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Microsatellite markers for the relict tree *Aextoxicon punctatum*: The only species in the Chilean endemic family Aextoxicaceae[1]

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- *Premise of the study:* We screened 10 microsatellite loci for the dioecious, rainforest tree *Aextoxicon punctatum*, a species belonging to a monotypic family and genus, endemic to southwestern South America (30–43°S).
- *Methods and Results:* Polymorphisms were evaluated in 108 adult trees from four populations, including the northern and southern extremes of the geographic range of *Aextoxicon* in Chile. All 10 microsatellites revealed polymorphic variation. A total of 69, 57, 59, and 69 alleles were found in 40 (Fray Jorge), 19 (Santa Ines), 21 (Quebrada del Tigre), and 28 (Guabun) individual trees, respectively. The mean expected heterozygosity per population ranged from 0.70 to 0.72.
- *Conclusions:* These polymorphic microsatellites will be useful in assessing the genetic structure and conservation status of *Aextoxicon* throughout its historically fragmented geographic range. Parentage analysis will provide additional insights into the key historical and contemporary processes that have mediated population differentiation in this species.

Key words: avian seed dispersal; Chilean temperate rainforests; dioecious tree; historical geographic range fragmentation.

Aextoxicon punctatum Ruiz & Pav. is the only member of the phylogenetically isolated (Savolainen et al., 2000) and Tertiary relict family Aextoxicaceae (Nishida et al., 1988). The species, which is largely endemic to Chile, grows over the entire range of temperate rainforests in the humid western margin of southern South America. It is a dioecious, fleshy-fruited tree, characterized as shade tolerant, often rising to heights of 25–30 m in the forest canopy. The present latitudinal range of *Aextoxicon punctatum* (30–43°S) extends northwards into the Chilean semiarid region (30–32°S), where it occurs on fog-inundated coastal hilltops. Biogeographers have explained the presence of these rainforest outposts as remnants from a formerly continuous forest community developed during wetter times in the late Tertiary. This forest became gradually fragmented by the process of climatic aridization that took place in north-central Chile since the Plio-Pleistocene period (Villagrán et al., 2004). According to genetic analysis using RAPD markers, *Aextoxicon punctatum* trees from the northernmost populations in Chile have become differentiated from those of the main geographic range of the species, 35–43°S (Núñez-Ávila and Armesto, 2006). However, the levels of heterozygosity present in these populations remain unknown.

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METHODS AND RESULTS

To assess genetic diversity and gene flow among *Aextoxicon punctatum* populations, we evaluated 10 microsatellite markers (Table 1). An enriched library was made by Ecogenics GmbH (Zurich, Switzerland) from size-selected genomic DNA ligated into SAULA/SAULB-linker (Armour et al., 1994) and enriched by magnetic bead selection with biotin-labeled $(CT)_{13}$, $(GT)_{13}$, $(GTAT)_7$, and $(GATA)_7$ oligonucleotide repeats (Gautschi et al., 2000a, b). Of 378 recombinant colonies screened, 130 gave a positive signal after hybridization. Plasmids from 32 positive clones were sequenced, and primers were designed for 10 microsatellite inserts, all of which were tested for polymorphism using the procedure described by Schuelke (2000). All loci were amplified in an Applied Biosystems (model 9700) thermal cycler by using the following components: 10 μL final volume, 2 μL of DNA (0.5 ng/μL), 1 μL of buffer PCR (10×), 1 μL dNTPs (2 mM), 0.4 μL of Forward primers (2 μM), 0.8 μL of Reverse primers (2 μM) and 0.8 μL of FAM labeled-M13 (–21) universal primers-3′ (2 μM), 3.5 μL ultrapurified water (GIBCO Life Technologies, Carlsbad, California, USA), 0.6 μL $MgCl_2$ (25 mM), and 0.1 μL *Taq* polymerase (5 U/μL, HotStart–Qiagen, Valencia, California, USA). The thermocycle consisted of 15 min of initial denaturation at 94°C, followed by 30 cycles of 30 s at 94°C, 45 s at TA°C and 45 s at 72°C, followed by 8 cycles of 30 s at 94°C, 45 s at 53°C and 45 s at 72°C with a final extension of 10 min at 72°C. For genotyping, 1 μL of the PCR product was added to 22 μL formamide and 0.5 μL LIZ-400 size standard. The mixture was run on the ABI PRISM 310

