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New microsatellite loci in the dwarf yams *Dioscorea* group Epipetrum (Dioscoreaceae)[1]

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- *Premise of the study:* Microsatellite loci were isolated and characterized from enriched genomic libraries of two taxa of the Chilean Epipetrum group of *Dioscorea* to assess their levels of genetic diversity and population differentiation.
- *Methods and Results:* Eleven microsatellite loci were identified. Six out of nine microsatellites from *D. biloba* amplified in *D. humilis*, and the two microsatellites from *D. humilis* amplified in both taxa. Two different sets of eight loci amplified in each of the two tested taxa, *D. biloba* and *D. humilis*. The average number of alleles was 5.75 and 5 for *D. biloba* and *D. humilis*, respectively. Higher levels of mean genetic diversity were found in *D. biloba* (H_E = 0.639) than in *D. humilis* (H_E = 0.414).
- *Conclusions:* These microsatellite primers will be useful in population genetic studies and to establish conservation strategies in the endangered taxa of the Epipetrum group of *Dioscorea*.

Key words: Chile; conservation genetics; genetic diversity; population genetics; SSR.

Dioscorea L., with more than 600 species, is the most diverse genus of the Dioscoreaceae. Several monophyletic groups of species, some of them previously recognized as independent genera, have been recently included within *Dioscorea* based on molecular grounds (Caddick et al., 2002). One of these species groups was initially recognized as the genus *Epipetrum* Phil. Morphological studies have accepted two species with two subspecies each, namely *Dioscorea biloba* (Phil.) Caddick & Wilkin subsp. *biloba*, *D. biloba* subsp. *coquimbana* Viruel, Segarra-Moragues & Villar, *D. humilis* Colla subsp. *humilis* and *D. humilis* subsp. *polyanthes* (F. Phil.) Viruel, Segarra-Moragues & Villar (Viruel et al., 2010). These taxa are dioecious, predominantly diploid geophytes endemic to Chile (Viruel et al., 2008, 2010). Because of their fragmented distribution and small population, they are considered to be taxa of conservation concern (Marticorena et al., 2001). Here we characterize 11 microsatellite markers that will be used to assess population genetic diversity levels and genetic structure across their distribution range and to inform the design of future conservation plans.

METHODS AND RESULTS

Total DNA was extracted from silica-gel-dried young leaves using the DNeasy plant minikit (Qiagen, Barcelona, Spain). Microsatellite isolation followed the protocol of Zane et al. (2002) with some modifications. Total DNA was digested with an excess of *Mse*I (New England Biolabs, Herts, UK) and purified with the PCR purification kit (Qiagen, Barcelona, Spain). This step removes small fragments, and thus avoids further size-selection steps of fragments. Purified digested products were ligated to *Mse*I adaptors in 20-μl volume using LigaFast™ DNA Ligation System (Promega, Madison, Wisconsin, USA), incubated overnight at 4°C, diluted 1 : 10 and used as a template for subsequent PCR amplification. The 50-μl PCR cocktail included: 1×*Taq* Buffer (Biotools, Madrid, Spain), 2 mM $MgCl_2$, 0.4 mM of each dNTP, 125 pmol of MseI-N adaptor-specific primer, 2.5 U of *Taq* polymerase (Biotools) and 5 μl of the 1 : 10 ligation dilution as template. The PCR program consisted of one cycle of 2 min at 72°C to fill the nicks, followed by 4 min at 94°C for DNA melting, and then 30 cycles each of 94°C, 30 s; 53°C, 1 min and 72°C 1 min; and a final extension of 7 min at 72°C and kept at 4°C. PCRs were carried out in a PE2720 thermal-cycler (Applied Biosystems, Madrid, Spain). PCRs were systematically repeated to obtain several hundred ng of amplified products. PCR amplifications were combined and precipitated with Ethanol-Ammonium Acetate and resuspended in 50 μl of distilled water.

