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TECHNICAL NOTE

Characterization of polymorphic microsatellite markers isolated from *Dendrobenthamia japonica* var. *chinensis* (Cornaceae)

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Abstract *Dendrobenthamia japonica* var. *chinensis* is an evergreen, broad-leaved, woody species of the Cornaceae family. Here we isolated 14 codominant compound microsatellite markers from *D. japonica* var. *chinensis* using an improved technique. Overall, the number of alleles ranged from 2 to 13, with an average of 7.21 alleles per locus. These markers would be the useful tools for analysing questions concerning population genetic structure and genetic diversity of *D. japonica* var. *chinensis*.

Keywords *Dendrobenthamia japonica* var. *chinensis* · Genetic diversity · Microsatellite markers

Dendrobenthamia japonica var. *chinensis* is an evergreen, broad-leaved, woody species of the Cornaceae family, which is disjunct between chinese mailand and Taiwan island. Because of the small number of populations, *D. japonica* var. *chinensis* is treated as “endangered” in Taiwan island (Editorial Board of Flora of Taiwan 2nd edition 1993). To date, most of the efforts have been focused on the germplasm, breeding and cultivation of this species (Yi et al. 2010). The study of genetic diversity, population ecology and conservation of this species is insufficient and limited, which makes the development of genetic markers in *D. japonica* var. *chinensis* important and necessary. Here, we identified 14 polymorphic compound microsatellite markers from genome of *D. japonica* var. *chinensis* using a recently developed isolation technique (Lian et al. 2006).

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), and the restricted fragments were ligated to an unequal-length adaptor. Then, fragments flanked by a microsatellite at one end were amplified from the *Ssp*I DNA library using compound SSR primer $(AC)_6(AG)_5$ 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) and transformed into JM109 competent cells (Takara). 289 clones were analyzed using M13 primers to amplify the complete microsatellite-containing insert. A total of 178 positive clones were obtained and sequenced on an ABI Prism 3730 automated DNA sequencer (Applied Biosystems, USA). 103 sequences were found to contain $(AC)_6(AG)_n$ compound SSR motifs. Only 61 sequences containing $(AC)_6$(AG)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 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). Reaction conditions: one cycle of 95°C for 5 min; 35 cycles of 95°C

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for 30 s, the optimized annealing temperature (Table 1) for 45 s, 72°C for 90 s; 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 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-four individuals of *D. japonica* var. *chinensis* collected from Lushan Mountain (LS), Tiantang Mountain (TT) and Qiyun Mountain (QY) were used to test the polymorphism of the microsatellite primers. The number of alleles (*Na*), observed (*Ho*) 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. japonica* var. *chinensis*, The mean number of alleles per locus (*Na*) was 5.50 (range 2–8), 6.07 (range 2–10) and 6.14 (range 2–10) for populations in LS, TT and QY, respectively (Table 2). On average, the observed heterozygosities (*Ho*) were 0.799 (range 0.727–0.909), 0.780 (range 0.667–0.917) and 0.766 (range 0.545–0.909), respectively (Table 2). The expected heterozygosities (H_e) were 0.785 (range 0.519–0.896), 0.795 (range 0.518–0.913) and 0.788 (range 0.519–0.918), respectively (Table 2). The PIC were 0.704 (range 0.373–0.839), 0.721 (range: 0.373–0.863) and 0.711 (range 0.373–0.863),

Table 1 Characteristics of 14 compound microsatellite loci developed for *D. japonica* var. *chinensis*

