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Applications in Plant Sciences

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Primer Note

Characterization and transferability of microsatellites for the Kangaroo Paw, *Anigozanthos manglesii* (Haemodoraceae)[1]

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- *Premise of the study:* Microsatellites were developed for the future assessment of population genetic structure, mating system, and dispersal of the perennial kangaroo paw, *Anigozanthos manglesii* (Haemodoraceae), and related species.
- *Methods and Results:* Using a Personal Genome Machine (PGM) semiconductor sequencer, ca. 4.03 million sequence reads were generated. QDD pipeline software was used to identify 190,000 microsatellite-containing regions and priming sites. From these, 90 were chosen and screened using PCR, and 15 polymorphic markers identified. These sites amplified di-, tri-, and pentanucleotide repeats with one to 20 alleles per locus. Primers were also amplified across congeners *A. bicolor*, *A. flavidus*, *A. gabrielae*, *A. humilis*, *A. preissii*, *A. pulcherrimus*, *A. rufus*, and *A. viridis* to assess cross-species transferability.
- *Conclusions:* These markers provide a resource for population genetic studies in *A. manglesii* and other species within the genus.

Key words: *Anigozanthos*; Catspaw; Haemodoraceae; Kangaroo Paw; microsatellite primers.

Anigozanthos manglesii D. Don (Haemodoraceae), the Red and Green Kangaroo Paw, is a perennial wildflower endemic to the Southwest Australian Floristic Region. Flowering occurs between July and November, with large inflorescences of red and green tubular flowers on stems up to a meter tall. These flowers are visited by nectar-feeding birds and invertebrates seeking nectar and pollen (Hopper, 1993). Differences in the foraging behavior of vertebrates and invertebrates are predicted to have a significant impact on pollen dispersal patterns, multiple paternity, genetic diversity, and fitness of offspring (Krauss et al., 2017). Manipulation of pollinator access to inflorescences and paternity assignment of the resulting seeds allows for the quantification of pollen dispersal patterns by specific pollinators. Here, we describe the development of microsatellite markers that will facilitate future research on the genetic consequences of pollen dispersal by bird and invertebrate pollinators of *A. manglesii*. In particular, we will use these markers for mating system and paternity assignment following pollinator manipulation studies to test hypotheses of high paternal diversity for plants pollinated by nectar-feeding birds (Krauss et al., 2017). The degree of congeneric cross-transferability of the markers was also assessed in eight other species, covering over 80% of the genus.

METHODS AND RESULTS

DNA was extracted from a leaf sample collected in Kings Park, Perth, Western Australia (Appendix 1), using the extraction method of Carlson et al. (1991), modified with the addition of potassium acetate after lysis incubation, a 5 M NaCl step, and an additional ethanol precipitation after the isopropanol precipitation. One hundred grams of DNA was sheared to approximately 300–400 bp using an S2 sonicator (Covaris, Woburn, Massachusetts, USA), and a single barcoded library was prepared using a NEBNext Ultra DNA Library Prep Kit (New England Biolabs, Ipswich, Massachusetts, USA). Inserts sized 330–360 bp were selected by gel excision (E-Gel, Invitrogen/Thermo Fisher Scientific, Waltham, Massachusetts, USA), and the libraries were produced, assessed, and quantified using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA). The final library was diluted to 9 pM using a OneTouch 2 Template 400 kit (Life Technologies, Carlsbad, California, USA) and enriched. A Personal Genome Machine (PGM) semiconductor sequencer (Life Technologies) using 850 flows on a 316 sequencing chip produced approximately 350–400 bp read lengths. Signal processing, base-calling, and quality trimming were conducted using the default settings on Torrent Suite 4.0 (Thermo Fisher Scientific),

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Table 1. Characteristics of 15 polymorphic microsatellite loci developed for *Anigozanthos manglesii.*

