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PRIMER NOTE

CHARACTERIZATION OF 13 MICROSATELLITE MARKERS FOR *DIURIS BASALTICA* (ORCHIDACEAE) AND RELATED SPECIES[1]

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- *Premise of the study: Diuris basaltica* (Orchidaceae) is an endangered forb on the Victorian grasslands and has many close relatives. Microsatellite markers have been developed to facilitate assessment of population structure within *D. basaltica* and among related taxa within the species complex.
- *Methods and Results:* Twenty-five microsatellite markers (13 polymorphic and 12 monomorphic) were developed from *D. basaltica* using 454 pyrosequencing, and all primer pairs were amplified in *D. gregaria* and *D. chryseopsis*. For the set of polymorphic markers, the number of alleles per locus ranged from one to 10, two to nine, and two to 18 for *D. basaltica*, *D. gregaria*, and *D. chryseopsis*, respectively. The expected and observed heterozygosities ranged from 0.18 to 0.95 and 0.14 to 0.86, respectively.
- *Conclusions:* The microsatellite markers developed in this study can be used to analyze the population genetic structure of *D. basaltica* and other *Diuris* species.

Key words: conservation; *Diuris* species; orchid; polyploidy.

The Natural Temperate Grassland of the Victorian Volcanic Plain (VVP) of Victoria, Australia, has been under constant anthropogenic pressure over the past century (McDougall and Kirkpatrick, 1994). Remnants in the VVP house the remaining endemic and naturally occurring species, many of which are in decline. *Diuris basaltica* D. L. Jones (Orchidaceae) occurs within this region and is only known to occur in three locations (Backhouse and Lester, 2010); however, the erratic flowering patterns make this difficult to confirm even though it was once "relatively frequent" in areas just east of Melbourne (Jones, 2006). At least three species closely related to *D. basaltica* occur in the region and have subtle morphological differences (*D. chryseopsis* D. L. Jones, *D. gregaria* D. L. Jones, and *D. behrii* Schltdl.). It has been established in other Orchidaceae genera that genetics do not always follow current species delimitations (Devey et al., 2008, 2009), and the *Diuris* complex may be no different. Devey et al. (2009) also conclude that gene flow between geographically close populations is more likely than between morphologically similar ones at greater distances. This suggests that the morphology used to delimit Orchidaceae species may not necessarily reflect the genetic composition of populations and may mislead conservation efforts because the inclusion of poorly substantiated taxa could be a hindrance to conservation plans. Therefore, the use of current genetic techniques and morphological measurements should both be used to delimit species within Orchidaceae genera.

Genetic markers are needed to investigate the legitimacy of *Diuris* species, understand how populations are connected, and aid in the conservation of *D. basaltica*. The markers were developed using 454 pyrosequencing because next-generation sequencing reduces total cost, provides rapid development in nonmodel organisms, is not limited to particular repeat motifs, and locates thousands of possible microsatellite loci, which increases the success of finding polymorphic loci (Schoebel et al., 2013). In addition to assessment of current species delimitation, the markers will help with future reintroduction efforts by enabling any changes in genetic structure, such as possible genetic erosion, to be monitored over time.

METHODS AND RESULTS

Diuris basaltica was sampled from known collections that were cultivated in a greenhouse at Royal Botanic Gardens Melbourne. Of the >100 cultivated plants, 18 were sampled for this survey. The other three populations used in this study were identified as *D. gregaria* (Chatsworth-Woorndoo) and *D. chryseopsis* (Dunkeld; Inverleigh Common).

Genomic DNA was extracted and isolated from 300 mg of silica-dried leaf tissue from one *D. basaltica* individual using a DNeasy Plant Maxi Kit (QIAGEN, Hilden, Germany). The genomic DNA library was prepared according to Roche's Rapid Library Preparation Method Manual and quantified by PicoGreen fluorimetry (Quant-iT PicoGreen dsDNA Assay Kit; Invitrogen, Carlsbad, California, USA). The DNA was nebulized and cleaned with a QIAGEN MiniElute purification kit. Fragments underwent end repair and subsequent barcoded adapter ligation with the 454 Adapter-A and Adapter-B (Lib-L). Small fragments were removed using Agencourt AMPure Beads (Beckman Coulter, Lane Cove, New South Wales, Australia). The final quality of the library was assessed using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) and quantitative PCR (qPCR) using KAPA reagents (KAPA Biosystems, Woburn, Massachusetts, USA). DNA was sequenced for primer development using a Roche GS FLX machine for a 1/8 coverage (454 Life Sciences, Branford, Connecticut, USA). The program QDD was used to search for tandem repeats and to design microsatellite primers (Meglécz et al., 2009), resulting in the isolation of 11,480 possible microsatellite loci. A total of 96

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TABLE 1. Microsatellite loci information based on 13 primer pairs developed in *Diuris*.

