

PRIMER NOTE

Isolation, characterization and cross-species amplification of microsatellite loci in the cycad genus *Dioon* (Zamiaceae). Potential utilization in population genetics studies of *Dioon edule*

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Abstract

Dioon edule (Zamiaceae) is an endemic Mexican cycad. Nineteen microsatellite loci were isolated from three enriched genomic libraries of *D. edule* var. *angustifolium*, *D. tomasellii*, and *D. caputoi*. Seven of these loci showed polymorphisms in *D. edule*. Levels of polymorphism were assessed using 16 individuals from each of seven populations throughout the range of this species. The number of alleles per locus ranged from two to five and the observed and expected heterozygosities ranged from 0.0 to 0.9821 and from 0.0088 to 0.6318, respectively. All loci show significant linkage disequilibrium. Three loci depart significantly from Hardy–Weinberg equilibrium.

Keywords: cycads, *Dioon*, Mexico, microsatellites, population genetics, SSR

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Cycads are among the oldest of extant seed plants and are geographically restricted to the tropics and subtropics of the New and Old World (Jones 1993). The Neotropical genus *Dioon* Lindl. (Zamiaceae) is composed of 13 taxa (Hill *et al.* 2004). *Dioon* occurs in restricted pockets throughout Mexico, with one species (*D. mejiae* Standl. & L. O. Williams) from Honduras (Sabato & De-Luca 1985; Jones 1993; Moretti *et al.* 1993). *Dioon edule* Lindl. is listed as near threatened ('nt' category) in the wild by the World Conservation Union – IUCN. Found along eastern Mexico, it forms a species complex with two varieties *D. edule* var. *edule* and *D. edule* var. *angustifolium* (Miq.) Miq. (González-Astorga *et al.* 2003a). Recent studies suggest that these two varieties be recognized as distinct species (González-Astorga *et al.* 2003a, b).

Microsatellites or simple sequence repeats (SSRs) have been previously reported for one other cycad genus, *Zamia* L. (Zamiaceae) (Meerow *et al.*, in press). In the present study, SSRs were developed to ascertain the genetic structure of

D. edule in order to address taxonomic, conservation and biogeographical questions.

Three microsatellite enriched libraries were constructed following a protocol developed at the USDA-ARS-SHRS, Chapman Field, Miami, Florida. The protocol is modified from that of Edwards *et al.* (1996). Genomic DNA was extracted from fresh leaf material from: *D. edule* var. *angustifolium*, *D. tomasellii* De Luca, Sabato & Vazq. Torres, and *D. caputoi* De Luca, Sabato & Vazq. Torres following a modified protocol by Dellaporta *et al.* (1983). The DNA was digested, linked with adaptors and amplified. The amplified regions were enriched twice with biotin-labelled oligoprobes and separated with streptavidin-coated magnetic beads. After enrichment, products were separated using a Sepharose CL-4B SizeSeptember 400 Spun Column (Amersham Pharmacia Biotech), cloned with a TOPO TA Cloning Kit (Invitrogen) and screened via sequencing using ABI PRISM BigDye Terminator version 3.1 (PerkinElmer) and sequenced on an ABI sequencer (Applied Biosystems).

A total of 480 colonies were obtained with 53 of them containing useable repeats (11%). The sequences were imported into SEQUENCHER version 4.1 (Gene Codes) to generate consensus sequences. The preponderance of

Table 1 Primer sequences and related information for seven microsatellite loci from *Dioon edule*

