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Isolation and characterization of microsatellite markers in an endangered species *Dracaena cambodiana* (Liliaceae)[1]

Lu Zhang[2,3] and Qiao-Ming Li[2,4]

[2]Laboratory of Plant Phylogenetics and Conservation, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming,Yunnan 650223, China; and Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun, Yunnan 666303, P.R. China; [3]The Graduate School of the Chinese Academy of Sciences, Beijing 100049, China

- *Premise of the study:* The development of microsatellite primers in the endangered species *Dracaena cambodiana* will be the foundation for genetic and conservation studies of *D. cambodiana* and several *Dracaena* species.
- *Methods and Results:* A total of 26 microsatellite markers were developed in Chinese populations of *D. cambodiana*, using the Fast Isolation by AFLP of Sequences Containing Repeats (FIASCO) protocol. Among them, sixteen primer pairs generated polymorphic loci (fourteen of them successfully amplified in other four *Dracaena* species) and ten primer pairs produced monomorphic loci.
- *Conclusions:* These microsatellite markers could be used in the further investigation of population genetics of *D. cambodiana* and other *Dracaena* species.

Key words: cross-species amplification; *Dracaena*; endangered species; microsatellite markers.

The genus *Dracaena* includes more than 50 species, which are mainly distributed in tropical and subtropical regions of Asia and Africa. *Dracaena cambodiana* is a narrow-distributed species, which is found in Yunnan, Guangxi, and Hainan provinces in China, Laos, Vietnam, Thailand and Cambodia (Chen and Turland, 2000). In 2001, *D. cambodiana* was listed as an endangered species due to its highly restricted distribution and overexploitation (Fu, 1992). Considering its medicinal, ornamental and ecological values, an appropriate conservation program is urgently needed to prevent further loss of *D. cambodiana*. Therefore, it is essential to develop molecular markers for the population genetic analysis of *D. cambodiana* to provide essential information for the development of a management and conservation strategy.

Due to the reproducibility, multiallelic nature, codominant inheritance, relative abundance and wide genome coverage, microsatellite markers have proven to be highly efficient molecular tools for population genetic studies. In the current study, we (1) developed microsatellite markers for *D. cambodiana* and assessed the polymorphism of these loci in 22 samples collected from 11 geographically distinct populations ($N = 2$ for each) distributed in Guangxi, Yunnan, and Hainan Provinces in China, and (2) performed cross-amplification tests in four other *Dracaena* species (*D. thalioides*, *D. reflexa*, *D. marginata*, and *D. gracilis*). Voucher specimens were deposited in Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences.

METHODS AND RESULTS

Total genomic DNA was extracted from leaf tissues of *D. cambodiana* using a modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). A dinucleotide-enriched microsatellite genomic library was constructed and screened using a fast isolation by AFLP of sequences containing repeats (FIASCO) protocol with some modifications (Zane et al., 2002). Approximately 250 ng of total genomic DNA was digested with *Mse* I (New England BioLabs, Beverly, MA), and then ligated to the adapters (5′ - TACTCAGGACTCAT - 3′/5′ - GACGATGAGTCCTGAG - 3′) using T4 DNA ligase (Fermentas, Burlington, Ontario, Canada) and diluted (1:10). Then 5 μL digestion-ligation DNA fragments were amplified with 1 μL *Mse* I-N primers (5′ - GATGAGTCCTGAGTAAN - 3′) (25 μM) in a 20 μL volume, using a thermal cycling program of initial denaturation of 3 min at 95°C, followed by 20 cycles of 30 s at 94°C, 1 min at 53°C, 1 min at 72°C, and a final extension at 72°C for 10 min. Approximately 500–1000 ng of amplified DNA was hybridized with 200 pmol of 5′- biotinylated $(AG)_{15}$ oligonucleotide in a total volume of 250 μL of SSC 4.2 × and SDS 0.07%. The mixture was incubated at 95°C for 5 min, followed by annealing at 58°C for 2 h and cooled to room temperature. The hybridization mixture was enriched using 600 μL of streptavidin-coated beads (Promega, Madison, WI), and three nonstringent and three stringent washes were carried out following Zane et al. (2002). DNA containing repeats were amplified by 30 cycles with *Mse* I-N primers. The polymerase chain reaction (PCR) product was purified with the E.Z.N.A.[1] Gel Extraction Kit (Omega, Bio-Tek, Winooski, VT) and ligated into a pGEM-T plasmid vector (Promega, Madison, WI) and transformed into $DH5^{\alpha}$ competent cells.

