	0.813	0.882
CGY012 *	4	0.726	0.458	0.374	0.439	CGY066 *	2	0.156	0	1.000	1.044
CGY028	4	0.482	0.292	0.401	0.357	CGY073 *	2	0.337	0	1.000	1.044
CGY029 *	3	0.635	0.083	0.871	0.928	CGY075 *	3	0.511	0	1.000	1.044
CGY031 *	2	0.350	0.083	0.766	0.847	CGY083 *	3	0.624	1	−0.624	−0.489
CGY036	3	0.254	0.125	0.514	0.531	CGY091 *	2	0.042	0.042	—	—
CGY043	3	0.669	0.583	0.131	0.155	CGY110	3	0.465	0.417	0.107	−0.068
CGY053 *	3	0.566	1	−0.795	−0.489						

N_A : number of alleles; H_E : expected heterozygosity; H_O : observed heterozygosity; F_{is} : estimates of inbreeding coefficient; W&C: Weir and Cockerham's method; R&H: Robertson and Hill's method; * statistically significant deviation from Hardy-Weinberg equilibrium (HWE) ($P < 0.01$).

3. Experimental Section

3.1. Isolation of Microsatellite Loci

Genomic DNA was extracted from silica-gel-dried leaves by following a CTAB method and the microsatellite loci were isolated by using the fast isolation by AFLP of sequences containing repeats (FIASCO) protocol [10,11]. Approximately 500 ng of total genomic DNA was digested with *MseI* enzyme (New England Biolabs, Beverly, MA, USA), and then fragments were ligated to the *MseI* AFLP adaptor pair (5'-TACTCAGGACTCAT-3'/5'-GACGATGAGTCCTGAG-3') at 37 °C for 2 h with T₄ DNA ligase (Fermentas, Burlington, ON, Canada).

A diluted digestion-ligation mixture (1:10) was amplified with the adaptor-specific primers *MseI*-N (5'-GATGAGTCCTGAGTAAN-3') by following the program: 95 °C for 3 min, 30 cycles of 94 °C for 30 s, 53 °C for 60 s, 72 °C for 60 s followed by an elongation step of 5 min at 72 °C. Amplified fragments with a size range of 200–800 bp were enriched for microsatellite repeats by magnetic bead selection with 5'-biotinylated (AC)₁₅, (AG)₁₅, and (AAG)₁₀ probes. Captured fragments were re-amplified with adaptor-specific primers. Polymerase chain reaction (PCR) products were purified by using EZNA Gel Extraction Kit (Omega Bio-Tek, Guangzhou, China).

The purified PCR products with enriched microsatellite repeats were ligated into the pGEM-T vector (Promega, USA), and transformed into DH5 α cells (TaKaRa, Dalian, China). Recombinant clones were screened by blue/white selection and the positive clones were tested by PCR with (AC)₁₀/(AG)₁₀/(AAG)₇ and T₇/Sp₆ primers. The clones with positive inserts were sequenced with an ABI PRISM 3730XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). The program Oligo 6.0 was used to design locus-specific primers for those microsatellite sequences found to contain sufficient flanking regions [12].

3.2. Detection of Polymorphism

Polymorphisms of microsatellite loci were evaluated in 24 wild individuals of *Cardiocrinum giganteum* var. *yunnanense* from Gaoligong Mountains (24°40'–28°30' N, 98°11.2'–98°47.5' E) in western Yunnan province. Polymerase chain reactions (PCR) were performed in 20 µL of reaction containing 30–50 ng genomic DNA, 0.6 µM of each primer, 7.5 µL 2× *Taq* PCR MasterMix [Tiangen (Tiangen, Beijing China); 0.1 U *Taq* Polymerase/µL, 0.5 mM dNTP each, 20 mM Tris-HCl (pH = 8.3), 100 mM KCl, 3 mM MgCl₂]. PCR amplifications were conducted under the following program: 95 °C for 3 min followed by 30–36 cycles at 94 °C for 30 s, with the annealing temperature optimized for each specific primer (Table 1), for 30 s, 72 °C for 60 s, and a final extension step at 72 °C for 7 min. The amplified fragments were separated on 6% polyacrylamide denaturing gels with a 20-bp ladder molecular size standard (Fermentas, Burlington, Ontario, Canada) by silver staining.

