1-1-2015

Microsatellite primers for vulnerable and thriving acacia (Fabaceae) species from Australia's arid zone

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Abstract

Premise of the study: Microsatellite markers were developed for the common arid Australian shrub Acacia ligulata (Fabaceae) and the threatened overstory trees A. melvillei and A. pendula. Methods and Results: DNA sequence data generated by 454 sequencing were used to identify microsatellite nucleotide repeat motifs. Including previously developed primer sets, we report on the development of 10 polymorphic microsatellite loci for each species. Six of these were novel for A. melvillei and A. ligulata, and five were novel for A. pendula, while five more each were transferred from primers developed for related species (A. carneorum and A. loderi). We found three to 17 alleles per locus for each species, with high multilocus genotypic diversity within each of two A. ligulata and A. pendula stands, and one A. melvillei population. A second A. melvillei stand appeared to be monoclonal. Conclusions: These markers will allow assessment of population genetics, mating systems, and connectedness of populations of these and possibly other arid-zone acacias.

Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

Publication Details

Forrest, C. N., Roberts, D. G., Denham, A. J. & Ayre, D. J. (2015). Microsatellite primers for vulnerable and thriving acacia (Fabaceae) species from Australia's arid zone. Applications in Plant Sciences, 3 (4), 1400121-1 - 1400121-5.

Microsatellite Primers for Vulnerable and Thriving *Acacia* (Fabaceae) Species from Australia's Arid Zone

Author(s): Cairo N. Forrest, David G. Roberts, Andrew J. Denham, and David J. Ayre
Source: Applications in Plant Sciences, 3(4)
Published By: Botanical Society of America
DOI: http://dx.doi.org/10.3732/apps.1400121
URL: http://www.bioone.org/doi/full/10.3732/apps.1400121

Applications in Plant Sciences

Applications in Plant Sciences 2015 3(4): 1400121

Primer Note

Microsatellite primers for vulnerable and thriving *Acacia* (Fabaceae) species from Australia's arid zone[1]

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- *Premise of the study:* Microsatellite markers were developed for the common arid Australian shrub *Acacia ligulata* (Fabaceae) and the threatened overstory trees *A. melvillei* and *A. pendula*.
- *Methods and Results:* DNA sequence data generated by 454 sequencing were used to identify microsatellite nucleotide repeat motifs. Including previously developed primer sets, we report on the development of 10 polymorphic microsatellite loci for each species. Six of these were novel for *A. melvillei* and *A. ligulata*, and five were novel for *A. pendula*, while five more each were transferred from primers developed for related species (*A. carneorum* and *A. loderi*). We found three to 17 alleles per locus for each species, with high multilocus genotypic diversity within each of two *A. ligulata* and *A. pendula* stands, and one *A. melvillei* population. A second *A. melvillei* stand appeared to be monoclonal.
- *Conclusions:* These markers will allow assessment of population genetics, mating systems, and connectedness of populations of these and possibly other arid-zone acacias.

Key words: *Acacia*; Fabaceae; genetic diversity; perennial plant; recruitment failure; sexual and asexual reproduction.

Several Australian arid-zone acacias are threatened by habitat loss, degradation, and fragmentation resulting from agricultural activities and exotic herbivores (Morton et al., 1995), although others, including *Acacia ligulata* A. Cunn. ex Benth., are thriving. Two long-lived and potentially clonal species facing a variety of potential threats are *A. melvillei* Pedley and *A. pendula* A. Cunn. ex G. Don. Both of these latter species likely suffer from infrequent seed production and chronic recruitment failure (Batty and Parsons, 1992). Moreover, there is some debate about the origin and taxonomy of stands of *A. pendula* found in the Hunter region of New South Wales (Bell et al., 2007), the extreme eastern range edge of its distribution and a notable anomaly for this species, given its predominate semiarid/arid distribution in four Australian states. A clear understanding of the factors underlying the variation in the performance of these three species is hampered by a lack of genetic tools that allow assessment of the mating and dispersal and genetic diversity of remaining stands.

