CHARACTERIZATION OF MICROSATellite PRIMERS 
IN THE ENDANGERED ORCHID P. AUSTRALIS AND 
CROSS-AMPLIFICATION TO P. BERNAYSII (ORCHIDACEAE) 

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METHODS AND RESULTS

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 (Orchidaceae), occurring 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.

Phaius australis

P. bernaysii

Congener Rowland ex Rchb. f. is currently listed as endangered and is a desirable species for collection, resulting in its federal “endangered” listing (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 species collected from the east coast of Australia using Illumina HiSeq genome sequencing technology.

Phaius australis

Phaius Tancarvilleae

Phaius bernaysii

Polymorphic microsatellite markers were developed to study genetic diversity and population structure for conservation and restoration purposes. The study received financial support from the Jani Haenke Charitable Trust and the Queensland Heritage. This study received financial support from the Jani Haenke Charitable Trust and the Queensland Heritage. This study received financial support from the Jani Haenke Charitable Trust and the Queensland Heritage. This study received financial support from the Jani Haenke Charitable Trust and the Queensland Heritage.

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

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 (BR). 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° (°C) | Fluorescent label | GenBank accession no. |
|---------|-------------------------------------------------|--------------|------------------------|---------|------------------|-----------------------|
| ml-Pa02 | F: TGAGCCCAAGGATGACAA R: GGAAGATATGATTTGAGC   | (AT)\(_8\)   | 198–202                | 56      | PET              | KR698089              |
| ml-Pa03 | F: TAGGCTAATCCAGGCTTCT    R: ATATTGGAACCACTCGAGG | (CT)\(_7\)   | 232–240                | 51.8    | FAM              | KR698090              |
| ml-Pa12 | F: TGGTTGCTCTCCTCTCTG   R: TGAAGGGCTCTTAATCTGGGC | (AT)\(_8\)   | 315–317                | 59.7    | FAM              | KR698093              |
| ml-Pa14 | F: CAAACTAAAGGAGGTGACC   R: TTCAGGCACTTGACAGCA | (ACT)\(_5\)  | 276–290                | 59.7    | NED              | KR698094              |
| ml-Pa19 | F: GAGATCTGCACTGATCTACCA | (AT)\(_5\)   | 298–300                | 56      | FAM              | KR698095              |
| ml-Pa21 | F: CCATACATAGGGTACATCCCA R: GATGCTGCTGCTCCGCC | (TA)\(_3\)(TC)\(_3\) | 158 | 59.7 | VIC              | KR698096              |
| ml-Pa24 | F: ACGTGCCAGGAGGAGAAGT  R: TCTCTAAAGATGCTGAGCAAAA | (TTC)\(_{11}\) | 235–241                | 56      | FAM              | KR698097              |
| ml-Pa27 | F: CAAACTAAAGGAGGTGACC   | (AT)\(_7\)   | 209–215                | 56      | NED              | KR698098              |
| ml-Pa31 | F: TAGGGTGATCTCAGCGAGA  R: CCCTACCGATCGATTGAACA | (CT)\(_3\)   | 231                    | 56      | VIC              | KR698106              |
| ml-Pa41 | F: GCTCTTAAGAAGCCTCTAGCTCAA  R: CCAAACTCTCTCTTCAATC | (AT)\(_6\) | 192                    | 56      | PET              | KR698103              |
| ml-Pa44 | F: CCATGGTCTATCTCCCTTG  | (AT)\(_5\)   | 188–192                | 56      | NED              | KR698104              |
| ml-Pa46 | F: TTAGAGCCGGAGAGCCGAGA R: GCCATTGGTGAGTAGGCAGA | (CT)\(_3\) | 231                    | 56      | VIC              | KR698106              |
| ml-Pa49 | F: GATCCAGAAGGGAGAACAG | (CT)\(_3\)   | 195–199                | 56      | VIC              | KR698107              |
| ml-Pa57 | F: TCTCCACTCCACAATTTGGA  R: ATATGGGAACACTCGACGG | (ACT)\(_3\) | 157–159                | 56      | PET              | KR698110              |
| ml-Pa59 | F: ACCCAATTAGGCAACACTTGGAGA  R: TGCTGATGACACTCCTATGGGG | (AT)\(_5\) | 228–232                | 56      | NED              | KR698111              |