Locus	Primer sequences (5'-3')	Repeat	N	Size	H _O	H _E	P
Ed3	F: GCATGAGGAGCTTGTTCCTG R: CTGTGAACTCCTGAAAGCATC	(CT) ₁₉	2	123–127	0.1476	0.2254	0.5526
Ed5	F: AGGCATAAATGGCTAAGCATAAC R: GCATTTCTAGTGGACAAACCAG	(AG) ₁₆	5	136–148	0.9821	0.6318	1.000
Ed6	F: ATGCAGATGAAACACACCC R: TCCTAACCATCCATCACTACC	(TGG) ₈	2	239–242	0.0089	0.0086	0.4103†
Ed9	F: CCTTGTGTTACTTTGAGCACC R: CAACAATGTAAGTGATGATGCC	(CAT) _{9int}	5	244–268	0.0805	0.1545	0.0282*
Cap5	F: CACTACCACCCCTATACCAC R: GACTTGAGCTTGTCTTTGTTG	(CT) ₂₃	3	225–241	0.8392	0.6092	1.000
Tom5	F: CGTTTCCATTTGGAGAGACAAG R: CCATCCAAGTGAGTGATACAAG	(TC) ₁₀	2	224–226	0.00	0.1317	0.000*
1660	F: GGTGCTGAAGAGGAAGAAGAA R: AGGGGAGAAGACATAACAAAGT	(GAA) ₁₆	4	194–230	0.0710	0.0863	0.0420*

N, number of alleles; size, allele size range (bp); H_O, observed heterozygosity; H_E, expected heterozygosity; P, P-values for the HWE tests (H₁, heterozygote deficiency) represent global average across seven populations; *significance at P < 0.05; †test was performed on only one population showing allelic polymorphism; the seven loci's GenBank Accession nos: DQ441409–DQ441415.

repeats was dinucleotides (61%), followed by trinucleotides (36%) and complex (3%). The dinucleotide motif with the highest frequency was GA/CT (88%), followed by AC/TG (12%). Trinucleotide repeats fell into five categories, each with equal frequency: CTT/GAA, TGG/ACC, CAT/GTA, TAA/ACC and CAA/GTT. One complex SSR was found – (AG)(TG).

Only 46 sequences were suitable for primer design. Primers were designed from the flanking regions using GCG Prime (Wisconsin package). Forty-six primers were tested on eight individuals from three populations each of our target species, *D. edule*. The polymerase chain reactions (PCRs) were performed with a PTC-200 Peltier Thermal Cycler (MJ Research) in 10 µL volumes. Each reaction contained 6.25 µL dH₂O, 1 µL 10× Buffer with 15 mM MgCl₂, 0.8 µL 10 mM dNTP, 0.3 µL 10 mM fluorescent 5'-end labelled forward primer, 0.3 µL 10 mM reverse primer, 0.05 µL AmpliTaq DNA polymerase (5 U/µL) (Applied Biosystems), and 1 µL template DNA (15–20 ng/µL). Samples were amplified using the following program: 5 min at 94 °C, 30 s at 94 °C, 1 min at 60 °C, steps 2 and 3 repeated 24 times and then a final extension of 2 min at 72 °C followed by storage at 4 °C. Products were loaded in an ABI sequencer and the data was analysed using GENESCAN version 3.1 (ABI PRISM) and GENOTYPER software. Population genetic analyses were performed using GENEPOP 3.4 (Raymond & Rousset 1995). All values reported were calculated using Fisher's exact method.

Nineteen primers yielded PCR amplifications; however, only seven of those primers captured polymorphisms in *D. edule* (Table 1). The seven primers were tested on 112 individuals, 16 individuals from each of the seven populations throughout the range of *D. edule* in Mexico (Table 1).

The number of alleles per locus ranged from two to five, and both the observed and the expected heterozygosities ranged from 0.0 to 0.9821 and from 0.0088 to 0.6318, respectively. Three loci (Ed9, Tom5, 1660) departed significantly from Hardy–Weinberg equilibrium. Those loci showed significant heterozygote deficiency (Table 1). This may be due to the presence of null alleles or the Wahlund effect. Linkage disequilibrium was detected for all pairs of loci in all populations where the test could be carried out. This linkage disequilibrium is most likely due to high levels of inbreeding (Flint-Garcia *et al.* 2003).

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