MICROSATELLITE PRIMERS FOR VULNERABLE AND THRIVING ACACIA (FABACEAE) SPECIES FROM AUSTRALIA’S ARID ZONE

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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.

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### A. melvillei

**CPUH4**
- **F:** AGATGCATTGACTGAGACGG (AT)$_{13}$ 6-FAM 40 112–115 Al, Alig, Ap KF776129
  - **R:** AGATTGAGGAGGATTATGTGAAGACG
- **C51M0**
  - **F:** CTCGAAATCGTTTCTCAAGGC (CTTT)$_{6}$ 6-FAM 20 175–182 Al, Ac, Alig, Ap KF776130
  - **R:** AGCAGATGGCAGACATCC
- **BBY8**
  - **F:** TGGGCAAATCGTTTCTCAAGGC (GT)$_{11}$ VIC 20 126–146 Ac, Alig, Ap KF776131
  - **R:** CACACAGACTGCGACCTCT
- **A9GR**
  - **F:** CACACAGACTGCGACCTCT (AT)$_{14}$ PET 10 185–200 Ac, Alig, Ap KF776132
  - **R:** CTCCGGTGGTTGCAAGGC
- **BA1R8**
  - **F:** GTGGCTTTTCCCCACCTCTC (GAA)$_{6}$ NED 10 245–258 Al, Ac, Alig, Ap KF776133
  - **R:** TCTCGGTCTTCATGCGAAG
- **CIDYF**
  - **F:** CACACTTAATGGAGATGTTTGC (AAT)$_{14}$ VIC 20 290–340 Al, Ac, Alig, Ap KF776134
  - **R:** AGCTAGAGAAGTGATCAGGGGAT

### A. ligulata

**BTTYH**
- **F:** TCTTACTTCCCCAACAACCGC (AT)$_{12}$ 6-FAM 60 192–235 Am, Al, Ap KF776134
  - **R:** AACAAGACAGTTGGAAGGG
- **APZI**
  - **F:** ACATCAACACTCACAACACAC (AC)$_{11}$ VIC 20 222–250 Am, Al, Ap KF776135
  - **R:** ACAACCGTTTGCTCGTCGTT
- **A7K4**
  - **F:** CGATCGGAGAGGAGTGAGGAG (AT)$_{10}$ 6-FAM 20 228–252 Am, Al, Ap KF776136
  - **R:** ACCAACCAGTCCAACTCC
- **BBY8**
  - **F:** TGGGCAAATCGTTTCTCAAGGC (GT)$_{11}$ PET 20 139–159 Am, Ac, Ap KF776131
  - **R:** TGGGCAAATCGTTTCTCAAGGC
- **AO12**
  - **F:** AAAACAGAAGAGGAGGATGTC (AT)$_{12}$ 6-FAM 20 280–350 Al, Ac, Ap KF776128
  - **R:** TCTGAGAAACGACAGCAAGAC
- **CU0E**
  - **F:** ACCACACATCCTACACCCAC (GGGA)$_{7}$ 60 190–220 Al, Ac, Ap KF776137
  - **R:** TCGGCGGTCTTCCCCACTCAAC

### A. pendula

**ACPU**
- **F:** GTCTGACGCTAGTGAAGTGC (AC)$_{12}$ (AT)$_{10}$ PET 20 151–191 Am, Alig, Ap KF161852
  - **R:** GTCTGACGCTAGTGAAGTGC
- **BA1R8**
  - **F:** GTGGCTTTTCCCCACCTTC (GAA)$_{6}$ VIC 20 240–256 Al, Ac, Ap KF776133
  - **R:** TCTCGGTCTTCATGCGAAG
- **BBY8**
  - **F:** TGGGCAAATCGTTTCTCAAGGC (GT)$_{11}$ VIC 20 135–173 Am, Al, Ap KF776131
  - **R:** TGGGCAAATCGTTTCTCAAGGC
- **C51M0**
  - **F:** CTCGAAATCGTTTCTCAAGGC (CTTT)$_{6}$ NED 20 170–190 Al, Ac, Ap KF776130
  - **R:** AGCAGATGGCAGACATCC
- **CUDI**
  - **F:** AACCTCACTTCCTACCCAC (AC)$_{11}$ VIC 40 426–454 Al, Ap KF161853
  - **R:** ACACGGTCTGCATACATGC
- **DBGX4**
  - **F:** CCTCCCTCTTCTTCTTCTTCT (AG)$_{10}$ PET 20 239–273 Al, Ac, Ap KF161854
  - **R:** AGAAGGCGATGATGGAACCG
- **DNZTA**
  - **F:** GTGCCCAAGACACCGCT (AG)$_{10}$ 6-FAM 20 171–221 Al, Ac, Ap KF161855
  - **R:** AGAAGGCGATGATGGAACCG
- **CQ63**
  - **F:** TGGAGTTCTGCTAGTTCCTC (AT)$_{11}$ VIC 60 177–225 Al, Ac, Ap KF161856
  - **R:** AGAAGGCGATGATGGAACCG
- **DE1HP**
  - **F:** GCGAGGGTGAGAAGGAGTGCT (AAT)$_{14}$ PET 40 167–203 Al, Ac, Ap KF161857
  - **R:** CTCTCGCAGCAAGATGAGAC

