CHARACTERIZATION OF MICROSATELLITE LOCI FOR AN AUSTRALIAN EPiphytic ORchid, Dendrobium Calamiforme, USING ILLUMINA SEQUENCING

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Methods and Results: Nineteen microsatellite loci were identified from an Illumina paired-end shotgun library of D. calamiforme. Polymorphism and genetic diversity were assessed in 24 individuals from five populations separated by a maximum distance of ~80 km. All loci were polymorphic with two to 14 alleles per locus, expected heterozygosity ranging from 0.486 to 0.902, and probability of identity values ranging from 0.018 to 0.380.

Conclusions: These novel markers will serve as valuable tools for investigation of levels of genetic diversity as well as patterns of gene flow, genetic structure, and phylogeographic history.

Key words: Dendrobium calamiforme; Dockrillia calamiformis; genetic diversity; Orchidaceae; phylogeography; simple sequence repeat (SSR) markers.

Molecular phylogeographic approaches can provide potent tests of historical biogeographic hypothesis, such as the influence of historical barriers to gene flow on evolutionary diversification. The tropical rainforests of northeastern Australia harbor a diverse flora rich in basal angiosperm lineages that has long been thought to have been assembled principally through ecological filtering of relic Gondwanan stock and exchange of lineages with Malesia and Southeast Asia (e.g., Webb and Tracey, 1981; Crayn et al., 2015). However, the role of in situ diversification in this old biome may be underappreciated. Within this biome, a congruent genetic discontinuity has been found in various fauna groups (e.g., Schneider et al., 1998) and tree species (Rossetto et al., 2009) across the biogeographic barrier known as the Black Mountain Corridor (BMC), located between Cairns and Cape Tribulation. To better understand the processes that gave rise to this pattern, and the significance of in situ diversification to the origins and maintenance of tropical rainforest diversity, we aim to determine the phylogeographic structure of a codistributed epiphytic orchid. These orchids release tiny, wind-borne seeds high in the air column, where they can be picked up by wind currents and potentially transported great distances.

Dendrobium calamiforme Lodd. ex Lind., commonly known as the pencil orchid in reference to the long, terete leaves, had been renamed Dockrillia calamiformis (Lodd. ex Lind.) M. A. Clem. & D. L. Jones (Clements and Jones, 1996); however, this was rejected by Adams (2011). This orchid is indigenous to coastal tropical Queensland, Australia, ranging from Badu Island in the Torres Strait to Mount Elliott near Townsville, with nearly continuous distribution in its habitat across its range. It is a canopy and subcanopy epiphyte that grows in vine forest, swamp forest, beach forest, and riparian forest but is uncommon in ever-wet closed canopy rainforest. Although it can occur on large boulders, populations reach their highest density in large mature trees and can be locally abundant. Individuals become reproductive within five years and can live for several decades. Dendrobium calamiforme flowers in the dry season (July to September) and, while the pollination syndrome has not been verified, Hymenoptera, Coleoptera, and birds have been observed visiting flowering plants.

The development of highly polymorphic microsatellite markers will allow insights into the levels and partitioning of neutral genetic variation in this common epiphytic orchid. With these markers, patterns of seed dispersal, colonization, and genetic connectivity across the BMC will be investigated. Based on the dispersal ability of D. calamiforme, we predict low genetic

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structure among populations straddling the BMC; however, biogeographic disjunctions have been found in Costa Rican epiphytic orchids (Trapnell and Hamrick, 2004; Kartzinel et al., 2013; Trapnell et al., unpublished) that appear to be maintained by cryptic processes.

METHODS AND RESULTS

Total DNA was extracted from one individual of *D. calamiforme*, following the cetyltrimethylammonium bromide (CTAB) protocol of Doyle and Doyle (1990). After shearing 1 μg of genomic DNA with a Covaris S220 Focused-ultrasonicator (Covaris, Woburn, Massachusetts, USA), a paired-end shotgun library was prepared with the Illumina TruSeq DNA Library Kit (Illumina, San Diego, California, USA). During library preparation, a multiplex identified adapter was incorporated as multiple species were run together on an Illumina MiSeq using 100-bp paired-end reads. The program PAL_FINDER_v0.02.03 (Castoe et al., 2012) was used to examine 5 million reads and identify those containing microsatellite repeats; positive reads were targeted for primer design (Castoe et al., 2012) was used to examine 5 million reads and identify those containing microsatellite repeats; positive reads were targeted for primer design using Primer3 (version 2.0.0; Rozen and Skaletsky, 1999). The frequency of de-

