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PRIMER NOTE

MICROSATELLITE PRIMERS FOR THE RARE SHRUB *ACACIA ADINOPHYLLA* (FABACEAE)[1]

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- *Premise of the study:* Microsatellite primers were developed for the rare shrub *Acacia adinophylla* (Fabaceae) to assess genetic diversity and its spatial structuring.
- *Methods and Results:* Shotgun sequencing on an Illumina MiSeq produced 6,372,575 reads. Using the QDD pipeline, we designed 60 primer pairs, which were screened using PCR. Seventeen loci were developed, of which 12 loci were identified that were polymorphic, amplified reliably, and could be consistently scored. These loci were then screened for variation in individuals from three populations. The number of alleles observed for these 12 loci ranged from three to 18 and expected heterozygosity ranged from 0.13 to 0.85.
- *Conclusions:* These markers will enable the quantification of genetic impact of proposed mining activities on the short-range endemic *Acacia adinophylla*.

Key words: *Acacia adinophylla*; Fabaceae; microsatellite primers; shotgun sequencing; South West Australian Floristic Region (SWAFR).

Acacia adinophylla Maslin (Fabaceae) is a short-range shrub species (to 1.5 m high but often prostrate) of the Helena and Aurora Range (Maslin, 1999), a small and ancient banded iron formation within the South West Australian Floristic Region (SWAFR). The SWAFR harbors an extraordinarily rich and endemic flora, with a substantial number of naturally rare species (of which *A. adinophylla* is one) with highly disjunct and fragmented populations (Hopper and Gioia, 2004). Due to its isolation, low population numbers, and proximity to mining activity, *A. adinophylla* (Conservation status P1) is protected under the *Wildlife Conservation Act 1950* (Western Australia). Here, we report the isolation and characterization of 12 polymorphic microsatellite loci from *A. adinophylla*, using low-coverage shotgun sequencing. Next-generation sequencing has greatly increased the contribution that molecular tools can make to conservation and restoration genetics (Williams et al., 2014), in this case, through the efficient development of microsatellite markers. The markers developed here will be used to examine spatial genetic structure across the species range and quantify the genetic impact of proposed mining.

METHODS AND RESULTS

We used the NucleoSpin Plant II method (Macherey-Nagel GmbH and Co., Düren, Germany) to extract genomic DNA from fresh phyllode material of one individual from population AA 5 (Universal Transverse Mercator [UTM] coordinates 759053E, 6638224N; collector no. Nevill 100; voucher held at the University of Western Australia Herbarium [UWA], Crawley, Western Australia, Australia). DNA was sent to the Australian Genome Research Facility node in Melbourne, Victoria, for shotgun sequencing and identification of DNA sequences containing microsatellites. Briefly, 200 ng of genomic DNA was sheared in a volume of 50 µL using a Covaris E220 Focused-ultrasonicator (Covaris, Woburn, Massachusetts, USA). After shearing, sequencing libraries were prepared using Illumina's TruSeq Nano DNA Library Preparation Kit (Life Technologies, San Diego, California, USA), following the manufacturer's protocol. Libraries were assessed by gel electrophoresis (Agilent D1000 ScreenTape Assay; Agilent, Santa Clara, California, USA) and quantified by qPCR (KAPA Library Quantification Kits for Illumina; KAPA Biosystems, Wilmington, Massachusetts, USA). Sequencing was performed on the Illumina MiSeq system (Life Technologies) with 2 × 250-bp paired-end reads using the MiSeq Reagent Kit version 2, 500 cycles. FASTAQ sequences were taken from the MiSeq sequencing run, and sequences were stitched using the PEAR assembler (Zhang et al., 2013) before processing. Shotgun sequencing produced 6,372,575 reads. The QDD version 3.1.2 pipeline (Meglécz et al., 2014) with default parameters was used to screen the raw sequences for ≥6 di-, tri-, tetra-, and penta-base repeats, remove redundant sequences, and design primers. The resultant sequences were filtered to ensure that the primer was not overlapping the repeat sequence, there were no poly-'A' or poly-'T' runs for more than seven base pairs within the sequence, and that there was only one repeat motif between the primers.