Table 1. Characterization of 10 microsatellite loci isolated from the dioecious tree *Aextoxicon punctatum*. Forward and reverse sequences, repeat array, allele size range, optimal PCR annealing temperature (TA), and GenBank accession numbers are shown for each primer pair.

Locus	Primer sequences (5′-3′)	Repeat Array	Size range (bp)	TA (°C)	GenBank Accession No.
Ap 2	F: GGGACCAAACCTTTCTCCTC R: TCGTTCATAGCTGGAGATTGC	$(TC)_{11}(AC)(TC)_9$	146–200	56	HQ398306
Ap 3	F: TGGTCATGACGTAATGGAAAAG R: CGGGAAATGGTAAGGACTAGC	$(CT)_{19}$	145–183	60	HQ398307
Ap 8	F: TCAGCACCCACTTTGTGTTC R: AACTAGGGTTCCACCGAAGC	$(TC)_{11}(CT)_{13}$	100–143	60	HQ398308
Ap 9	F: TCCTTTCCAAAGAAAATTCCTC R: ACGGAACCTAGGCCTCACTC	$(GT)_6(AT)(GA)_{22}$	144–176	56	HQ398309
Ap 10	F: TGGATAAGATATGGGGAATAGCC R: TCATGCACAAACCTTTAATAACC	$(GT)_9\ (GA)_{16}$	118–136	56	HQ398310
Ap 11	F: GCTTGGTTTCATCATCACCTG R: ATGACCTGGCCTTGGGTAG	$(CT)_6(CTAT)_8$	128–148	60	HQ398311
Ap 12	F: GGACACCTTCGTCGTCCTC R: ATAGACAGGCGCATTCTTGC	$(TC)_{21}$	200–242	56	HQ398312
Ap 18	F: TGCCAACTTGATGGTTGATG R: TTTCGATTTTTAGAGAGGGAGAC	$(CT)_{19}$	140–166	56	HQ398313
Ap 21	F: CTCTTTCGTCCACCTTGATTG R: GGTGTATTAGAATTTGTTCGACTTTG	$(GT)_{18}(GA)_8(GT)_{15}$	144–206	56	HQ398314
Ap 22	F: AACGCATTGGAAAGGATTTG R: GGCCTCTCTCAAAGACATCG	$(GA)_{14}$	193–215	56	HQ398315

(Applied Biosystems, Foster City, California, USA), and analyzed using Peak Scanner Software version 1.0 (Applied Biosystems).

Polymorphisms of the genetic markers were evaluated using leaf samples collected from a total of 108 *Aextoxicon* individuals from four natural populations: Fray Jorge ($N = 40$), Santa Inés ($N = 19$), and Quebrada del Tigre ($N = 21$) in semiarid Chile, and Guabun ($N = 28$) near the wetter southern limit of the species range (Table 2). Genomic DNA from leaf samples was extracted using the QIAGEN DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA). Linkage disequilibrium and deviations from Hardy–Weinberg equilibrium were calculated with Arlequin 3.1 (Excoffier et al., 2005) using the Markov chain method (chain length 100 000).