Locus	Primer sequences (5′–3′)	Fluorescent label	Repeat motif	Allele size (bp)	T_a (°C)	GenBank accession no.
AM1	F: TACTGAGATCGAACCACTTCTTG R: GGGATGGAGGTAGGACTGTT	VIC	$(AG)_{15}$	190	61.5	KY853194
AM2	F: GTTGTGTTGTACTCGCTGGG R: GTCCTTGCTCTAGCCACCAA	VIC	$(AT)_7$	91	65.5	KY853195
AM8	F: AACATGACTTAAGCTTCACTTTCG R: ACTTAGCCTTTCTGGCAAATG	6-FAM	$(ATC)_{15}$	140	56	KY853196
AM11	F: AGTCGGACTAACTTGGCAGC R: CCACAACGATGTTGTCTTGC	6-FAM	$(AAT)_8$	290	57	KY853197
AM13	F: TTGAGTAACGATGGCAAACTT R: TGACTTACCTTCATTCGCCA	PET	$(ACAT)_8$	241	57	KY853198
AM20	F: CAACTCAAGAACAAAGAAGGAAG R: TGGTTTCTCTATCTGAGTTTGGAT	6-FAM	$(AGC)_8$	193	57	KY853199
AM23	F: CTCGCTCTCCACAATCCACT R: TGGAATTCTCCTGCCTTCAC	VIC	$(AG)_{14}$	120	60	KY853200
AM28	F: TGGTTTATCAATGGAACAATATAAGA R: CAAATGATGATAAATGAATGAATAAGA	NED	$(AG)_9$	94	56	KY853201
AM29	F: TCCACCATATCCTACCGTGA R: GCTGCATTCACATCCTCAGA	PET	$(AGC)_{11}$	119	57	KY853202
AM56	F: GGAAGTTGAAGAGGAGCTGGT R: ACAAGACAGTCAATTATTCATTCATTA	VIC	$(AG)_{24}$	120	55	KY853203
AM60	F: TTTCCGGAACTGAAGGAAAG R: CCTGGCGAGGTTATTAAGCA	VIC	$(AT)_{10}$	176	55	KY853204
AM71	F: AATCCGGAGCAAAGTATCCA R: TTGGGAGAGGAGACGCTTTA	PET	$(AAG)_8$	263	64.5	KY853205
AM75	F: CAATGCATGACAGAAGGTTCA R: TTCTGCATGATCAGGGTAGTTG	NED	$(AAG)_8$	300	65.5	KY853206
AM79	F: AACAATCACGGCTCCCTTT R: GAGATTGTTCCTCTCGCTGC	6-FAM	$(AAG)_{12}$	237	64.5	KY853207
AM82	F: CTTTCCCATTCCCTCCCAT R: AGCTCCTTGACCAAGCACTG	PET	$(AAG)_8$	177	65.5	KY853208

Note: T_a = annealing temperature.

and library-specific FASTQ files were generated. This resulted in 4.03 million reads with a modal read length of 354 bp and 2.4 Gb of data (National Center for Biotechnology Information [NCBI] Sequence Read Archive Bioproject no. PRJNA390010).

Using QDD 3.1 software, all reads were screened for microsatellite-containing regions (Meglecz et al., 2014). A total of 190,000 were identified. Thirty primer pairs were chosen for screening at a time. Primers chosen were all categorized as design A (no homopolymers, no other target microsatellites in flanking region, no nanosatellite in primer or flanking regions, pure not compound microsatellites), were unable to form a hairpin, had a low PCR align score, had a >20-bp distance between primer and microsatellite, had higher microsatellite repeats, and had similar annealing temperatures, but had a variety of PCR product lengths (Meglecz et al., 2014; http://net.imbe.fr/~emeglecz/qdd.html#choice). Each assay had a final volume of 10 µL and contained 5 µL of SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories, Hercules, California, USA), 0.3 µM of forward and reverse primers, and 5–10 ng of genomic DNA. PCR was conducted on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). Using a single sample, DNA was amplified across a range of

Table 2. Genetic properties of 15 polymorphic microsatellite loci for three populations of *Anigozanthos manglesii*.[a]

