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PRIMER NOTE

CHARACTERIZATION OF MICROSATELLITE PRIMERS IN THE ENDANGERED ORCHID *PHAIUS AUSTRALIS* AND CROSS-AMPLIFICATION TO *P. BERNAYSII* (ORCHIDACEAE)[1]

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- *Premise of the study:* The swamp orchid, *Phaius australis* (Orchidaceae), is nationally endangered due to illegal collection and habitat loss and fragmentation, resulting in a disjunct distribution in spring and coastal wetland ecotones along Australia's east coast. Polymorphic microsatellite markers were developed to study genetic diversity and population structure for conservation and restoration purposes.
- *Methods and Results:* Illumina HiSeq high-throughput sequencing was used to develop 15 nuclear microsatellite markers, including 10 polymorphic markers for *P. australis*. Polymorphism at each marker was evaluated using 90 individuals from four natural populations. The number of alleles per locus ranged from one to three, and the observed and expected heterozygosity varied from 0.036 to 0.944 and from 0.035 to 0.611, respectively. These markers transferred successfully to congener *P. bernaysii*.
- *Conclusions:* The microsatellite markers will be useful for revealing levels of genetic diversity and gene flow for *P. australis* and may inform future conservation efforts.

Key words: Australia; conservation; microsatellite primers; orchid; Orchidaceae; *Phaius australis*; population genetics.

The family Orchidaceae is the most successful flowering plant group, with over 26,500 recognized species worldwide (Dixon et al., 2003). Australia's tallest terrestrial orchid is the swamp orchid, *Phaius australis* F. Muell. (Jones, 2006). It occurs in disjunct populations in marginal swamp wetlands and littoral rainforests distributed across a 2000 km latitude gradient along the east coast of Australia and is thought to be a primarily self-pollinating species (Benwell, 1994; Jones, 2006). The species occurs in habitat that is vulnerable to climate change. Populations have become highly fragmented by urban and regional development and it is a desirable species for collection, resulting in its federal "endangered" listing (Benwell, 1994). Congener *P. bernaysii* Rowland ex Rchb. f. is also listed as "endangered" in Australia, and both species are very closely related to Southeast Asian *P. tancarvilleae* (L'Hér.) Blume (Benwell, 1994; Jones, 2006).

Microsatellite (simple sequence repeat [SSR]) markers are widely used in plant population genetic and genetic diversity studies because of their high levels of polymorphism, stability, and codominance. High-throughput and low-cost next-generation sequencing has accelerated the identification of microsatellite markers for plant species (Rico et al., 2013). The development of microsatellite loci to assess genetic diversity, genetic structure, and gene flow among populations will be beneficial for developing conservation strategies for *P. australis* and may be useful for identifying molecular differences between three species (*P. australis*, *P. bernaysii*, *P. tancarvilleae*). In this paper, we report the development of microsatellite markers for *Phaius* Lour. species collected from the east coast of Australia using Illumina HiSeq genome sequencing technology.

METHODS AND RESULTS

Leaf material (10 × 10 cm) was sampled from four wild populations of *P. australis* for the development of genetic markers and one extant wild population of *P. bernaysii* to test for congeneric cross-amplification (Appendix 1). The population geographic location was recorded using a handheld GPS, and leaf and flower material were collected from a plant at each population and vouchered at the Queensland Herbarium (BRI). Approximately 30–50 mg of dried plant tissue and a 3-mm tungsten bead were frozen using liquid nitrogen for 30 s and ground using a Retsch MM200 Tissue Lyser grinding mill (QIAGEN, Valencia, California, USA). Total genomic DNA was extracted from the leaf tissue using QIAGEN DNeasy Plant Mini Kits (QIAGEN) following manufacturer's instructions as described in Shapcott et al. (2015). DNA (5 µg) from four individuals was sent to the Australian Genome Research Facility (AGRF, Brisbane, Australia; http://agrf.org.au/) for next-generation 454 pyrosequencing and used to construct a random library that was paired-end sequenced using GS-FLX Titanium chemistry Illumina HiSeq (Roche Applied Science, Mannheim, Germany). Sequences were trimmed for length and quality using the CLC