### Table 1. Characteristics of 19 polymorphic microsatellite loci developed for *Dendrobium calamiforme*.a

| Locus  | Primer sequences (5’–3’) | Repeat motif | Allele size range (bp)b | TDc |
|--------|--------------------------|--------------|-------------------------|-----|
| Doca5  | F: *GAAGTGGTAGTGGCAGAGC* | ATC          | 252–297                 | TD65|
|        | R: AACCTGAAACACACCAAGGC  |              |                        |     |
| Doca6  | F: *AGTGTGAAGCAATGCTAGGC* | ATT          | 219–273                 | TD65|
|        | R: AAGGTCTAAATTGCTCTAGGGC |              |                        |     |
| Doca10 | F: *TGCTCCTCTCTCTGCAAATAGC* | ATCT         | 212–222                 |     |
|        | R: AGAGAGTGGAGGGCTCTAGAGTC |              |                        |     |
| Doca11 | F: *GCCTTGCTGACAAGGCTG*  | ATT          | 194–209                 |     |
|        | R: AGGAGACGTCCTGAGGTTG   |              |                        |     |
| Doca13 | F: *CCACCACGCCCTGATATCC* | TTC          | 175–211                 |     |
|        | R: CAAACGCAAGGAGCTCCTCCG |              |                        |     |
| Doca14 | F: *AAAGGATAAGCAGCATAAAGGGC* | ATT         | 251–293                 |     |
|        | R: CAACCAATCGGCACTGAGGC   |              |                        |     |
| Doca15 | F: *GGAAGCTGCTGGATCTGCG* | TTC          | 184–206                 |     |
|        | R: CATCCCTCAGGCTCCATCC    |              |                        |     |
| Doca16 | F: *CATTTGACGATAGTCGCGG* | ATT          | 145–181                 |     |
|        | R: CCAAAGACCCTCCTGAGAG   |              |                        |     |
| Doca18 | F: *CATATGAGCTGTTCTGCTACC* | ATCT        | 260–338                 |     |
|        | R: CTTGAGGCACCTGAGGC     |              |                        |     |
| Doca19 | F: *GAGCAGAAGATCTGAGGGG*  | TTC          | 222–271                 |     |
|        | R: GACATAGAGCCTGAGGAAAGC  |              |                        |     |
| Doca25 | F: *GACCTAAACTCTTACATCTGAGCC* | ATT   | 219–264                 |     |
|        | R: GCTCTCTGATGCAAAATAAGGCG |              |                        |     |
| Doca27 | F: *CTCTCAATTCCCCGAGAGCC* | ATCT        | 173–175                 |     |
|        | R: GGAGCTAGGAGGAGAGG     |              |                        |     |
| Doca28 | F: *TGCAATCTGCTACACACATCC* | TTC       | 283–313                 |     |
|        | R: GCTCTCAAGGATGAGGGCC  |              |                        |     |
| Doca33 | F: *CATATAAGAGCTGATAAAGACTGCACGGG* | AATC   | 204–212                 |     |
|        | R: TACCACTACAGGCGCCTGCG |              |                        |     |
| Doca37 | F: *GCGAGAAGAGAAGAGAAGGGG* | ATCT  | 193–299                 |     |
|        | R: TCTCTCAAAACCCCTCCTCC   |              |                        |     |
| Doca38 | F: *GAGAGAGACAAAGAGAGG*  | ATCT        | 216–276                 |     |
|        | R: TCTCTGATACCCCTCCTGCC  |              |                        |     |
| Doca39 | F: *AGGAGAGCCGGAGAGG*    | ATCT        | 244–267                 |     |
|        | R: TCTCTCTTCTCCCTCTCTCC  |              |                        |     |
| Doca40 | F: *GGATATGAGTGAAGCAAGAGATGG* | ATT   | 402–477                 |     |
|        | R: TCTCTCTTATAGCTACAACTGAGG |              |                        |     |
| Doca41 | F: *CGCTTGAAGACCTCAAAATGCC* | ATCC | 272–299                 |     |
|        | R: TGAAAAGGCGGCTCCTATCC  |              |                        |     |

a The GenBank accession number for all loci is SAMN03437177.
b Includes the length of the CAG tag.
  c Touchdown protocol used for PCR (see Methods and Results section).
  d Indicates CAG tag (5’–CAGTCGGGCGTCATA–3’) label.

http://www.bioone.org/loi/apps
and tetranucleotide (7 loci) repeat motifs (Table 1). The remaining 29 loci did not amplify well and therefore were not used.

We assessed the variability of these 19 loci in 24 specimens of *D. calamiforme* collected from five sites, spanning a distance of 79.7 km (Appendix 1). Vouchers from each site were deposited at the Australian Tropical Herbarium (CNS) (Appendix 1). Each site consisted of a small number of individuals in each of two to five host trees. We used GenAlEx version 6.4 (Peakall and Smouse, 2006) to estimate the number of alleles per locus (*A*), observed heterozygosity (*H*), expected heterozygosity (*H*), and the probability of identity (*P*). To test for deviations from Hardy–Weinberg equilibrium (*HWE*) and for linkage disequilibrium, GENEPOP version 4.0 (Rousset, 2008) was used.