Enrichments were performed using streptavidin-coated M-280 magnetic beads (Invitrogen, Barcelona, Spain) attached to $(CTT)_8GC$, 5′-biotinylated oligonucleotide, which has given positive results in other Dioscoreaceae (Segarra-Moragues et al., 2003, 2004). The beads were washed with 1×B&W buffer to remove unbound oligonucleotides, resuspended in 100 μl of 3×Saline Sodium Citrate (SSC, 20×SSC: 6M NaCl, 0.6M Na-citrate, pH 7), 0.1% SDS and 2% PEG-6000 and kept at room temperature in continuous rotation. Fifteen μl of the PCR products were denatured 5 min 94°C, hybridized to the oligonucleotides at room temperature for 30 min and washed at 40°C with three decreasing salt-concentration solutions [2×SSC, 1×SSC, 0.5×SSC, respectively, plus 0.1% SDS, and 1.6 μM of *Mse*I-N primer] to remove unbound fragments and unspecific hybridizations. Fragments were released at 94°C, 5 min in 100 μl 0.2×SSC and desalted with Qiaex II (Qiagen). Two μl of enrichment were used for 50 μl PCR with the same conditions as above but a final elongation step of 12 min at 72°C was added to generate 3′-A overhangs required for cloning. Fragments were purified (Qiagen), cloned into pGEM-T easy vector system (Promega), transformed into XL1-blue *E. coli* competent cells (Stratagene), and recombinants screened by PCR (Lunt et al., 1999). Clones were sequenced using BigDye Terminator Kit v. 3.1 and electrophoresed on a 3730 DNA sequencer (Applied Biosystems).

From a total of 167 and 79 sequenced clones from, respectively, the *Dioscorea biloba* and *D. humilis* libraries, 190 clones (77.24%) contained microsatellites. After discarding clones with too short flanking sequences, primers

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TABLE 1. Characteristics of 11 microsatellite loci developed in *Dioscorea* group Epipetrum. For each locus the primer pair sequences, repeat motif, size of the original fragment (bp), annealing temperature (T_a), and Genbank accession numbers are shown. 6FAM, NED, PET, and VIC are fluorescent dyes from Applied Biosystems. †, loci isolated from *D. biloba* and ‡, loci isolated from *D. humilis*.

Locus	Primer sequence (5′–3′)	Repeat motif	Size	T_a	GenBank Accession No.
B204†	F: NED-CATTATAGTTGACGGATCCAACG R: TCAGATCTTCAGGAATCGGTAA	$(CAT)_6(CTT)_8TTT(CTT)_6$	160	56	HQ117937
B209†	F: PET-GTCTTGCCTTTGTCCGTCAT R: GGCTGAACTGGGTATATGGAAAC	$(CTT)_9$	256	51	HQ117938
B322†	F: NED-AAGGAAGAGGTTGACACCATT R: AATACACAAGGAGATAAAAGAGCA	$(GAA)_8$	199	61	HQ117939
B531†	F: 6FAM-AGTGACATGGATGCTGATTGT R: GGGAGTATGCCGATGAAATG	$(CTT)_{15}$	201	56	HQ117940
B628†	F: NED-AGCTTCAATGCCTTCTCCATTT R: ACGGAGTTATCGGTTGCAGAG	$(CCT)_5$	149	51	HQ117941
B633†	F: PET-CAAGCGATGTGGTCCTCTC R: GGCGATTGAACAATGTCATCA	$(GAA)_4GTT(GAA)_7$	139	56	HQ117942
B744†	F: VIC-GAATAAGGAATAAGCCCGATCC R: GCCGATTGAACAATGTCATAA	$(GAA)_5$	230	56	HQ117943
B809†	F: VIC-GGTGGGAGAAGGGTTCAGTAG R: AGAGTTCCATCCGCAATGAC	$(GAA)_{10}$	169	56	HQ117944
B812†	F: PET-AAGCCACAAGGGATACATCA R: TGATGATGAGAACTGGATGAACT	$(GAA)_5$	210	51	HQ117945
H422‡	F: VIC-CTAGTAGTATGCACCGCCTCC R: GGCTTGGACGTCATGAACAG	$(CTT)_5$	122	51	HQ117946
H442‡	F: 6FAM-ATGGGATAAGGGATTGAGGA R: ATGATCTTCAGAGAAATGAGAGC	$(GA)_6$	161	51	HQ117947

were designed for 16 and 10 clones from *D. biloba* and *D. humilis*, respectively, using PRIMER3 (Rozen and Skaletsky, 2000). Fifteen of the 26 primer pairs produced clear amplicons of the expected size on agarose gels and were selected for analysis on automated sequencers. PCRs were performed in a 20-μl mix containing 1× Surf*Taq* Buffer (StabVida), 2 mM $MgCl_2$, 0.4 mM of each dNTP, 5 pmol each of the fluorescent-labeled (forward) and unlabeled (reverse) primers, 1 U of Surf*Taq* polymerase (StabVida) and 1 μl of DNA. The PCR program consisted of one step of 4 min at 94°C followed by 39 cycles each of 1 min at 94°C, 1 min at annealing temperature (Table 1) and 1 min at 72°C, and a final step of 7 min at 72°C. Products were run on an ABI 3730 automated sequencer using LIZ500 as the internal lane size standard. Fragment lengths were assigned to allelic classes with GENEMARKER v. 1.71 software (Soft-Genetics, State College, Pennsylvania, USA). After an initial screening of individuals, nine loci from *D. biloba* and two from *D. humilis*, respectively (Table 1), were selected to genotype the whole sample set.