	Kattidj (n = 20)			Korung National Park (n = 25)			Lovekin (n = 24)		
Locus	A	H_o	H_e[b]	A	H_o	H_e[b]	A	H_o	H_e[b]
AM1	4	0.342	0.745*	6	0.539	0.799**	20	0.650	0.931*
AM2	18	0.389	0.767**	7	0.350	0.499^{ns}	9	0.636	0.809*
AM8	16	0.500	0.914**	18	0.773	0.914**	11	0.333	0.880***
AM11	9	0.579	0.799^{ns}	9	0.864	0.825**	9	0.708	0.773^{ns}
AM13	14	0.600	0.880*	8	0.714	0.787^{ns}	9	0.556	0.778**
AM20	8	0.313	0.783***	13	0.500	0.869***	8	0.286	0.810***
AM23	1	0.000	0.000	1	0.000	0.000	15	0.318	0.916***
AM28	6	0.375	0.727***	8	0.238	0.833***	6	0.833	0.842*
AM29	9	0.529	0.808^{ns}	7	0.545	0.705*	10	0.522	0.817*
AM56	2	0.133	0.124^{ns}	2	0.200	0.180^{ns}	5	0.261	0.363^{ns}
AM60	3	0.421	0.342^{ns}	10	0.696	0.593^{ns}	1	0.000	0.000
AM71	12	0.579	0.856***	14	0.909	0.874***	8	0.739	0.772^{ns}
AM75	15	0.333	0.895***	9	0.750	0.834***	18	0.591	0.862*
AM79	9	0.474	0.838***	7	0.391	0.797***	15	0.952	0.901^{ns}
AM82	11	0.700	0.818^{ns}	6	0.182	0.419***	11	0.667	0.747*

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

[a] Voucher and locality information are provided in Appendix 1.

[b] Statistically significant deviation from Hardy–Weinberg equilibrium is indicated as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ns = not statistically significant.

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TABLE 3. Results of cross-amplification (allele size ranges) of microsatellite loci isolated in *Anigozanthos manglesii* and tested in five individuals across eight congeneric taxa. *Anigozanthos manglesii* is included for comparison.

Locus	*A. manglesii*	*A. bicolor*	*A. flavidus*	*A. gabrielae*	*A. humilis*	*A. preissii*	*A. pulcherrimus*	*A. rufus*	*A. viridis*
AM1	152–258	—	—	—	—	—	—	—	—
AM2	89–109	87–89	—	81–89	81–89	89	89	89	89
AM8	84–195	—	—	—	—	—	—	—	—
AM11	209–320	295–307	294	307–309	301–357	294	302–307	294–304	306–311
AM13	240–280	—	247	—	180	—	177	—	—
AM20	178–230	179–184	190–197	188	151–190	—	—	185–194	177–194
AM23	105–159	102–125	106–131	128–130	103–153	104–122	116–140	103–145	—
AM28	84–115	—	—	—	—	—	—	—	—
AM29	82–132	117–126	114–117	120	112–132	117–126	117–129	123–126	106–129
AM56	84–107	—	—	—	—	—	—	—	—
AM60	162–187	—	—	—	—	—	—	—	—
AM71	258–322	271–294	—	265–271	262–286	—	276–290	276–294	274–290
AM75	295–362	—	—	—	—	—	—	289	—
AM79	215–260	—	—	—	—	—	—	—	—
AM82	174–302	179–189	180–194	—	179–182	—	—	—	—
Total		7	6	6	8	4	6	7	5

Note: — = unsuccessful amplification.

temperatures to determine an appropriate annealing temperature. To test for polymorphism, eight individuals were amplified at the chosen best temperature and analyzed using Precision Melt Analysis (Bio-Rad Laboratories). The forward primer of primer pairs that amplified consistently across all eight individuals were each tagged with a fluorescent label (6-FAM, NED, VIC, or PET) compatible with the ABI 3500 sequencer (Life Technologies). This process was repeated three times, until 15 reliable primer pairs were produced. All other primer pairs failed to amplify consistently and/or cleanly (i.e., they displayed stuttering and allelic patterns were difficult to distinguish) across different DNA samples.