These 19 loci were highly polymorphic with mean per locus values of *A* = 8.4 (range = 2–14), *H* = 0.754 (0.486–0.902), and *H* = 0.496 (0.043–0.957). Mean population values were *A* = 3.7, (range 2.5–5.0), *H* = 0.591 (0.484–0.693), *H* = 0.489 (0.432–0.588), and *P* = 0.259 (0.161–0.370) (Table 2). After Bonferroni correction for multiple comparisons, 14 loci showed significant deviation from expectations under HWE (Table 2). Linkage disequilibrium was detected for 61 of the 171 pairs of loci comparisons, which is not surprising considering that our samples came from multiple small populations.

### CONCLUSIONS

The 19 novel microsatellites developed for *D. calamiforme* revealed high levels of polymorphism and genetic diversity and thus should prove valuable for elucidating levels and patterns of genetic variation in future population genetic and phylogeographic investigations of this species in northeastern Australia. These highly variable markers may also be useful for discerning species boundaries among *D. calamiforme* and the putative taxa *D. basiyavanum* St. Cloud and *D. xfoederatum* St. Cloud, which have in the past been recognized as occurring in the Cairns area of northeastern Australia (Field and Zich, 2012).

### LITERATURE CITED

Adams, P. B. 2011. Systematics of Dendrobinae (Orchidaceae), with special reference to Australian taxa. Botanical Journal of the Linnean Society 166: 105–126.

Castoe, T. A., A. W. Poolé, A. P. J. de Koning, K. L. Jones, D. F. Tomback, S. J. Oyler-McCann, J. A. Fire, et al. 2012. Rapid microsatellite identification from Illumina paired-end genomic sequencing in two birds and a snake. PLoS ONE 7: e30953.

Clements, M. A., and D. L. Jones. 1996. New species of Dendrobinae (Orchidaceae) from Papua New Guinea. Lasiocarpa 1: 8–25.

Crayn, D. M., C. Costion, and M. G. Hambrook. 2015. The Sahul–Sunda floristic exchange: Dated molecular phylogenies document Cenozoic intercontinental dispersal dynamics. Journal of Biogeography 42: 11–24.

DeWoody, A. J., J. Schupp, L. Kenefic, J. Busch, L. Murffit, and P. Kemb. 2004. Universal method for producing ROX-labeled size standards suitable for automated genotyping. BioTechniques 37: 348–350.

Don, R. H., P. T. Cox, B. J. Wainwright, K. Baker, and J. S. Mattick. 1991. ‘Touchdown’ PCR to circumvent spurious priming during gene amplification. Nucleic Acids Research 19: 4008.

Doyle, J. J., and J. L. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus (San Francisco, Calif.) 12: 13–15.

Field, A. R., and F. A. Zich. 2012. Types of enigmatic north-Queensland orchids from the Dockrill herbarium. Australobata 8: 696–698.

Kartzenel, T. R., R. P. Shefferman, and D. W. Trapnell. 2013. Relative importance of pollin and seed dispersal across a Neotropical mountain landscape for an epiphytic orchid. Molecular Ecology 22: 6048–6059.

Peakall, R., and P. E. Smouse. 2006. GenAlEx 6: Genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6: 288–295.

Rossetto, M., D. Crayn, A. Ford, R. Mellick, and K. Sommerville. 2009. The influence of environment and life-history traits on the distribution of genes and individuals: A comparative study of 11 rainforest trees. Molecular Ecology 18: 1422–1438.

Rousset, F. 2008. GENEPOP007: A complete re-implemention of the GENEPOP software for Windows and Linux. Molecular Ecology Resources 8: 103–106.
APPENDIX 1. Geographic locations and voucher information for *Dendrobium calamiforme* samples collected from five sites in Queensland, Australia, and deposited in the Australian Tropical Herbarium (CNS) by Ashley R. Field (ARF).

| Geographic coordinates | Site description | CNS primary collector no. |
|------------------------|------------------|---------------------------|
| 16°40′52.6″S, 145°10′47.0″E | Font Hill Station in Baker and Blue Mountain Range | ARF4151 |
| 16°57′02.1″S, 145°44′36.8″E | Bruce Hwy. and Toogood Rd. intersection | ARF4154 |
| 17°06′00.3″S, 145°47′12.0″E | Bruce Hwy. crossing of Mulgrave River | ARF4155 |
| 16°52′29.7″S, 145°40′40.8″E | Barron River off of Stony Creek Rd. | ARF5152 |
| 16°54′08.6″S, 145°45′08.3″E | Centenary Lakes, on track to Flecker Botanical Garden | ARF4150 |