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PERMANENT GENETIC RESOURCES

Isolation and characterization of microsatellite markers for *Cycas debaoensis* Y. C. Zhong et C. J. Chen (Cycadaceae)

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Abstract

***Cycas debaoensis* is one of the critically endangered cycad species endemic to China. In this study, we described the development of six microsatellite markers from the genome of *C. debaoensis* using the protocol of fast isolation by AFLP of sequences containing repeats (FIASCO) and two microsatellite markers derived from the database of expressed sequence tags (dbEST). Polymorphism of each locus was assessed in 60 adult individuals of the cycad. The average allele number of the microsatellites was 2.6 per locus, ranging from two to five. The observed and expected heterozygosities varied from 0.0833 to 0.7333 and from 0.0805 to 0.7188, respectively. Despite its rarity, only one locus (Y177) deviated from Hardy–Weinberg equilibrium due to the excessive homozygosity. The marker transferability of the eight primer pairs was tested on other four congeneric species that also occur in China.**

Keywords: *Cycas debaoensis*, dbEST, FIASCO, microsatellite markers, polymorphism

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Cycas debaoensis Y. C. Zhong & C. J. Chen is a critically endangered cycad species endemic to China (Zhong & Chen 1997). One previous study has assessed the genetic diversity using intersimple sequence repeat (ISSR) markers (Xie *et al*. 2005). Compared to the ISSR fingerprinting, miscrosatellites show numerous advantages since they are locus-specific, codominant, highly reproducible and usually highly polymorphic (Powell *et al*. 1996). However, no nuclear microsatellite primers have been reported for cycads. In order to devise adequate conservation and management strategies for the rare species and its sisters, we developed simple sequence repeat (SSR) microsatellite markers from *C. debaoensis* by using the fast isolation by AFLP of sequences containing repeats (FIASCO) protocol of Zane *et al*. (2002), and by screening EST-SSR markers derived from the GenBank database.

Genomic DNA samples were extracted from dry leaf tissue, which was ground in liquid nitrogen, using a CTAB methodology (Doyle & Doyle 1987). Total genomic DNA derived were completely digested with *Mse*I and then ligated to an *Mse*I AFLP adaptor. A diluted digestion–ligation mixture (1:10) was amplified with adaptor-specific primers (5′-GATGAGTCCTGAGTAAN-3′). Amplified DNA fragments, with a size range of 200–800 bp, were enriched for repeats by magnetic bead selection with a 5′-biotinylated $(AC)_{15}$ and $(AG)_{15}$ probe, respectively. Enriched fragments were amplified again with adaptor-specific primers. Polymerase chain reaction (PCR) products were purified using an EZNA Gel Extraction Kit (Omega Bio-Tek). Purified DNA fragments were ligated into the pMD18-T vector, and transformed into JM 109 cells. Positive clones were tested by PCR using $(AC)_{10}$/$(AG)_{10}$ and $M13^{+}$/$M13^{-}$ as primers, respectively. In total, 82 clones with positive inserts were sequenced with an ABI PRISM 3100 DNA sequencer.

A total of 69 (84%) sequences were found to contain microsatellite repeats, and 34 of them were suitable for designing locus-specific primers, using the PRIMER 5.0 program (Clarke & Gorley 2001). In addition, 260 expressed sequence tags (ESTs) of *Cycas rumphii* from GenBank were screened with SPUTNIK (http://www.cbi.labri.fr/outils/Pise/sputnik.html) to select sequences containing microsatellites. Five ESTs (GenBank Accession nos DR063256, DR063250, DR063232, DR063135 and DR063107) were identified as containing SSR, and primers were designed as described above. Polymorphisms of these loci were assessed in 60 individuals collected from four populations. All

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Table 1 Specific primer sequences and characterization for eight microsatellite loci isolated from *Cycas debaoensis*