To amplify microsatellite regions, PCR was performed on a Veriti Thermocycler (Life Technologies), either individually or in multiplex. Individual microsatellite loci (Am1, Am2, Am8, Am23, Am28, Am56, Am60, and Am79) were amplified using 10–20 ng of DNA with 2 µL of 5× buffer containing dNTPs (Fisher Biotec, Wembley, Western Australia, Australia), 2 mM $MgCl_2$, 0.16 µM of both reverse and fluorescently labeled forward primers, and 0.05 µL of 5.5 units/µL *Taq* polymerase (Fisher Biotec) in a 10-µL reaction. The amplification cycle began with a 1-min denaturation at 95°C; followed by 35 cycles of denaturation at 95°C for 10 s, annealing (at variable temperatures, see Table 1) for 30 s, and extension at 72°C for 45 s; and a final extension of 15 min at 72°C. The remaining seven primer pairs were amplified across two multiplex mixes (primer mix 1 contained Am11, Am13, Am20, and Am29, all at 0.2 µM; primer mix 2 contained Am2 [0.1 µM], Am75 [0.4 µM], and Am82 [0.3 µM]). All multiplex reactions used 6 µL of 2× Multimix (QIAGEN, Hilden, Germany), 2 µL of 5× Q-solution (QIAGEN), 1.25 µL of primer mix, and 2.75 µL of 10–20 ng DNA in a final 12-µL reaction. The amplification cycle began with 15-min denaturation at 95°C; followed by 30 cycles of denaturation at 94°C for 30 s, annealing (at variable temperatures, see Table 1) for 90 s, and extension at 72°C for 90 s; and a final extension of 30 min at 60°C. PCR products were separated by capillary electrophoresis on an ABI 3500 Genetic Analyzer (Life Technologies), and allele sizes scored using Geneious version 7.1 (Biomatters Ltd., Auckland, New Zealand; http://www.geneious.com/).

Primers were tested on leaf samples collected from three populations of *A. manglesii* (Appendix 1, Table 2). All 15 markers were polymorphic in at least one population. Analysis for observed heterozygosity, expected heterozygosity, and Hardy–Weinberg equilibrium was completed with GenAlEx (Peakall and Smouse, 2006, 2012). Observed and expected heterozygosities ranged from 0.182 to 0.950 and 0.133 to 0.931, respectively. A significant departure from Hardy–Weinberg equilibrium was recorded in different loci across the three populations (Table 2). MICRO-CHECKER (van Oosterhout et al., 2004) identified the possibility of null alleles in some loci, but not consistently across populations. No stuttering or large allele dropouts were identified.

Using the same extraction and amplification methods as above, the primers were tested on DNA extracted from five individuals from each of *A. bicolor* Endl., *A. flavidus* DC., *A. gabrielae* Domin, *A. humilis* Lindl., *A. preissii* Endl., *A. pulcherrimus* Hook., *A. rufus* Labill., and *A. viridis* Endl. Success varied, with four to eight markers successfully amplified across different species (Table 3).

CONCLUSIONS

Fifteen microsatellite markers have been developed for *A. manglesii*. Without changing any of the amplification conditions, between four and eight of these markers successfully amplified in each of eight congeneric species. This suggests that with further species-specific refinement, these markers will provide a valuable resource for population genetic studies of the genus.

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Appendix 1. Voucher information for *Anigozanthos* species used in this study.[a]

Species	Voucher specimen accession no.	Collection locality (Population ID)	Geographic coordinates	*N*
A. manglesii D. Don	PERTH 2027925	Kings Park and Botanic Gardens, Perth (Kattidj)	–31.9602, 115.8323	20
A. manglesii	PERTH 2028069	Canning Mills Rd., Perth Hills (Korung National Park)	–32.06666, 116.03333	25
A. manglesii	PERTH 2883961	Kings Park, Perth (Lovekin)	–31.9697, 115.8294	24
A. bicolor Endl.	KPBG 20040828	Mogumber, 37.7 km N from turnoff from Bindoon to Moora	–31.05555, 116.043889	5
A. flavidus DC.	PERTH 4661192	Mount Barker	–34.766570, 117.4454163	5
A. gabrielae Domin	KPBG 20060052	Mount Arid, slopes ca. 1 km due SW of summit	–34.273611, 115.269722	5
A. humilis Lindl.	CANB 701.549.1	Muchea	–31.48345, 115.9333	5
A. preissii Endl.	KPBG 20120597	17.2 km S of the Mt. Barker to Denmark rd.	–49.976667, 117.620833	5
A. pulcherrimus Hook.	KPBG 20091116	1.1 km from the Cockleshell Gully, Dandaragan	–30.1414722, 115.0975833	5
A. rufus Labill.	PERTH 5746167	Mullet Lake Nature Reserve	–33.47261, 121.5949.2	5
A. viridis Endl.	KPBG 20000308	5 km S Brennans bridge Scott River National Park	–34.273611, 115.269722	5

Note: *N* = number of individuals.

[a]Vouchers are stored in the Western Australian Herbarium (PERTH), Perth, Western Australia; the Kings Park and Botanic Gardens Herbarium (KPBG), Perth, Western Australia; and the Australian National Herbarium (CANB), Canberra, Australian Capital Territory.

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