Note: T° = 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., 2009). Default settings on Primer3 software (Rozen and Skaletsky, 1999) were used to develop the primers flanking the microsatellite loci. 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 25 μL.

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

| Locus   | Atherton Tableland (n = 29) | Atherton Tableland 2 (n = 28) | Byfield (n = 3) | Blackdown Tableland (n = 30) | Myora Conservation Park (n = 9) |
|---------|--------------------------|-----------------------------|----------------|-----------------------------|-------------------------------|
| ml-Pa02 | A: 0.185                 | H\(_e\): 0.456             | 1: 0.000       | 0.000                       | 2: 0.000                      |
| ml-Pa03 | 0.828                    | 0.599                       | 3: 0.714       | 0.471                       | 1: 0.889                      |
| ml-Pa12 | 0.000                    | 0.000                       | 1: 0.000       | 0.000                       | 0: 1.000                      |
| ml-Pa14 | 0.440                    | 0.497                       | 2: 0.933       | 0.498                       | 2: 0.944                      |
| ml-Pa19 | 1.000                    | 0.500                       | 2: 0.933       | 0.498                       | 2: 0.944                      |
| ml-Pa21 | 0.000                    | 0.000                       | 1: 0.000       | 0.000                       | 1: 0.000                      |
| ml-Pa24 | 0.231                    | 0.473                       | 2: 0.286       | 0.477                       | 2: 0.286                      |
| ml-Pa27 | 0.038                    | 0.375                       | 2: 0.222       | 0.346                       | 1: 0.333                      |
| ml-Pa31 | 0.107                    | 0.484                       | 2: 0.231       | 0.453                       | 1: 0.333                      |
| ml-Pa41 | 0.000                    | 0.000                       | 1: 0.000       | 0.000                       | 1: 0.000                      |
| ml-Pa44 | 0.207                    | 0.285                       | 3: 0.286       | 0.304                       | 3: 0.308                      |
| ml-Pa46 | 0.000                    | 0.000                       | 1: 0.000       | 0.000                       | 1: 0.000                      |
| ml-Pa49 | 0.000                    | 0.000                       | 1: 0.000       | 0.000                       | 1: 0.000                      |
| ml-Pa57 | 0.000                    | 0.000                       | 1: 0.000       | 0.000                       | 1: 0.000                      |
| ml-Pa59 | 0.107                    | 0.101                       | 2: 0.250       | 0.219                       | 2: 0.333                      |

Note: A = number of alleles sampled; H\(_e\) = expected heterozygosity; H\(_o\) = observed heterozygosity; n = number of individuals sampled.

*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 (NH₄)₂SO₄, 0.45% Triton X-100, 0.2 mg/mL gelatin), 200 μM of each dNTP, 2 mM MgCl₂, 0.2 μM bovine serum albumin (BSA), 0.5 units 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 multi-locus genotypes were used to characterize the microsatellites by calculating allelic frequencies, mean number of alleles per locus, mean expected heterozygosity (Hₑ), and mean observed heterozygosity (Hₒ) for each locus using GenAIEx 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 $Hₑ$ 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 $Hₒ$ 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 congeners, which is not surprising given the species was conserved migrations or population enhancements (Gustafsson, 2000; Kingsford, 2011). The evolutionary relationships that may inform future as-

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. | 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. bernaysii* Rowland ex Rchb. f. | 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.*

*aOne voucher per population deposited at the Queensland Herbarium (BRI), Brisbane, Australia.*

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