Recombinant clones were detected by PCR amplification using T7/SP6 and $(AG)_{10}$ as primers at the following thermal cycling conditions: initial denaturation of 3 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, 60 s at 72°C, and a final extension at 72°C for 8 min. A total of 100 clones with foreign inserts and relatively long flanking regions were sequenced using an ABI PRISM 3730 DNA sequence analyzer (Applied Biosystems, Foster City, CA). A total of 74 sequences containing microsatellites were obtained by SSR Hunter 1.3 software huntering (Li and Wan, 2005) and then subjected to primer designing using both PRIMER 3 (Rozen and Skaletsky, 2000) and software OLIGO 6.0 (National Biosciences). About 40 primer pairs were designed and

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[4] Author for correspondence: lqm@xtbg.ac.cn

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TABLE 1. Characteristics of the 16 polymorphic and 10 monomorphic microsatellite markers developed in *Dracaena cambodiana*.

Locus	Primer sequence(5′→3′)	Repeat motif	*T*a(°C)	No. of cycles	Allele size (bp)	GenBank Accession No.
DC003	F:AGAAAGGGAGGTGACAGG	$(AG)_{10}..(AG)_5A(AG)_5$	54	30	169–212	HM215604
	R:GTCAAAGAGCCCAAACAA					
DC006	F:GTTTCTAGTTCAAGAACCCAA	$(AG)_{16}$	54	30	188–200	HM215605
	R:TTCCTCCTCTTTCTCATCCT					
DC128	F:CCTGAGTAATGGGACTATA	$(CT)_9$	46	30	182–206	HM215606
	R:TGTGAGACCCAAATGCTA					
DC136	F:CAGGATCACAAGTGCTAT	$(GA)_{10}$	50	35	222–231	HM215607
	R:CCTGAGTAAGGAGTACAA					
DC138	F:GCAAGGGTTGATGATAGA	$(AG)_{13}$	50	30	160–184	HM215608
	R:TGAGAACAGCAATGATTAG					
DC140	F:AGCGTATTCAAAATGTCC	$(TCA)_6TG(GA)_9$	48	35	140–158	HM215609
	R:TCTGTTGCTATCGTGATC					
DC317	F:TCGGTCTTAGCTTTCTCC	$(CT)_6C_2AC_4T(TC)_{12}$	48	30	166–202	HM215610
	R:AGTAAGGTTAGGGCTTGA					
DC318	F:GAGAATCAGGTTATGGTC	$(AG)_{12}$	50	30	152–170	HM215611
	R:GTAATCCCCAATTACTCG					
DC435	F:AACCCCTTACTTGTCCAC	$(AG)_{12}$	52	35	121–137	HM215612
	R:CCAACTTACCTTTTGCTAC					
DC437	F:TGGAGCTAAGAAGAATAGAA	$(GT)_8(GA)_8$	48	30	109–117	HM215613
	R:GTAAGGAGCAGGAGGTGT					
DC448	F:TAAGGGAGATCCAAGGAA	$(TG)_5A(GT)_8(GA)_{11}$	46	30	229–258	HM215614
	R:AACCTCCAATTTGAAACC					
DC456	F:CGCTTTGCTTTGTGAATC	$(TC)_{18}ACT(CA)_{11}$	60	30	315–342	HM215615
	R:CTCCAACTATGGCACCTC					
DC460	F:AACAACAGCAGCAACGAC	$(CT)_{13}$	52	30	159–167	HM215616
	R:GGCTTGGCTTCTGGGAAA					
DC501	F:CCCACTAACTCAAAGAAG	$(GA)_{23}$	48	30	187–213	HM215617
	R:AACACGAATGTGAAAAGG					
DC516	F:GGGGAATTTCCGTTGCTT	$(GA)_8..(AG)_{23}$	54	40	165–195	HM215618
	R:CTGGACGTGAGTATTTGG					
DC616	F:TAGAAAGTTTGTAGCCAA	$(CT)_9(CA)_9$	50	30	172–184	HM215619
	R:GAGATTGTGAGCCATAAA					
DC121	F:AATAAGGGCAGAGGAGGA	$(AAT)_5..(AG)_{15}$	50	30	167	HM581792
	R:CGTGGGATTTGTACTTGC					
DC124	F:GCCCTGTATCTGCCACTT	$(TC)_{10}$	54	35	184	HM581793
	R:CACCTTCAACATGCACCC					
DC305	F:TTAGGGCTGGTTTGCTCA	$(AG)_9$	54	40	439	HM581794
	R:GTAAAACGGGCCGGAAGG					
DC319	F:ATTGTGGATTGGATGAAG	$(CT)_6..(TC)_{12}$	48	30	273	HM581795
	R:GCTAGTACCAAACAGGAA					
DC402	F:GACTGGCTAAAGAAATGGA	$(GA)_7GC(GA)_7$	50	35	197	HM581796
	R:TGGGAGGTAGCTGAAAGA					
DC415	F:AAGACTGGGAGAAGAGCA	$(GA)_5..(GA)_8$	54	30	414	HM581797
	R:TGAGTAAGAAATAAAACGGA					
DC450	F:GAGGCACATCGGCAAGTT	$(AG)_8..(TG)_8(GA)_6$	56	40	358	HM581798
	R:CTTCTTCCATCTCCCACC					
DC465	F:TCCCATAAATGCTCCTCA	$(CT)_9$	48	35	162	HM581799
	R:TCAAGCTATGCATCCAAC					
DC522	F:GTAAGAAGAAAAGAGGAAGA	$(GA)_{20}$	52	40	146	HM581800
	R:AGGGAATCTGTCACTTGT					
DC623	F:TGGTTTGGATGCAGGGAG	$(GA)_8$	52	35	224	HM581801
	R:CGTGACACCACCAGAAGAA					