Locus	Repeat	Primer sequence (5′–3′)	Size range (bp)	*Ta* (°C)	*Na*	GenBank accession no.
Si1	$(AC)_6(AG)_7$	F: ACACACACACACAGAGAGAGAG R: TTTGCTGTCTTTGGATTGTTA	172–198	56	10	JN225435
Si2	$(AC)_6(AG)_7$	F: ACACACACACACAGAGAGAGAG R: CTGGTTTTTTTGTGACCTCGTA	142–158	55	5	JN225436
Si3	$(AC)_6(AG)_6$	F: ACACACACACACAGAGAGAGAG R: GAAAGGTTCTCAGTTCTATGGG	141–187	56	12	JN225437
Si4	$(AC)_6(AG)_8$	F: ACACACACACACAGAGAGAGAG R: AAATGTATTAGTAAATGTTAGGAAA	151–179	55	10	JN225438
Si5	$(AC)_6(AG)_5$	F: ACACACACACACAGAGAGAGAG R: GATTATCACCCATAACCCAAGT	185–240	55	9	JN225439
Si6	$(AC)_6(AG)_5…(TG)_7$	F: ACACACACACACAGAGAGAGAG R: TTGGATTAAGAGCTTAATGAAA	296–338	56	9	JN225440
Si7	$(AC)_6(AG)_5$	F: ACACACACACACAGAGAGAGAG R: TTAGGGCACTTCCTGATTGT	316–342	56	9	JN225441
Si8	$(AC)_6(AG)_5$	F: ACACACACACACAGAGAGAGAG R: TATATTTATGTATGTGGGCTCC	156–168	53	4	JN225434
Si9	$(AC)_6(AG)_{14}$	F: ACACACACACACAGAGAGAGAG R: ATTGCTTTTAACTTTTTTTCAT	123–161	55	13	JN225442
Si10	$(AC)_6(AG)_{10}$	F: ACACACACACACAGAGAGAGAG R: AAAAAGGAGAACCAAGAACTAC	191–229	56	5	JN225443
Si11	$(AC)_6(AG)_5…(TTC)_5$	F: ACACACACACACAGAGAGAGAG R: GAACTTAGCCCATACATACATT	217–257	56	5	JN225444
Si12	$(AC)_6(AG)_5(AC)_3$	F: ACACACACACACAGAGAGAGAG R: TAAGACCTTGAAATGAAACAGT	213–235	55	3	JN225445
Si13	$(AC)_6(AG)_7…(AG)_4$	F: ACACACACACACAGAGAGAGAG R: TTTAAACTTTGATCATAAAGAT	146–162	55	5	JN225433
Si14	$(AC)_6(AG)_7$	F: ACACACACACACAGAGAGAGAG R: ATTAAAGTAATAGCGATGGAGC	152–158	54	2	JN225446

Ta the optimized annealing temperature, *Na* total number of alleles per locus

Table 2 Results of initial primer screening in three populations of *D. japonica* var. *chinensis*

Locus	Population LS (11)				Population TT (12)				Population QY (11)			
	Na	*Ho*	*He*	PIC	*Na*	*Ho*	*He*	PIC	*Na*	*Ho*	*He*	PIC
Si1*	8	0.818	0.896	0.839	8	0.667	0.873	0.817	9	0.909	0.870	0.813
Si2	4	0.909	0.766	0.683	4	0.750	0.728	0.641	5	0.636	0.818	0.745
Si3*	8	0.727	0.879	0.820	10	0.917	0.913	0.862	10	0.818	0.918	0.863
Si4*	8	0.818	0.874	0.814	8	0.667	0.895	0.841	8	0.545	0.896	0.838
Si5	7	0.727	0.835	0.769	7	0.917	0.844	0.783	8	0.818	0.835	0.775
Si6	7	0.818	0.866	0.803	7	0.833	0.884	0.828	6	0.636	0.823	0.753
Si7	5	0.818	0.827	0.756	8	0.750	0.877	0.821	8	0.818	0.879	0.818
Si8	3	0.909	0.697	0.591	3	0.750	0.670	0.570	4	0.727	0.697	0.607
Si9*	7	0.727	0.870	0.808	10	0.667	0.913	0.863	10	0.818	0.905	0.849
Si10	5	0.818	0.736	0.653	5	0.833	0.790	0.721	5	0.727	0.736	0.653
Si11	5	0.818	0.788	0.709	5	0.750	0.786	0.713	4	0.909	0.710	0.623
Si12	3	0.727	0.688	0.583	3	0.833	0.649	0.550	3	0.909	0.680	0.574
Si13	5	0.818	0.745	0.657	5	0.833	0.786	0.713	4	0.727	0.749	0.663
Si14	2	0.727	0.519	0.373	2	0.750	0.518	0.373	2	0.727	0.519	0.373

Na number of alleles per locus, *He* expected heterozygosity, *Ho* observed heterozygosity, *PIC* polymorphism information content

* With locus means significant departures from HWE ($P < 0.05$)

respectively (Table 2). Significant heterozygote deficiencies ($P < 0.05$) were detected in 4 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. JN225433–JN225446). Details about the 14 microsatellite loci and their variability across the 34 individuals were summarized in Table 1.

These polymorphic microsatellite markers of *D. japonica* var. *chinensis* should represent a useful tool to study the genetic diversity and population genetic structure of *D. japonica* var. *chinensis*. We are currently using these microsatellite primers together with chloroplast DNA markers to assess patterns of geographical molecular variation in *D. japonica* var. *chinensis* at the population level and across the species' ranges in China mainland and Taiwan Island.

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