*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. carneaorum* 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 below. For each species, 24 locus-specific primer sets were synthesized by Sigma-Aldrich.

Each PCR assay contained 0.2 mM dNTP, 1× ImmolBuffer (Bioline, Alexandria, Australia), 1.5 mM MgCl$_2$, 100 ng/μL bovine serum albumin (BSA; Sigma-Aldrich), 75 mM 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 of genomic DNA (~10 ng/μL) 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 sufficient to be as robust as other loci, or polymorphic enough for further use.

We developed new polymorphic primers that had consistently clean amplification profiles, six each for *A. melvillei* and *A. ligulata*, and five for *A. pendula* (Table 1).
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. 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 2. Multiplex PCR combinations achieved and fluorescent dyes used.

| Species        | Multiplex no. | Fluorescent dye |
|----------------|---------------|-----------------|
| *Acacia melvillei* | CPUH4 / CSIM0 / BNQS6 | FAM |
|                 | BBY8P / DZ709 / CIDYF | VIC |
|                 | AV9GR / BAIR8 | PET |
|                 | DCLOC / DSGN5 | NED |
| *Acacia ligulata* | DCLOC / BVWHY / AO12C | FAM |
|                 | C03PC6 / APZIZ | VIC |
|                 | BBY8P / A4IKI | NED |
| *Acacia pendula* | BBY8P / BAIR8 | 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* (DCLOC, A035A, DSIGN5, BNQS6, and DZ709). A. ligulata (A4IKI, AQBUV, DCLOC, ARC19, and C03PC6), and A. pendula (ACP7, BAIR8, BBY8P, CSIMO, 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 multiple 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, ... ) 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).

### 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. To date, there have been no studies on the reproductive biology of *Acacia* species to determine the reproductive mode (sexual vs. asexual) in natural populations.

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

| Locus          | *A* | *H*~e~ | *F*~IS~ |
|----------------|-----|--------|---------|
| CPUH4_a        | 4   | 0.71   | 0.48    |
| CSIM0_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    |
| DSIGN5_a       | 13  | 0.86   | 0.23    |
| VIC            | 9   | 0.72   | 0.40    |
| NED            | 9   | 0.68   | 0.36    |
| Average across all loci | 8.9 ± 1.29 | 0.70 ± 0.05 | 0.38 ± 0.04 |

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

| Locus          | *A* | *H*~e~ | *F*~IS~ |
|----------------|-----|--------|---------|
| DCLOC_a        | 11  | 0.85   | 0.20    |
| BVWHY_a        | 7   | 0.77   | 0.42    |
| C03PC6_a       | 11  | 0.86   | 0.34    |
| APZIZ_a        | 10  | 0.86   | 0.30    |
| BBY8P_a        | 16  | 0.91   | 0.27    |
| A4IKI_a        | 4   | 0.63   | 0.27    |
| AQBUV_a        | 15  | 0.88   | 0.20    |
| A4IKI_a        | 8   | 0.75   | 0.42    |
| C03PC6_a       | 10  | 0.80   | 0.30    |
| AO12C_a        | 10  | 0.82   | 0.28    |
| Average across all loci | 10.2 ± 1.11 | 0.81 ± 0.02 | 0.29 ± 0.04 |

Note: *A* = number of alleles per locus; *F*~IS~ = inbreeding within populations; *H*~e~ = expected heterozygosity; *N* = number of individuals sampled.

*Significant deviation from Hardy–Weinberg equilibrium for all loci at *P* < 0.05.

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phenomenon which, if widespread, may influence the choice of conservation actions. For the threatened \textit{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 \textit{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 \textit{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 melanoxylon* | 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 melanoxylon* | 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.*