The three target species have partially overlapping ranges. "*Acacia melvillei* shrubland" endangered ecological community occurs in semiarid and arid eastern Australia. This community is considered threatened primarily because of senescence of the overstory (dominated by *A. melvillei*), infrequent seed set, and recruitment failure due to overgrazing (NSW Scientific Committee, 2008). *Acacia pendula* is more widespread, occurring throughout the eastern semiarid zone, but is considered threatened within the Hunter Valley (NSW Scientific Committee, 2008). In contrast, *A. ligulata* is one of the most widespread *Acacia* species, occurring throughout arid Australia. Seed set occurs annually in this species, recruits are common (personal observation), and most stands appear to be thriving (personal observation). For each of these species, we developed primers that amplify microsatellite loci. By comparing and contrasting the genetic structure of populations of these species with partially overlapping distributions and perceived variation in reproductive success, we aim to gain insights into the impact of anthropogenic disturbance on their genetic structure and diversity and, together with demographic assessments, will seek to use these data to predict the resilience of remaining stands.

METHODS AND RESULTS

We used GS FLX Titanium sequencing (Roche Diagnostics Corporation, Sydney, Australia) to generate databases of DNA sequences for *A. melvillei* and *A. pendula*. Specimens of each species were sourced from stands located in western New South Wales. Genomic DNA was extracted using a DNeasy Plant Mini Kit (QIAGEN, Melbourne, Australia). Multiple DNA extracts from the same individual were pooled to obtain 5 µg of high-molecular-weight DNA for library construction. The library was prepared in accordance with the manufacturer's instructions (Roche Diagnostics Corporation), and the sequencing was performed at the Otago Genomic Sequencing Unit, University of Otago, New Zealand, using the GS FLX system with the GS FLX Titanium Rapid Library Preparation Kit (catalog no. 05608228001; Roche Diagnostics Corporation). Upon receipt of the DNA sequence databases from the University of Otago, we used the program MSATCOMMANDER version 0.8.1 (Faircloth, 2008) to detect DNA sequences containing di-, tri-, and tetranucleotide repeats, and to design microsatellite primers for PCR assays.

[1]Manuscript received 29 December 2014; revision accepted 17 February 2015.

This work was supported by an Australian Research Council (ARC) linkage grant to D.J.A. and A.J.D. and by the University of Wollongong Institute for Conservation Biology and Environmental Management.

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doi:10.3732/apps.1400121

Table 1. Novel microsatellite loci for *Acacia melvillei*, *A. ligulata*, and *A. pendula*.[a]