Locus	Repeat motif	Primer sequences (5′–3′)	T_a (°C)	Allele size (bp)	N	A	H_O	H_E	GenBank Accession no.
G45	$(TC)_{12}$	F: AGAGTCCAATCATCTACGAG	54	215–225	60	2	0.6167	0.4929	EU131885
		R: GACCTTTCATATCATTCCCA							
G46	$(TC)_{11}$	F: CAAAAGCCCCTTGAAACC	55	165–173	60	4	0.5833	0.5097	EU131886
		R: AAGCTCGACTTGTAGATGGA							
Y89	$(AC)_{11}$	F: CTGTAAGATAATGCCCCACT	48	132–142	60	2	0.4667	0.3608	EU131887
		R: ATCCACAGGCAAAGGTCAGA							
Y94	$(CA)_8TA(CA)_{11}$	F: ATATGCAACAAGGACGAAAG	56	210–220	60	2	0.3167	0.2688	EU131888
		R: AATGCTCAATAGTTAGGTGG							
Y106	$(AC)_{10}$... $(AC)_8$	F: TAGAAACCCCAACCATAGAA	56	203–213	60	2	0.0833	0.0805	EU131889
		R: GGTGGAATCCCTTGAGAAAA							
Y177	$(TG)_{11}TA(TG)_{17}TA(TG)_{12}$	F: ATTACACCTATCCTCCAAGT	54	300–310	60	2	0.1000	0.4840*	EU131890
		R: ACTAATGAAGGGACATAACC							
E001	$(CA)_{10}$	F: TGGGATTAATATTCCAGAAA	52	266–270	60	2	0.1500	0.1679	DR063256
		R: CGACGAGTCTGATGTAGGTAT							
E004	$(AT)_{11}$	F: CTATCATCAGAGCCTCATTC	54	116–132	60	5	0.7333	0.7188	DR063135
		R: AAGTCATACATGGACAGCAA							

*indicates that the observed heterozygosity is significantly different from the expected heterozygosity under Hardy-Weinberg equilibrium ($P < 0.01$); Ta, PCR annealing temperature; N, number of individuals; A, number of alleles revealed; H_O, observed heterozygosity; H_E, expected heterozygosity.

Table 2 Cross-species amplification of microsatellites cloned from *Cycas debaoensis* in other cycad species, *C. micholitzii*, *C. multipinnata*, *C. guizhouensis* and *C. hongheensis*

Species	Locus								
	N	G45	G46	Y89	Y94	Y106	Y177	E001	E004
C. micholitzii	14	P(2)	P(4)	M	M	P(2)	M	P(2)	P(4)
C. multipinnata	21	P(2)	P(4)	M	P(2)	P(2)	M	P(2)	P(2)
C. guizhouensis	9	M	P(2)	M	P(2)	—	M	M	P(3)
C. hongheensis	19	P(2)	M	M	P(2)	P(2)	P(2)	M	P(5)

N, number of individuals tested.
M, monomorphic.
P, polymorphic (number of alleles).
—, no specific product.

microsatellites were amplified in a total volume of 20 μL which contained 50 ng template DNA, 0.2 μM of each primer, 0.2 mM of each dNTP, 1× PCR buffer (Mg^{2+} free), 1.5 mM Mg^{2+}, and 1 U of *Taq* polymerase. PCR amplifications were conducted on an MJ PTC-200 Thermal Cycler under the following conditions: initial denaturing at 95 °C for 5 min; 36 cycles of 40 s at 94 °C, 40 s at the annealing temperature for each designed specific primer, 40 s at 72 °C; and finally 10 min at 72 °C. PCR products were then electrophoresed on 6% denaturing polyacrylamide gel and visualized using silver staining. Allele sizes were estimated using a 10-bp ladder molecular size standard (Invitrogen).

Twenty-six of the 34 primer pairs successfully amplified the target regions. Of them, six displayed polymorphisms (Table 1), while 20 loci were monomorphic. Two primer pairs derived from the database of ESTs (dbEST) — DR063256 and DR063135 — successfully genotyped all individuals. Population genetic analyses were performed using GENEPOP version 3.4 (Raymond & Rousset 1995). Over all, the number of alleles per locus ranged from two to five, with an average of 2.6. The expected and observed heterozygosities ranged from 0.0805 to 0.7188 and from 0.0833 to 0.7333, respectively. Only the locus Y177 was deviated significantly from Hardy–Weinberg equilibrium ($P < 0.01$) due to excessive homozygosity. No significant genotypic disequilibrium was detected for any pair of loci.

For cross-species application, these eight primer pairs were tested in other four cycad species, *C. micholitzii*, *C. multipinnata*, *C. guizhouensis*, and *C. hongheensis* (Table 2), which also occur in China. Seven of them were successfully amplified in all species, whereas the Y106 failed to amplify in *C.*

guizhouensis. The polymorphic microsatellite loci presented here would be useful for assessing the population genetic structure of *C. debaoensis* and its sisters.

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