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TECHNICAL NOTE

Microsatellite markers for vulnerable Australian aridzone Acacias

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Abstract Several Australian arid zone *Acacia* species are under threat because of decades of fruiting and recruitment failure that may reflect the loss of genetic diversity within small and isolated populations. We developed primers for eight microsatellite loci for *Acacia carneorum* and *Acacia loderi*. We detected high levels of clonality in each of two stands of *A. carneorum* (1 and 2 genets). In contrast, one stand of *A. loderi* was wholly clonal (1 genet), while in a second there were 30 unique genotypes. These loci allow assessment of the genetic diversity and connectedness of populations, the relative contribution of asexual reproduction to genotypic diversity and population structure, and use of paternity analysis to identify sires of seed within populations known to have set seed in past decades. This type of information may provide a basis for a recovery plan based on 'genetic rescue'.

Keywords Perennial plant · Recruitment failure · Genetic diversity · Sexual and asexual reproduction

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Australian arid zone plant communities have been modified considerably over the past 200 years through land clearing for agriculture, the introduction of exotic weeds, and grazing by domestic and feral herbivores. This has produced habitat loss, degradation, and fragmentation, with major effects on ecosystem function and biodiversity (Morton et al. 1995; Woinarski and Fisher 2003). In particular, several species of *Acacia* are threatened because of infrequent seeding and chronic recruitment failure (Crisp and Lange 1976; Auld 1993, 1995; Denham and Auld 2004) that may reflect low genetic diversity in now small and isolated populations. The failure to produce viable or fit seed may be due to the disruption of native pollination systems (Cunningham 2000a, b), or gamete wastage through pollen transfer among senescing or reproductively incompatible conspecifics (Pickup and Young 2008).

We developed primers for microsatellite loci for the 'vulnerable' purple-wood wattle, *Acacia carneorum* Maiden, and nealie, *A. loderi* Maiden, the dominant overstorey tree of the 'endangered *A. loderi* Shrublands' (New South Wales Threatened Species Conservation Act 1995). Both species are slow-growing perennials, capable of reproducing sexually and asexually (via vegetative suckers). *Acacia carneorum* occurs in western NSW and eastern South Australia, where it forms small, naturally fragmented stands, on red-sand dunes (Maslin 2001). For decades, seed production has been rare or absent in most populations, despite annual flowering (Auld 1993; Auld and Denham 2001). In partial contrast, *A. loderi* is distributed over the eastern margin of the semi-arid, with large stands occurring in western NSW, and isolated smaller stands in SA and Victoria (Chapman and Maslin 2001).

We used GS-FLX Titanium sequencing to generate a database of DNA sequence. Genomic DNA was extracted using a DNeasy Plant Mini Kit (QIAGEN), with multiple

Table 1 Microsatellite loci for *Acacia carneorum* and *A. loderi*[c]

Locus	Primer sequence (5′–3′) F, Forward: R, Reverse	Microsatellite repeat[c] copies of the repeated motif	Fluorescent dye	Primer concentration (nM)	Allele size range (bp)
A. carneorum					
C0F94	F: CAGGCTGGACTTGAGCGAG	(AC)[c]10	6FAM	40	210
	R: AGAGGACACAGGTGGTATGC				
C03P6	F: CTCTCTCACAATTTCCTCACCG	(AC)[c]10	VIC	20	128–130
	R: TAAGTTGTGCGTCCATATTGTTCG				
EF03V	F: GCCTACAAGAAAGAAGCCCAC	(CT)[c]12	VIC	40	226–228
	R: TCCCCTCCAAGGCTCAAAC				
CTFGS	F: AGTAGCGCGAGCATCCTTC	(AT)[c]12	PET	40	240–242
	R: CCCAGTCTCTTCCCAAGCC				
AQBUV	F: AGGAGCTGATGAAGTGGGG	(GT)[c]10	PET	20	209–215
	R: GGAATGTGTGCCAGCAACG				
ARU19	F: ACGAGCGGGTCCTAAATGC	(CT)[c]12	NED	20	232
	R: GGAGGAGGGTGAGTGAGTG				
CDRBZ	F: GCAACGGTGCTCCAATCTC	(AG)[c]10	NED	10	258–262
	R: TTCTCTTCCGCCTTCCACC				
EEHI7	F: AGGCATGGACAAATTTCACAC	(AT)[c]12	VIC	40	180–202
	R: ACGACGTAGTTTTGAGTGACTTG				
A. loderi					
A4IKI[a]	F: TCAACGACGACGACTTGGG	(AT)[c]15	6FAM	10	234–249
	R: TCGTTAGGGGTAGGTGGATG				
BKTKQ	F: CGGATTACCCTATGCGATGC	(AT)[c]12	NED	20	204–210
	R: TCCTGATTGGGCCGTCTAC				
BNQS6[b]	F: CACACTTATGGGATGGGTTGC	(AAT)[c]14	NED	40	281–339
	R: TCGATTCCTTTCACATGCCC				
DCL0C[b]	F: CAACTTGTGATTAAAGTCCACGG	(CT)[c]11	6FAM	10	128–160
	R: TGTGTTGAGACTTTGTGCTG				
DSGN5	F: ACCTGGTGGTGAAAGGAGAAG	(AG)[c]12	VIC	20	204–226
	R: TTCCATGGCCGTGTTTCTG				
A472O	F: AGGAAGGGAGGCTGAATGC	(AT)[c]12	PET	40	259–271
	R: CCGGGTCTAACCCAAGTCC				
DZ7O9	F: GCCACTTCCTACAGAAACCAAG	(GT)[c]12…(AG)[c]6	PET	20	196–218
	R: CCTAGGCAGGGCAGTAGTC				
AO35A	F: AACGAAAGGCCACAAAGGC	(GT)[c]13	6FAM	20	196–240
	R: TTCTCCCTCCCATTCGGTC				