Locus[b]	Primer sequences (5′–3′)	Repeat motif	Fluorescent dye	Primer conc. (nM)	Allele size range (bp)	Cross-species amplification[c]	GenBank accession no.
A. melvillei							
CPUH4^{Ac}	F: AGATGCATTGACTGAGACGG R: CGAATGAAGGAGATTTATGAAGAGAC	$(AT)_{13}$	6-FAM	40	112–115	Al, Alig, Ap	KF776129
C51M0^{Am}	F: CTGCAAATCGTTTCTTCAAGCC R: ACAGAAATGAGCATGACCCC	$(CTTT)_6$	6-FAM	20	175–182	Al, Ac, Alig, Ap	KF776130
BBY8P^{Al}	F: TTGGCAAATCCGCACAGTC R: TGCCATCGCAACATATAGCTTC	$(GT)_{11}$	VIC	20	126–146	Ac, Alig, Ap	KF776131
AV9GR^{Al}	F: CCAACGACAGTGGGCAGTC R: CTCCGGTGTTAGCAAAGGC	$(AT)_{14}$	PET	10	185–200	Ac, Alig, Ap	KF776132
BA1R8^{Am}	F: GGTGCTTTTCCCCACCTTC R: TCTCGCTTTTCATGTGCAAG	$(GAA)_8$	NED	10	245–258	Al, Ac, Alig, Ap	KF776133
CIDYF^{Am}	F: CACACTTATGGGATGGGTTGC R: AGCTAAGGAAAGTGTACGGGAAT	$(AAT)_{14}$	VIC	20	290–340	Al, Ac, Alig, Ap	
A. ligulata							
BVWHY^{Ac}	F: TCCTACTTCCCCAACACGC R: ACAAGCAGCCATTGGAAGG	$(AT)_{12}$	6-FAM	60	192–235	Am, Al, Ap	KF776134
APZIZ^{Ac}	F: ACACTACACTCACAACACACAC R: ACACGGTTTGCTTGGCTTG	$(AC)_{11}$	VIC	20	222–250	Am, Al, Ap	KF776135
A47K4^{Ac}	F: CGAATCGGGAGAGTGGGAG R: ACCCAACCCAGTCCAATCC	$(AT)_{10}$	6-FAM	20	228–252	Am, Al, Ap	KF776136
BBY8P^{Al}	F: TTGGCAAATCCGCACAGTC R: TGCCATCGCAACATATAGCTTC	$(GT)_{11}$	PET	20	139–159	Am, Ac, Ap	KF776131
AO12C^{Ac}	F: AAAACAAGAGAAGAGGACATGC R: TCGTAGAAACGACACGAAACG	$(AT)_{12}$	6-FAM	20	280–350	Am, Al, Ap	KF776128
CU0EQ^{Am}	F: ACCACCATCTTCACCTCCAC R: TCCGGCGTTTCCAACTAAC	$(GGGA)_7$	6-FAM	40	190–220	Al, Ac, Ap	KF776137
A. pendula							
ACPU7^{Al}	F: GTTCTACGGCTAGATGGTGC R: TGTCATACGGCCTCACAAAG	$(AC)_{12}(AT)_{10}$	PET	20	151–191	Am, Ac, Alig	KP161852
BA1R8^{Am}	F: GGTGCTTTTCCCCACCTTC R: TCTCGCTTTTCATGTGCAAG	$(GAA)_8$	VIC	20	240–256	Al, Ac, Alig	KF776133
BBY8P^{Al}	F: TTGGCAAATCCGCACAGTC R: TGCCATCGCAACATATAGCTTC	$(GT)_{11}$	VIC	20	135–173	Am, Ac, Alig	KF776131
C51M0^{Am}	F: CTGCAAATCGTTTCTTCAAGCC R: ACAGAAATGAGCATGACCCC	$(CTTT)_6$	NED	20	170–190	Al, Ac, Alig	KF776130
CYD8I^{Ap}	F: GACCTCAAGCAAGACAAGCC R: ACAACGCTGCTCATACATGC	$(AC)_{22}$	NED	40	426–454	Al, Ac	KP161853
DBGX4^{Ap}	F: CCTCCTCCCTTATTCCCTCAC R: AGAAGGCGATATGGACACCG	$(AG)_{10}$	PET	40	239–273	Al, Ac	KP161854
DNZTA^{Ap}	F: TGTCCACACAGAACCCGTC R: AGAGGCTCCGAAATCCAAGG	$(AG)_{10}$	6-FAM	40	171–221	Al, Ac	KP161855
C2Q63^{Ap}	F: TGCACAGTTCTAGGCTTCCC R: ACCCAAACCACCTACACCTC	$(AT)_{11}$	VIC	60	177–225	Al, Ac	KP161856
DE1HP^{Ap}	F: GCGGAGGTAGAAGGAGAGTC R: GCTCACGCCACAAGTATGAC	$(AAT)_9$	PET	40	167–203	Al, Ac	KP161857

[a]Annealing temperature for all primers is 55°C.

[b]Loci discovered in *A. melvillei*, *A. loderi*, *A. carneorum*, and *A. pendula* 454 sequencing data sets are identified as follows: *A. melvillei* = Am, *A. loderi* = Al, *A. carneorum* = Ac, *A. pendula* = Ap.

[c]Loci that were successfully cross-amplified in *A. melvillei* (Am), *A. loderi* (Al), *A. carneorum* (Ac), *A. ligulata* (Alig), and *A. pendula* (Ap), but not found to be as robust as other loci, or polymorphic enough for further use.