*T*a, annealing temperature.

used for the subsequent test. These primers are all free of dimer and hairpin structure in 3′-termini, the length of each primer was 18–20 bp, and the value of T_m are ranged from 48°C to 60°C.

The final SSR-PCR system used in screening was optimized by an orthogonal design experiment, and four levels of five factors (Mg^{2+}, dNTP, Primer, Taq polymerase, and DNA template) have been tested separately in this system. The results demonstrated the reaction efficiency was affected by these factors. Based on the results, a stable, productive and reproducible PCR system was obtained: 20 μL system containing 10 ng template DNA, 0.4 μM of forward and reverse primers, 2.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 2 μL 10×Taq buffer and 1.0 unit of Taq polymerase (Takara, Dalian, Liaoning, China). Cycling conditions were 94°C for 5 min followed by 30–40 cycles at 94°C for 30 s, 46°C–60°C for 30 s (number of cycles and annealing temperatures were locus-specific; see Table 1), and 72°C for 1 min, with a final extension step of 8 min at 72°C. The PCR product was then separated on a 8% denaturing polyacrylamide gel and visualized by silver staining. A 25 bp DNA ladder (Promega, Madison, WI) was used as standard for scoring.

A total of 26 of the 40 primers successfully amplified DNA fragments, with 16 showing polymorphism and 10 showing monomorphism. Preliminary population genetics analyses for these polymorphic loci were performed using GENEPOP version 4.0 (Rousset, 2008). The number of alleles per locus (*A*) was 6 to 14 with an average of 9.188, the expected heterozygosity (H_E) ranged from 0.668 to 0.940 and the observed heterozygosity (H_O) varied from 0.333 to 0.875 (Table 2) . Eight of the 16 polymorphic loci departed significantly from the Hardy-Weinberg equilibrium ($P < 0.01$), which was due to excess of homozygosites. Analysis of errors using Micro-Checker version 2.2.3 (van Oosterhout et al., 2004)

Table 2. Results of initial primer screening in 22 individuals of *Dracaena cambodiana* for 16 polymorphic loci. Number of alleles (*A*), observed heterozygosity (H_O), expected heterozygosity (H_E), fixation index (F) and *P* -values for the Hardy-Weinberg equilibrium (HWE) test are given for each marker.