All 10 markers tested revealed polymorphic variation at microsatellite loci (Table 2). No significant linkage disequilibrium was found among loci ($P > 0.05$). The locus Ap 10 was only tested for the population in Fray Jorge (Table 2). The total number of alleles found in each population was 69 (Fray Jorge), 57 (Santa Inés), 59 (Quebrada del Tigre), and 69 (Guabun). The mean expected heterozygosity values over all loci per population varied between 0.70 and 0.72 (Table 2). An excess of homozygotes was found for loci Ap 10 and Ap 12 ($P < 0.05$) in the population from Fray Jorge and for locus Ap 21 in the population from Quebrada del Tigre (Table 2).

CONCLUSIONS

This study describes the first polymorphic microsatellite markers for the South American endemic tree *Aextoxicon punctatum.* Using these markers, we can investigate how historical processes have molded plant biogeography in Chilean forests and quantify the genetic structure of *Aextoxicon punctatum* populations. Land cover change has greatly altered the patterns of tree recruitment in southern temperate rainforests in recent decades (Newton, 2007). New molecular tools will enable us to make useful comparisons between the impacts of historical processes (e.g., aridization, related fragmentation) and more recent human activities on native forests (e.g., forest cover loss and habitat fragmentation). Moreover, these microsatellite markers will prove useful in assessing processes that drive contemporary gene flow among *Aextoxicon punctatum* populations such as pollen and seed dispersal.

Table 2. Description of 10 microsatellite loci isolated from leaf samples of *Aextoxicon punctatum*. Samples were collected from four coastal populations along the latitudinal range of the species in Chile. The table shows the number of alleles (N_{all}) and the observed and expected heterozygosity (H_O and H_E) for each population. The probabilities (*P*-value) associated with deviations from Hardy–Weinberg equilibrium are also shown for each locus. Dashes in locus Ap 10 indicate that the locus was not tested in these populations.

	Fray Jorge (30°40′13″S, 71°40′37″W)				Santa Inés (32°09′38″S, 71°29′33″W)				Quebrada del Tigre (32°32′57″S, 71°25′53″W)				Guabun (41°47′21″S, 74°01′04″W)			
Locus	N_{all}	H_O	H_E	*P*-value	N_{all}	H_O	H_E	*P*-value	N_{all}	H_O	H_E	*P*-value	N_{all}	H_O	H_E	*P*-value
Ap 2	9	0.77	0.77	0.540	11	0.95	0.87	0.975	10	0.86	0.83	0.795	9	0.82	0.81	0.274
Ap 3	6	0.55	0.59	0.075	6	0.79	0.76	0.965	10	0.86	0.85	0.203	10	0.93	0.86	0.934
Ap 8	8	0.85	0.81	0.519	9	0.95	0.86	0.936	6	0.95	0.82	0.733	10	0.70	0.84	0.123
Ap 9	8	0.77	0.80	0.732	5	0.84	0.75	0.740	6	0.76	0.73	0.633	7	0.75	0.70	0.909
Ap 10	9	0.15	0.65	0.000*	—	—	—	—	—	—	—	—	—	—	—	—
Ap 11	5	0.77	0.73	0.443	5	0.63	0.69	0.971	4	0.43	0.40	0.757	4	0.28	0.38	0.199
Ap 12	4	0.22	0.60	0.000*	3	0.53	0.51	1.000	3	0.48	0.55	0.490	5	0.43	0.51	0.050
Ap 18	7	0.62	0.63	0.385	7	0.79	0.68	0.714	6	0.76	0.76	0.127	6	0.68	0.70	0.621
Ap 21	9	0.85	0.84	0.189	5	0.74	0.63	0.962	8	0.86	0.82	0.002*	11	0.86	0.85	0.668
Ap 22	4	0.70	0.71	0.319	6	0.53	0.52	0.305	6	0.76	0.72	0.434	7	0.75	0.82	0.064
Mean over loci		0.63	0.71			0.75	0.70			0.75	0.72			0.69	0.72	
SD		0.25	0.09			0.15	0.12			0.17	0.14			0.19	0.16	

*Indicates the observed heterozygosity departed significantly from the expected heterozygosity under Hardy–Weinberg equilibrium ($P < 0.05$).

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