To PCR amplify loci of interest, we used Multiplex-Ready Technology. This method was developed by Hayden et al. (2008) and is briefly described below. For each species, 24 locus-specific primer sets were synthesized by Sigma-Aldrich (Sydney, Australia). We also made use of existing primers (obtained in the same way) that amplify microsatellite loci in *A. carneorum* Maiden and *A. loderi* Maiden (Roberts et al., 2013) to potentially increase the number of microsatellites available for use in *A. melvillei*, *A. pendula*, and *A. ligulata*. Each respective forward and reverse primer had the nucleotide sequence 5′-ACGACGTTGTAAAA-3′ and 5′-CATTAAGTTCCCATTA-3′ attached to its 5′-end. Tag primers, tagF (5′-ACGACGTTGTAAAA-3′) and tagR (5′-CATTAAGTTCCCATTA-3′), were also synthesized, with tagF 5′-end labeled with one of Applied Biosystems' (Carlsbad, California, USA) proprietary fluorescent dyes (VIC, FAM, NED, and PET). Each PCR assay contained 0.2 mM dNTP, 1× ImmoBuffer (Bioline, Alexandria, Australia), 1.5 mM $MgCl_2$, 100 ng/μL bovine serum albumin (BSA; Sigma-Aldrich), 75 nM each of dye-labeled tagF and unlabeled tagR primer, 0.15 units of Immolase DNA polymerase (Bioline), and 2 μL of genomic DNA (~10 ng/μL). 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) and also was included within each 10 μL (total volume) assay. PCRs were conducted on either a Bio-Rad (Hercules, California, USA) or Eppendorf (Hamburg, Germany) thermocycler with a denaturing step at 95°C, primer annealing step of 63°C, and an extension step at 72°C repeated for 40 cycles. Genomic DNA was extracted from phyllodes from one individual from each of five stands across the range of each species using a standard cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). For each species, we genotyped eight individuals separated by at least 10 m, from each of five stands separated by at least 30 km. This initial sampling allowed us to assess levels of polymorphism within and between stands, before primers were deemed sufficiently polymorphic to characterize population genetic structure.

We developed new polymorphic primers that had consistently clean profiles, six each for *A. melvillei* and *A. ligulata*, and five for *A. pendula* (Table 1).

Table 2. Multiplex PCR combinations achieved and fluorescent dyes used. Primers listed in Table 1 but absent here were not successfully multiplexed.

Species	Multiplex PCR combinations	Multiplex no.	Fluorescent dye
Acacia melvillei	CPUH4 / C5IM0 / BNQS6	1	FAM
	BBY8P / DZ7O9 / CIDYF	2	VIC
	AV9GR / BAIR8	3	PET
	DCL0C / DSGN5	4	NED
Acacia ligulata	DCL0C / BVWHY / AO12C	1	FAM
	C03PC6 / APZIZ	2	VIC
	BBY8P / A4IKI	3	NED
Acacia pendula	BBY8P / BAIR8	1	FAM

We were also able to cross-transfer 15 previously optimized loci, 11 of which are described in Roberts et al. (2013). Specifically, five of 11 primer sets amplified successfully and had equally clear profiles on electropherograms for *A. melvillei* (DCL0C, AO35A, DSGN5, BNQS6, and DZ7O9), *A. ligulata* (A4IKI, AQBUV, DCL0C, ARU19, and C03P6), and *A. pendula* (ACPU7, BAIR8, BBY8P, C5IMO, and DCLOC), respectively. This resulted in a total of 11 working primers each for *A. melvillei* and *A. ligulata*, and 10 for *A. pendula*. All other primers tested did not amplify consistently or were difficult to score because of complex stuttering of the amplified product. These primer sets were discontinued. Combinations of successful primers were trialed together in multiplex PCRs to look for repeatable and clean assays. Successful combinations of primers as multiplex PCRs, which were subsequently used for all further genotyping, are presented in Table 2.

Following our initial screening of loci described above, we preceded to genotype plants from two New South Wales populations of each species (*A. melvillei*: AMEL1, AMEL2; *A. ligulata*: ALIG1, ALIG2; *A. pendula*: APEN1, APEN2; Appendix 1) using 10 of the primer pairs developed for each plant species (Tables 3–5). All loci amplified consistently in duplicate PCR assays and were polymorphic with between three and 17 alleles per locus.