Sixty potentially suitable microsatellite loci were identified and selected for initial screening using DNA from six individuals selected from different populations. Each marker was amplified in a 6-µL reaction volume containing PCR buffer, Bioline IMMOLASE DNA polymerase and dNTPs (Bioline Reagents Ltd., London, United Kingdom) based on the recommendations provided by Bioline, 1.5 mM $MgCl_2$, 0.06 µM of M13-labeled forward locus–specific primer, 0.13 µM of reverse locus–specific primer, 0.13 µM of fluorescently labeled M13 primer (FAM [Sigma-Aldrich, St. Louis, Missouri, USA]; NED, VIC, and PET [Invitrogen/Thermo Fisher Scientific, Waltham, Massachusetts, USA]), and 15 ng gDNA. The following PCR conditions were used: 94°C for 5 min; followed by 11 cycles at 94°C for 30 s, 60°C for 45 s (decreasing 0.5°C per cycle), and 72°C for 45 s; followed by 30 cycles at 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s; followed by 15 cycles at 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s; and a final elongation step at 72°C for 10 min. Thermocycling was performed with an Applied Biosystems 384-well Veriti Thermal Cycler (Life

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TABLE 1. Characteristics of 17 microsatellite loci developed in *Acacia adinophylla*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Fluorescent label[b]	GenBank accession no.
aacur4	F: ATGGCGGCGAAGATAGCTTT	$(AGA)_6$	120–146	VIC	Pr032816334
	R: GTTTGCATCTCACGCGTCTC				
aacur5	F: GCTCCAGCGACGATCATACA	$(TA)_6$	129–137	FAM	Pr032816335
	R: TGTCTGCATCACCAAGGACC				
aacur11	F: AACCAGGAAAGACCAGCAGG	$(GA)_6$	144–172	PET	Pr032816326
	R: TCAGTTGCCAGAGTAGCTCC				
aacur19	F: TGAACACCGAGGCGAGAATC	$(GA)_6$	167–189	PET	Pr032816327
	R: CCCTGTTCTTCAGCCTCCAG				
aacur20	F: TGGAAGGAGGGCATTTCAGG	$(GA)_6$	173–183	VIC	Pr032816328
	R: CCGAGTGGTGAAGGAGTTGG				
aacur21	F: CGATCTCACAACGTGGAGCT	$(GT)_6$	180–194	FAM	Pr032816329
	R: AGATGTGAGGCCACTTGAGC				
aacur25	F: CCTGAAACCAGGTGGAAGCT	$(AGA)_6$	183–193	FAM	Pr032816330
	R: CTGATCCTTCGGAGGCAGAC				
aacur26	F: CTGAAATTGGGCAGGGAGGA	$(AGA)_6$	178–205	NED	Pr032816331
	R: TCTTCAAGCTCGCCTGGATC				
aacur29	F: TCGGGCAAGGCATTCAGATT	$(CCA)_6$	192–204	FAM	Pr032816332
	R: GTGGCTGACATGTGGGAAGA				
aacur32	F: GCACAACCACAGTGATGCAT	$(AATT)_6$	202–214	VIC	Pr032816333
	R: TTCACCGTTTGGAGAGTGGA				
aacur52	F: ATACGAGCATGTGCAGTGCA	$(AC)_6$	255–285	VIC	Pr032816336
	R: CCATCGGGATTTGGGTAGCA				
aacur58	F: AGCCTCTGAAGGTGCATTCC	$(TCA)_6$	254–275	NED	Pr032816337
	R: AGTCCAGTAACAGAAATACCGTGA				
aacur9[c]	F: CCGAGCCCTGGTTATCTTCC	$(CT)_6$	129–151	FAM	Pr032816398
	R: AATCCTGGCCTTGCTACGAC				
aacur14[c]	F: GCGAGCATAGAGGACGACAA	$(AAG)_6$	142–161	NED	Pr032816394
	R: GGTTGTGGCCATGGATGAGA				
aacur34[c]	F: ACGAGTGAAGACGGTGATGG	$(AGG)_6$	208–235	NED	Pr032816395
	R: CCACCGTAACGAAATCTGGGA				
aacur36[c]	F: GGTTCAGAGCAGGAGGATGG	$(AAG)_6$	254–273	VIC	Pr032816396
	R: CCCTCAATCAATCATTTGGCCC				
aacur53[c]	F: ACACGGGCATCATCACAACA	$(TGT)_6$	251–279	FAM	Pr032816397
	R: CTTCCGATGGCGAGTTGAGT				

[a]An annealing temperature of 60°C was used for all loci.
[b]Forward 5′ label.
[c]Marker not selected; size range values based on six individuals (see Methods and Results section).