[a, b] Denotes loci discovered in *A. carneorum* and *A. melvillei*, respectively, and transferred successfully to *A. loderi*

[c] The DNA sequence from which each locus was isolated is contained in online resource 1 of the online supplementary material

extracts from a single individual pooled to provide 5 μg of high-molecular weight DNA for library preparation. The library was prepared in accordance with the manufacturer's instructions (Roche Diagnostics Corporation) except that a species-specific oligonucleotide barcode was ligated to the sheared DNA, allowing data collection for multiple species in a single sequencing run. The sequencing was performed at the Otago Genomic Sequencing Unit, University of Otago, New Zealand. The program MSATCOMMANDER v0.8.1 (Faircloth 2008) was used to detect DNA sequences containing microsatellites, and to design primers for PCR assays.

We screened loci with greater than 10 uninterrupted microsatellite repeat motifs in one individual from each of six stands distributed across the range of both species. To PCR amplify loci of interest we used Multiplex-Ready Technology (Hayden et al. 2008). The optimal primer concentration of each forward and reverse locus-specific

primer was determined in preliminary PCR assays varying the primer concentration between 5 and 120 nM (Table 1).

Polymorphism for the eight loci that consistently amplified a product of the expected size in duplicate PCR assays was tested within two NSW stands of each species (*A. loderi*: ALOD1/ALOD2; *A. carneorum*: ACAR1/ACAR2). We sampled phyllodes of 30 plants, separated by at least 10 m to reduce the probability of sampling identical genets. We used the program GenClone to estimate the probability that n (where $n = 1, 2, 3\ldots$,i) repeated identical multilocus lineage's (MLL's) were produced by distinct episodes of sexual reproduction, P_{sex} (Arnaud-Haond et al. 2007; Arnaud-Haond and Belkhir 2007). Where $P_{sex} < 0.05$ it is improbable that n repeated MLL's were derived by sex alone, indicating a contribution of asexual reproduction to the genotypic composition of each stand. We detected 30 distinct genets in ALOD1. In contrast, all 30 plants in ALOD2 were identical, with up to 7 ($P_{sex} = 0.073$) occurrences of this genet possibly the product of sexual reproduction. As for ALOD2, ACAR1 and ACAR2 were highly clonal, with 1 and 2 genets, respectively. All subsequent analyses of genetic diversity were conducted on the sexually derived MLL's.

We used GenAlEx 6.4 (Peakall and Smouse 2006) to estimate genetic diversity as the average number of alleles per locus (A), and observed and expected heterozygosity (H_o and H_e). For ALOD1 (the wholly sexually derived *A. loderi* stand), we tested for Hardy–Weinberg and linkage equilibrium (Rousset 2008; Raymond and Rousset 1995). A ($\pm$SE) was 8.8 ± 1.3 and 1.4 ± 0.2 for ALOD1 and ALOD2, respectively, while H_o and H_e was 0.488 ± 0.054 and 0.755 ± 0.041, and 0.375 ± 0.183 and 0.188 ± 0.091. In ALOD1, there were large deficits of heterozygotes, and all loci departed from HW equilibrium ($P < 0.05$), which suggests inbreeding. None of the pair-wise tests of linkage equilibrium were significant ($P > 0.05$). Estimates of genetic diversity were extremely low in both stands of *A. carneorum* (ACAR1: $A = 1.4 \pm 0.2$, $H_o = 0.375 \pm 0.183$, $H_e = 0.188 \pm 0.091$; ACAR2: $A = 1.6 \pm 0.2$, $H_o = 0.571 \pm 0.175$ $H_e = 0.301 \pm 0.089$).

These loci clearly provide molecular tools to assess the contribution of sexual and asexual reproduction to genotypic diversity and population structure, and to estimate a range of mating system parameters. This information will be crucial to any management or recovery plan that seeks to ensure the long-term conservation of these iconic *Acacia* spp. through translocation or augmentation, including 'genetic rescue' through experimental hand pollination.

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