Because *A. melvillei* reproduces both sexually and asexually, we used GenClone to estimate the probability that n (where $n = 1, 2, 3\ldots i$) copies of a multilocus genotype were produced by distinct episodes of sexual reproduction, P_{sex} (Arnaud-Haond and Belkhir, 2007). Where P_{sex} is less than 0.05, it is improbable that n multilocus genotype copies were derived by sex alone.

All 30 plants in AMEL1 were identical, which far exceeds the maximum number of replicates of that genotype ($n = 7$) that is expected to result from sexual reproduction ($P_{sex} = 0.073$) with all replicates of $n > 7$ identical genotypes associated with P_{sex} values less than 0.05. In contrast, we detected 26 distinct genets in AMEL2, and it was improbable that the $n = 4$ replicated genotypes were produced by independent episodes of sexual reproduction ($P_{sex} < 0.001$), implying that while the vast majority of distinct genotypes in this stand were founded sexually, the replicate genotypes were produced by asexual reproduction. All *A. pendula* and *A. ligulata* plants were genetically distinct, with the exception of one pair in ALIG2. Levels of genetic diversity and expected genotypic diversity expressed as the average number of alleles per locus (A) and expected heterozygosity (H_e), respectively, were generally high for AMEL2, APEN1, APEN2, ALIG1, and ALIG2 (Table 2). However, average inbreeding within populations (F_{IS}) scores across all loci indicated significant deficits of heterozygotes in all five populations, suggesting inbreeding is a common phenomenon in these species (Tables 3–5). None of the pairwise tests for linkage equilibrium revealed significant associations between loci ($P > 0.05$).

Table 3. Levels of genetic diversity and expected genotypic diversity for a nonclonal population of *Acacia melvillei*.

	AMEL2 ($N = 30$)		
Locus	A	H_e[a]	F_{IS}
CPUH4_a	4	0.71	0.48
C5IMO_a	5	0.44	0.54
BBY8P_a	8	0.54	0.23
DZ709_a	18	0.90	0.31
AV9GR_a	8	0.80	0.59
BAIR8_a	6	0.55	0.20
DCLOC_a	9	0.81	0.49
DSKN5_a	13	0.86	0.23
CIDYF_a	9	0.72	0.40
AO35A_a	9	0.68	0.36
Average across all loci	8.9 ± 1.29	0.70 ± 0.05	0.38 ± 0.04

Note: A = number of alleles per locus; F_{IS} = inbreeding within populations; H_e = expected heterozygosity; N = number of individuals sampled.
[a] Significant deviation from Hardy–Weinberg equilibrium for all loci at $P < 0.05$.

CONCLUSIONS

These polymorphic markers have proved effective in estimating levels of genetic diversity within populations of these three acacias (*A. pendula*, *A. ligulata*, and *A. melvillei*) and partitioning of variation within and among populations. Moreover, these primer sets can be used to compare levels of genetic diversity and structure within species as part of the process of investigating reproductive failure in *A. melvillei* and *A. pendula.* The amplification of DNA extracted from adult leaf material and the embryo of seeds enables estimation of mating system parameters and the assessment of the relative past contributions of sexual and asexual reproduction within and among populations and species. In this initial study, we found evidence of inbreeding in all three species, suggesting a history of isolation. We also identified a high degree of clonality in one population of *A. melvillei*, a

Table 4. Levels of genetic diversity and expected genotypic diversity for two nonclonal populations of *Acacia ligulata*.

	ALIG1 ($N = 30$)			ALIG2 ($N = 30$)		
Locus	A	H_e[a]	F_{IS}	A	H_e[a]	F_{IS}
DCLOC_a	11	0.85	0.20	6	0.79	0.39
BVWHY_a	7	0.77	0.42	5	0.29	0.43
CU3P6_a	11	0.86	0.34	10	0.85	0.55
AP212_a	10	0.86	0.30	9	0.84	0.35
BBY8P_a	16	0.91	0.27	15	0.90	0.39
A4IKI_a	4	0.63	0.27	6	0.61	0.40
AQBUV_a	15	0.88	0.20	9	0.81	0.62
A47K4_a	8	0.75	0.42	4	0.45	0.53
CU0EQ_a	10	0.80	0.30	8	0.71	0.45
AO12C_a	10	0.82	0.28	8	0.67	0.49
Average across all loci	10.2 ± 1.11	0.81 ± 0.02	0.29 ± 0.04	8.0 ± 0.99	0.69 ± 0.06	0.47 ± 0.04

Note: A = number of alleles per locus; F_{IS} = inbreeding within populations; H_e = expected heterozygosity; N = number of individuals sampled.
[a] Significant deviation from Hardy–Weinberg equilibrium for all loci at $P < 0.05$.