Locus	*A*	*Ho*	H_E	F	HWE
DC003	7	0.632	0.859	0.275	0.020
DC006	11	0.773	0.906	0.127	0.005*
DC128	7	0.333	0.756	0.597	0.000*
DC136	9	0.650	0.892	0.314	0.000*
DC138	13	0.875	0.897	–0.007	0.050
DC140	14	0.632	0.935	0.390	0.000*
DC317	13	0.750	0.899	0.144	0.053
DC318	7	0.682	0.668	–0.044	0.480
DC435	8	0.727	0.889	0.163	0.272
DC437	8	0.563	0.865	0.442	0.000*
DC448	7	0.625	0.853	0.413	0.000*
DC456	6	0.533	0.770	0.350	0.047
DC460	6	0.632	0.817	0.298	0.001*
DC501	11	0.364	0.940	0.795	0.000*
DC516	14	0.773	0.918	0.138	0.047
DC616	6	0.688	0.806	0.404	0.177

*Indicates the observed heterozygosity is departed significantly from the expected heterozygosity under Hardy-Weinberg equilibrium ($P < 0.01$). Voucher specimens of 11 *Dracaena cambodiana* natural populations: DCJC1568; DCPX1569; DCDF1570; DCSY1571; DCJG1572; DCMY1573; DCZK1574; DCML1575; DCXBL1576; DCMX1577; DCYW1578 were all deposited in Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences.

Table 3. Cross-amplification of 16 polymorphic microsatellite loci across other four *Dracaena* species.

Locus	*Dracaena thalioides* (*N* = 5)	*Dracaena reflexa* (*N* = 5)	*Dracaena marginata* (*N* = 5)	*Dracaena gracilis* (*N* = 5)
DC003	127, 137	141	143	131, 143
DC006	198	P(3)/196-206	204	204, 206
DC128	N	N	184	184
DC136	190	208, 210	204, 206	208, 210
DC138	N	140, 148	146	N
DC140	N	N	N	N
DC317	N	106, 108	109, 111	104, 106
DC318	N	176	176	176
DC435	P(6)/110-128	110	118	104, 106
DC437	N	N	N	N
DC448	N	N	196	196
DC456	320, 328	330, 332	332	N
DC460	P(3)/141-204	141	137, 141	161, 164
DC501	P(4)/166-191	161, 187	166, 180	168, 174
DC516	160	162, 164	144, 146	154, 156
DC616	138	156	158	158

n, Sample size; N, no amplification; P, polymorphic amplification, numbers in parentheses showed the number of alleles and the range of product size; Voucher specimen of four *Dracaena* species: DT1026 (*D. thalioides*); DR1027 (*D. reflexa*); DM1028 (*D. marginata*); DG1029 (*D. gracilis*) were all deposited in Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences.

suggested that these loci are likely to contain null alleles. No significant linkage disequilibrium was detected between locus pairs except for locus pair DC136 and DC140, DC435 and DC501, DC435 and DC516, DC003 and DC616, DC448 and DC616 ($P < 0.01$). These 16 loci further investigated for cross-species amplification in other four *Dracaena* (*D. thalioides*, *D. reflexa*, *D. marginata*, *D. gracilis*) species using five individuals for each species. In this test, 14 loci successfully amplified products, and 10 primer pairs among them showed polymorphism in at least one of these four *Dracaena* species (Table 3).

CONCLUSIONS

A total of 26 primer pairs in *D. cambodiana* could be used to assess the genetic diversity and genetic structure of *Dracaena* species for further evaluation and research.

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