Genotypic data were obtained for one population each of *D. biloba* and *D. humilis*, for 11 microsatellite loci (Table 2). Genetic diversity indices, deviations from Hardy–Weinberg equilibrium and linkage disequilibrium (LD) between pairs of microsatellite loci, using 1000 permutations, were calculated using GENEPOP v. 4.0 software (Rousset, 2008).

From the 11 assayed microsatellite loci, eight amplified in *D. biloba* and eight in *D. humilis*. The remaining loci, B633, B744, and B812 in *D. biloba*, and B204, B209, and B531 in *D. humilis*, produced either multibanding profiles or failed to amplify. None of the 28 pairwise comparisons between loci showed significant LD ($p < 0.05$) for the *D. humilis* population or for *D. biloba* after Bonferroni correction.

The number of alleles per locus ranged from 2 to 12 in both *D. biloba* and *D. humilis*, with a mean of 5.75 and 5 alleles in *D. biloba* and *D. humilis*, respectively. Observed and expected heterozygosities ranged from 0.167 to 0.833 and from to 0.274 to 0.867, respectively, in *D. biloba* and from 0.033 to 0.853 and from 0.033 to 0.832, respectively, in *D. humilis*. Mean observed and expected heterozigosities were higher in *D. biloba* (0.613–0.639) than in *D. humilis* (0.447–0.414), as a consequence of the lower allelic diversity of the *D. biloba* SSR loci when transferred to *D. humilis*. Both taxa showed mostly non-significant values of per locus estimates of F_{IS}s. Only one locus (B628) showed significant heterozygote excess in *D. humilis*, whereas in *D. biloba* two loci (B209 and H442) significantly deviated toward heterozygote deficiency. Overall F_{IS} values did not deviate from HW equilibrium in *D. humilis*, while in *D. biloba* they showed slight deviation toward heterozygote deficiency, which could be attributed to the presence of null alleles, or to population genetic sub-structuring.

TABLE 2. Results of initial primer screening in two populations of *Dioscorea* group Epipetrum. For each locus, number of alleles (*A*), observed (H_O) and unbiased expected (H_E) heterozygosities and F_{IS} values are reported for one population of *D. biloba* (Chile: Coquimbo, road from Chalinga to La Aguada, 30°47.875′S, 71°27.056′W, *N* = 30) and one population of *D. humilis* (Chile: Maule, road from Curicó to Sagrada Familia, 35°03.050′S, 71°31.054′W, *N* = 36). $^*p < 0.05$, $^{**}p < 0.01$; ns, not significant; —, no amplification.

	Dioscorea biloba				*Dioscorea humilis*			
	A	H_O	H_E	F_{IS}	*A*	H_O	H_E	F_{IS}
B204	10	0.767	0.819	0.065^{ns}	—	—	—	—
B209	4	0.167	0.274	0.396**	—	—	—	—
B322	2	0.667	0.499	-0.343^{ns}	8	0.853	0.832	-0.026^{ns}
B531	12	0.833	0.867	0.038^{ns}	—	—	—	—
B628	4	0.700	0.617	-0.137^{ns}	3	0.778	0.579	−0.349*
B633	—	—	—	—	2	0.033	0.033	0.000
B744	—	—	—	—	2	0.273	0.239	-0.143^{ns}
B809	6	0.667	0.709	0.060^{ns}	3	0.111	0.108	-0.029^{ns}
B812	—	—	—	—	3	0.057	0.057	-0.007^{ns}
H422	5	0.800	0.804	0.005^{ns}	7	0.722	0.726	$+0.005^{ns}$
H442	3	0.300	0.524	0.431**	12	0.750	0.739	-0.016^{ns}
Mean (±SD)	5.75	0.613 ± 0.244	0.639 ± 0.201	0.023*	5.0	0.447 ± 0.360	0.414 ± 0.338	-0.081^{ns}

CONCLUSIONS

The two sets of microsatellite loci will be adequate to conduct population genetic studies for both *Dioscorea* species. We will investigate whether the strong population disjunction found in *D. biloba* correlates with morphological and genetic differentiation of their populations and agrees with the recognition of the two subspecies (Viruel et al., 2010). Additionally, these markers will provide empirical evidence of the genetic effects of a more widespread distribution area (as in *D. humilis* subsp. *humilis*) compared to the more restricted and fragmented areas of the remaining taxa. The genetic information compiled for the whole Epipetrum group of *Dioscorea* will implement more efficient conservation programs on the three more endangered taxa.

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