TABLE 5. Levels of genetic diversity and expected genotypic diversity for two nonclonal populations of *Acacia pendula*.

	APEN1 (N = 30)			APEN2 (N = 30)		
Locus	A	H_e[a]	F_{IS}	A	H_e[a]	F_{IS}
ACPU7	12	0.861*	0.303	10	0.793*	0.370
BA1R8	3	0.633*	0.684	3	0.593**	0.606
BBY8P	15	0.898***	0.109	10	0.816^{NS}	–0.063
C51M0	5	0.634^{NS}	–0.157	3	0.559***	–0.311
DCL0C	10	0.850*	0.569	10	0.788^{NS}	–0.016
CYD8I	7	0.807*	0.445	8	0.651^{NS}	0.129
DBGX4	9	0.867^{NS}	0.039	11	0.818^{NS}	–0.100
DNZTA	8	0.782^{NS}	0.105	9	0.696*	0.569
C2Q63	9	0.808^{NS}	0.092	7	0.616***	0.189
DE1HP	7	0.718*	0.424	4	0.559^{NS}	0.285
Average across all loci	8.5 ± 1.1	0.786 ± 0.030*	0.261 ± 0.084	7.5 0.689 ± 0.034* 1.0	0.689 ± 0.034*	0.166 ± 0.094

Note: A = number of alleles per locus; F_{IS} = inbreeding within populations; H_e = expected heterozygosity; N = number of individuals sampled; NS = not significant.

[a] Significant deviations from Hardy–Weinberg equilibrium at *P < 0.001, **P < 0.01, ***P < 0.05.

phenomenon which, if widespread, may influence the choice of conservation actions. For the threatened *A. melvillei*, further landscape-level assessment of genetic diversity and structure, across a wider range of populations, will allow us to estimate historic levels of connectivity, identify populations containing novel genotypes, and assess the suitability of strategies such as genetic rescue. Ultimately, such strategies will inform management via translocation or augmentation. Our success in cross-amplifying markers among *Acacia* species implies that at least some of these primers will be transferable to other acacias. This study represents the first attempt to characterize the genetic structure of these three important overstory *Acacia* species.

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APPENDIX 1. Voucher and location information for *Acacia* spp. populations used in this study. All vouchers were deposited in the Janet Cosh Herbarium at the University of Wollongong, Australia.

Population reference	Species	Collection date	Locality	Geographic coordinates	*N*	Voucher no.	Herbarium ID
ALIG1	*Acacia ligulata*	25 September 2013	Big Dune, Kinchega National Park, New South Wales	32.53235°S, 142.16016°E	30	*AJD355*	10843
ALIG2	*Acacia ligulata*	25 September 2013	Near Lake Menindee, Kinchega National Park, New South Wales	32.37642°S, 142.39462°E	30	*AJD356*	10844
AMEL1	*Acacia melvillei*	6 January 2012	38 km SSW Barnato Lake on Tilpa Rd., New South Wales	31.93420°S, 144.87594°E	30	*AJD345*	10842
AMEL2	*Acacia melvillei*	15 September 2010	5 km W of Emmdale on the Barrier Hwy., New South Wales	31.66016°S, 144.25639°E	30	*AJD336*	10845
APEN1	*Acacia pendula*	2 March 2010	6 km NW of Tharbogang on road to Tabbita, New South Wales	34.20632°S, 145.95525°E	30	N/A	11111
APEN2	*Acacia pendula*	10 March 2010	30 km E of Hay on Sturt Hwy., New South Wales	34.50677°S, 145.17246°E	30	*AJD309*	11099

Note: *N* = number of individuals sampled.