3.3. Data Analysis

Standard genetic diversity parameters of polymorphic loci, e.g., the number of alleles (N_A), and expected (H_E) and observed levels of heterozygosity. We also estimated deviations from Hardy-Weinberg equilibrium (HWE) and genotypic linkage disequilibrium (LD) between pairs of loci using Chi-square tests.

4. Conclusions

The 21 microsatellite markers developed in this study are the first set of such markers for *Cardiocrinum giganteum* var. *yunnanense*. They should prove useful for further investigating the spatial genetic structure, genetic diversity, and levels of gene flow within and among populations of this species, which will help to develop viable strategies for the conservation and management of this important economic plant.

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References

1. Mabberley, D.J. *Mabberley's Plant-book: A Portable Dictionary of Plants, Their Classification and Uses*; Cambridge University Press: New York, NY, USA, 2008; p. 151.
2. Wu, Z.Y.; Lu, A.M.; Tang, Y.C.; Chen, Z.D.; Li, D.Z. *The Families and Genera of Angiosperms in China, A Comprehensive Analysis*; Science Press: Beijing, China, 2003; p. 256.

3. Liang, S.; Tamura, M.N. *Cardiocrinum*. In *Flora of China*; Wu, Z.Y., Raven, P.H., Eds.; Science Press: Beijing, China; Missouri Botanical Garden Press: St. Louis, MO, USA, 2000; Volume 24, pp. 134–135.
4. Zhang, J.Z.; Long, Y.Y.; Sun, G.F. Biodiversity of *Cardiocrinum giganteum* and observation during its introduction. *Acta Hortic. Sin.* **2002**, *29*, 462–466.
5. Li, M.; Ling, K.H.; Lam, H.; Shaw, P.C.; Cheng, L.; Tehen, N.; Khan, L.A.; Chang, Y.S.; But, P.P. *Cardiocrinum* seeds as a replacement for *Aristolochia* fruits in treating cough. *J. Ethnopharmacol.* **2010**, *130*, 429–432.
6. Li, H. *Cardiocrinum*. In *Flora Yunnanica*; Wu, Z.Y., Ed.; Science Press: Beijing, China, 1997; Volume 7, pp. 787–789.
7. Li, R.; Dao, Z.L.; Li, H. Seed plant species diversity and conservation in the northern Gaoligong mountains in western Yunnan, China. *Mt. Res. Dev.* **2011**, *31*, 160–165.
8. Molecular Ecology Resources Primer Development Consortium. Permanent genetic resources added to molecular ecology resources database 1 August 2009–30 September 2009. *Mol. Ecol. Resour.* **2010**, *10*, 232–236.
9. Powell, W.; Morgante, M.; Andre, C.; Hanafey, M.; Vogel, J.; Tingey, S.; Rafalski, A. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Mol. Breed.* **1996**, *2*, 119–122.
10. Doyle, J.J.; Doyle, J.L. A rapid DNA isolation procedure for small quantities of leaf tissue. *Phytochem. Bull.* **1987**, *19*, 11–15.
11. Zane, L.; Bargelloni, L.; Patarnello, T. Strategies for microsatellite isolation: a review. *Mol. Ecol.* **2002**, *11*, 1–16.
12. Offerman, J.; Rychlik, W. Oligo Primer Analysis Software. In *Introduction to Bioinformatics: A Theoretical and Practical Approach*; Krawetz, S., Womble, D., Eds.; Humana Press: Totowa, NJ, USA, 2003; pp. 345–361.