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TABLE 1. Characteristics of 15 microsatellite loci developed in *Phaius australis*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	Fluorescent label	GenBank accession no.
ml-Pa02	F: TGAAGCCAAAGGATGAACAA	$(AT)_8$	198–202	56	PET	KR698089
	R: GGAAGCATTATGATTGATGACG					
ml-Pa03	F: TAGAGGTAATCCAGGCCTCT	$(AT)_8$	232–240	51.8	FAM	KR698090
	R: ATAATATGTGAATTAGATAGCTTGGGC					
ml-Pa12	F: TTGGTTGCTCCTTCTTGACC	$(AT)_8$	315–317	59.7	FAM	KR698093
	R: TGAGGCCTCCTTAATCTCTAGC					
ml-Pa14	F: CAAACTAAAGGAAGGTGAGCCA	$(AT)_{10}$	276–290	59.7	NED	KR698094
	R: TCCAAGCCTTAGAAAGGGC					
ml-Pa19	F: GGAGCTATGCAACTGACTATCACA	$(AT)_5$	298–300	56	FAM	KR698095
	R: TCATTAGTTGTGTGCTCTTGC					
ml-Pa21	F: CCATACATAGGGTCATCAATCCA	$(TA)_3(TC)_4$	158	59.7	VIC	KR698096
	R: GTTAAGGCCTCCTATTCCCG					
ml-Pa24	F: ACGTGGCGGAGGAGAAGT	$(TTC)_{11}$	235–241	56	FAM	KR698097
	R: TCCTTAATAGATCTGAAGCACAAA					
ml-Pa27	F: CAAACTAAAGGAAGGTGAGCCA	$(AT)_7$	209–215	56	NED	KR698098
	R: GGCATTGGTGAGTAGGCAGA					
ml-Pa31	F: TAGAGGTAATCCAGGCCTCT	$(AG)_5$	221–229	59.7	PET	KR698098
	R: GATAGCTTGGGCCATTCAAA					
ml-Pa41	F: GCTCTTAAGAAGCACTTCAAGTCAA	$(AT)_6$	192	56	PET	KR698103
	R: CCCAAACTCCTCTTCCAATC					
ml-Pa44	F: CCATTGGGTCACTTCCTCTG	$(AT)_5$	188–192	56	NED	KR698104
	R: TCAGAGAACAACCATGCCAA					
ml-Pa46	F: TTAAAGGACGGAAACGCAGA	$(CT)_5$	231	56	VIC	KR698106
	R: GCCTACCGATCGATTGAACA					
ml-Pa49	F: GATGCAGGAATGGGAAACAG	$(CT)_5$	195–199	56	VIC	KR698107
	R: ATATTGGAACCACTCGACGG					
ml-Pa57	F: TGACCATCCCACAATGTTGA	$(ACT)_5$	157–159	56	PET	KR698110
	R: TCTCCACTACCATAACCACCAC					
ml-Pa59	F: ACCCAATTAGAAGCAAACTTGAAGA	$(AT)_5$	228–232	56	NED	KR698111
	R: TGGTTAGAGAACATCTCATTGGG					

Note: T_a = annealing temperature.

Genomics Workbench version 6 software (QIAGEN, Aarhus, Denmark). A total of 53,176 reads were obtained with an average length of 381 bp and searched for microsatellite loci having a minimum of six repeats for dinucleotides and four repeats for tri- and tetranucleotides, using the QDDv2b pipeline (Meglécz et al., 2010) (data available from the Dryad Digital Repository: doi:10.5061/dryad.j380c; Simmons et al., 2017). Default settings on Primer3 software (Rozen and Skaletsky, 1999) were used to develop the primers flanking the microsatellite loci. A resulting 2102 loci were sorted based on PCR product size, repeat class, repeat length, GC content, and multiplexing potential; 61 unlabeled microsatellite primer pairs were selected for evaluation of amplification and optimization using 15 *P. australis* DNA samples in the initial trials.

Fifteen primer pairs that consistently amplified single bands within the expected size range were end-labeled directly with one of four fluorescent dyes (VIC, NED, PET [Applied Biosystems, Scoresby, Victoria, Australia]; FAM [GeneWorks, Thebarton, South Australia, Australia]) and multiplexed in fragment analysis. PCR amplification was performed using reaction volumes of

TABLE 2. Genetic properties of the 15 newly developed microsatellites of *Phaius australis* and cross-amplification to *P. bernaysii*.[a]

	Phaius australis												*Phaius bernaysii*		
	Atherton Tableland (n = 29)			Atherton Tableland 2 (n = 28)			Byfield (n = 3)			Blackdown Tableland (n = 30)			Myora Conservation Park (n = 9)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
ml-Pa02	2	0.185	0.456	2	0.115	0.440	1	0.000	0.000	1	0.000	0.000	2	0.000	0.198
ml-Pa03	3	0.828	0.599	3	0.714	0.471	3	1.000	0.611	2	0.889	0.499	2	1.000	0.500
ml-Pa12	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
ml-Pa14	2	0.440	0.497	2	0.154	0.311	2	0.000	0.444	2	0.036	0.035	1	0.000	0.000
ml-Pa19	2	1.000	0.500	2	0.933	0.498	2	0.667	0.444	2	0.944	0.498	2	1.000	0.500
ml-Pa21	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
ml-Pa24	2	0.231	0.473	2	0.286	0.477	2	0.000	0.444	1	0.000	0.000	1	0.000	0.000
ml-Pa27	2	0.038	0.375	2	0.222	0.346	1	0.000	0.000	2	0.036	0.101	2	0.000	0.375
ml-Pa31	2	0.107	0.484	2	0.231	0.453	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
ml-Pa41	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
ml-Pa44	2	0.207	0.285	3	0.286	0.304	2	0.333	0.278	3	0.308	0.370	2	0.333	0.401
ml-Pa46	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
ml-Pa49	2	0.000	0.067	1	0.000	0.000	2	0.333	0.278	1	0.000	0.000	1	0.000	0.000
ml-Pa57	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
ml-Pa59	2	0.107	0.101	2	0.250	0.219	2	0.333	0.278	2	0.154	0.142	2	0.333	0.278

Note: A = number of alleles sampled; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.
[a]Locality and voucher information are available in Appendix 1.