Technologies). For a given panel, the markers were pooled together for each sample, 1 µL of pooled sample was then applied to a 10-µL mixture of Applied Biosystems Hi-Di Formamide and GeneScan 500 LIZ Size Standard (Life Technologies). This was then heated at 95°C for 5 min. Capillary electrophoresis of the product was performed by an Applied Biosystems 3730 DNA Analyzer (Life Technologies). Running time for a 96-well plate was approximately 1 h (230 V, 32 A). Allele sizes were determined using Geneious version 7.1 (Biomatters Ltd., Auckland, New Zealand). Of these 60 loci, 17 produced readable electropherograms, but five were excluded from further analyses because they amplified inconsistently or were difficult to score accurately (Table 1). Subsequently, 12 loci were selected to complete the study using the conditions described above. We tested for linkage disequilibrium among loci using FSTAT version 2.9.3.2 (Goudet, 1995), and sequential Bonferroni corrections were applied to alpha values in the determination of significance to correct for multiple comparisons of linkage disequilibrium (Rice, 1989). Departure from Hardy–Weinberg equilibrium was assessed for each locus by χ^2 tests in GenAlEx version 6.5 (Peakall and Smouse, 2006), and the possibility of null alleles was checked using MICRO-CHECKER version 2.2.3 (van Oosterhout et al., 2004). Standard measures of genetic variation including observed and expected heterozygosity and the number of alleles were calculated using GENODIVE (Meirmans and Van Tienderen, 2004).

We did not find any evidence of linked loci after Bonferroni corrections, and there was no consistent departure from Hardy–Weinberg equilibrium or evidence for null alleles, for any locus, across all sites. Overall we observed 3–18 alleles per locus, and observed and expected heterozygosities ranged from 0.14 to 0.92 and 0.13 to 0.85, respectively (Table 2). Assessment of cross transferral of loci to closely related taxa was not possible given project resources, timelines,

TABLE 2. Results of primer screening of 12 polymorphic loci identified in three populations (AA 5, AA 10, and AA 11) of *Acacia adinophylla*.[a]

	AA 5			AA 10			AA 11		
Locus	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e
aacur4	10	0.79	0.84	9	0.81	0.82	9	0.85	0.84
aacur5	4	0.29	0.48	3	0.33	0.58	3	0.27	0.64*
aacur11	8	0.92	0.81	7	0.90	0.81	5	0.85	0.78
aacur19	8	0.79	0.82	8	0.67	0.83	8	0.85	0.85
aacur20	4	0.21	0.19	2	0.14	0.13	4	0.23	0.21
aacur21	5	0.58	0.68	6	0.57	0.72	4	0.47	0.57
aacur25	3	0.58	0.50	3	0.55	0.45	2	0.62	0.57
aacur26	4	0.35	0.52	6	0.57	0.72	4	0.62	0.51
aacur29	4	0.25	0.29	3	0.19	0.18	2	0.15	0.26
aacur32	4	0.83	0.70	4	0.57	0.69	4	0.77	0.65
aacur52	9	0.68	0.74	10	0.81	0.76	8	0.77	0.76
aacur58	6	0.82	0.78	4	0.62	0.66	4	0.62	0.71

Note: *A* = number of alleles sampled; H_e = expected heterozygosity; H_o = observed heterozygosity.

[a]Values are based on samples from three populations in the Helena and Aurora Range of Western Australia. Twenty individuals were genotyped from each population (AA 5 UTM coordinates: 759053E, 6638224N; AA 10 UTM coordinates: 759142E, 6639102N; AA 11 UTM coordinates: 761276E, 6643346N).

* Significant deviation from Hardy–Weinberg equilibrium ($P < 0.001$).

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and the geographic distribution of suitable related taxa. The sequences of the microsatellite loci have been deposited in GenBank.

CONCLUSIONS

The 12 microsatellite loci developed for *A. adinophylla* in this study will enable the quantification of the potential impact of mining on genetic variation within the species and establish a baseline for future management of genetic variation. These markers add to molecular tools that are available to examine banded iron formation species (e.g., Nevill et al., 2010), and ultimately they will facilitate the expansion of our understanding of the genetics of the short-range endemic flora of this habitat and inform restoration strategies, should mining proceed.

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