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12 µL containing approximately 25 ng of *P. australis* genomic DNA, 1× reaction buffer (67 mM Tris-HCl [pH 8.8], 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg/mL gelatin), 200 µM of each dNTP, 2 mM $MgCl_2$, 0.2 µM bovine serum albumin (BSA), 0.5 units F1 *Taq* polymerase (all reagents Fisher Biotech, Brisbane, Australia), and 0.2 µM forward primer, 0.2 µM reverse primer, with the forward primer of each pair. Amplification was performed on an Eppendorf Mastercycler Nexus Gradient with the following cycling conditions: denaturation at 95°C for 3 min; 35 cycles of 94°C for 30 s, specific annealing temperature (Table 1) for 30 s, 72°C for 45 s; and a final elongation step at 72°C for 10 min.

PCR products were multiplexed according to dye sets and size ranges to avoid overlap and then separated on an AB 3500 Genetic Analyzer (Applied Biosystems). Fragment sizes were determined relative to internal lane standard (GeneScan 600 LIZ; Applied Biosystems), and then banding patterns were manually checked in GENEMAPPER version 4.1 software (Applied Biosystems) that scored bands fitted within the expected size range. A resultant 15 primers (Table 1) were scored for *P. australis*. The presence of null alleles, scoring errors, and large allele dropouts were checked for all loci using MICRO-CHECKER version 2.2.3 (van Oosterhout et al., 2004). Linkage disequilibrium was tested using Fisher's exact tests in GENEPOP version 1.2 (Laboratoire de Genetique et Environnement, Université Montpellier II, Montpellier, France), and a sequential Bonferroni correction was applied for multiple tests. The multilocus genotypes were used to characterize the microsatellites by calculating allelic frequencies, mean number of alleles per locus, mean expected heterozygosity (H_e), and mean observed heterozygosity (H_o) for each locus using GenAlEx 6.5 (Peakall and Smouse, 2012; Table 2).

Ten of the 15 loci successfully developed for *P. australis* were polymorphic (Table 2) with an average of 1.57 alleles per locus across all populations (SE ± 0.071; Table 2). Mean H_o was 0.210 (SE ± 0.081), 0.213 (SE ± 0.071), 0.178 (SE ± 0.079), and 0.110 (SE ± 0.083) at the Atherton Tableland, Atherton Tableland 2, Byfield, and Blackdown Tableland populations, respectively (Table 2). Mean H_e was 0.256 (SE ± 0.061), 0.235 (SE ± 0.055), 0.185 (SE ± 0.057), and 0.110 (SE ± 0.048) at the Atherton Tableland, Atherton Tableland 2, Byfield, and Blackdown Tableland populations, respectively (Table 2). All SSR sequences have been deposited in GenBank (Table 1). All 15 loci were cross-compatible to the congener *P. bernaysii* using the PCR conditions described above (Table 2). Six loci were polymorphic, fewer than in *P. australis*, potentially due to the smaller sample size from the limited population of this species. There was an average of 1.573 alleles per locus (SE ± 0.071), mean H_o of 0.187 (SE ± 0.035), and mean H_e of 0.187 (SE ± 0.025) across all loci for *P. bernaysii* (Table 2).

CONCLUSIONS

In this study, we developed 15 microsatellite markers in *P. australis*, 10 of which were polymorphic. The optimization of 10 polymorphic microsatellite markers for *P. australis* will be useful for revealing levels of genetic diversity, range expansion, and the evolutionary relationships that may inform future assisted migrations or population enhancements (Gustafsson, 2000; Vallee et al., 2004; Kingsford and Watson, 2011). The successful cross-amplification to *P. bernaysii* indicates a similarity between the congeners, which is not surprising given the species was suggested to be a color morph of *P. australis* (Nicholls, 1950; Jones, 2006). These microsatellite markers may be useful in clarifying the genetic relatedness between Australian *P. australis*, *P. bernaysii*, and Southeast Asian *P. tancarvilleae* (Harrison et al., 2005).

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Appendix 1. Voucher information for *Phaius* species used in this study.

Species	Voucher specimen accession no.[a]	Collection locality	Geographic coordinates	*n*
P. australis F. Muell.	BRI AQ0890327	Atherton Tableland	−17.108, 145.426	29
P. australis	BRI AQ0890325	Atherton Tableland 2	−17.125, 145.367	28
P. australis	BRI AQ0890339	Byfield	−22.800, 150.800	3
P. australis	BRI AQ0890329	Blackdown Tableland	−23.904, 149.193	30
P. bernaysii Rowland ex Rchb. f.	BRI AQ0890313	Myora Conservation Park	−27.475, 153.419	9

Note: *n* = number of individuals sampled.

[a]One voucher per population deposited at the Queensland Herbarium (BRI), Brisbane, Australia.

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