Locus	GenBank accession no.	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Size range (bp)	Fluorescent label
db003	KF318193	F: CCCTATTCACATCCTCCATCC R: TGGAGGTCGTCGGAGTTAGT	$(AG)_8$	60	104–120	NED
db012	KF318194	F: TTTCCCAAAGTTGGAAATAATGA R: CAGCGGCTTTGCAGATTTAT	$(AAT)_7$	60	129–174	PET
db025	KF318195	F: TTTGCTCTTTCACCAACCGT R: CGTGTCTTCGCCAAGAGTATC	$(AT)_6$	60	160–196	NED
db028	KF318196	F: ATGCGGCAACAACTGCTAAT R: CGACAATGAGGAATTCAGTGG	$(AG)_7$	60	159–187	VIC
db047	KF318197	F: GCAACTGTTCTATCCCACGC R: GAGCCAGTGTCGCAATAGGT	$(AAG)_5$	60	194–221	FAM
db051	KF318198	F: ATGTCCATCGTATCCATGCC R: TGGACGAGTGCTTCAGTACTACC	$(AAC)_5$	60	190–238	PET
db052	KF318199	F: CAAACCATCCCTTCAATCCT R: CTTCGGTTGGGACTTGATATG	$(AAG)_6$	60	218–230	NED
db053	KF318200	F: TTCTCACGAAGAGCTTGCTAAA R: CGGAAACAACGATGAGGAGA	$(AG)_6$	60	206–238	VIC
db066	KF318201	F: AACTGGACCGGATCATCGT R: TGGGAGTCCACGAAATGAGT	$(AC)_8$	60	204–284	FAM
db080	KF318202	F: TCAACTTCACTGGAGCACGA R: GCTGTGGTTTCCATGCTAGG	$(ACC)_5$	60	285–300	FAM
db081	KF318203	F: TTTGCATGGTACTCAGACATCTTC R: TCTGCGATTCTTGTATGCATCT	$(AT)_6$	60	290–326	PET
db086	KF318204	F: GGTTGTTGTCCTGCCACATT R: GCATCTAAGCCGCTAAACCA	$(AC)_8$	60	311–405	NED
db094	KF318205	F: GGGCTGGTGCATAAGAGTTT R: TCATCATCGTGACATGGAAA	$(AAT)_6$	60	390–416	PET

Note: T_a = annealing temperature.

loci were selected for screening based on penalty score (lowest score indicates "best" primer pair at the locus), size of DNA fragment (100–400 bp), and motif repeat size. The 96 primer pairs were manufactured by IDT (Integrated DNA Technologies, Coralville, Iowa, USA) and screened for amplification and variability in three *D. basaltica*, two *D. chryseopsis*, and two *D. gregaria* individuals using Blacket et al.'s (2012) three primer approach. This method enabled efficient amplification of loci in multiplex PCRs using all four fluorochromes (FAM, VIC, NED, PET), because each fluorochrome contained a unique universal "tail" (Blacket et al., 2012). Locus-specific forward primers were allocated to one of the four tails so each reaction was multiplexed with four different primer pairs. PCRs consisted of 10-μL reactions consisting of 20 ng of DNA, 5 μL Master Mix microsatellite multiplex Type-it Microsatellite PCR Kit (QIAGEN), 0.1 μM of each forward primer, 0.2 μM of each reverse primer, and 0.1 μM of fluorescently tagged universal primer (FAM, VIC, NED, PET). All PCRs were run with the same conditions: 5 min denaturation at 95°C, followed by 28 cycles of 95°C for 30 s, annealing at 60°C for 1 min 30 s and 72°C for 30 s, and a final extension at 60°C for 30 min. Of the 96 primer pairs screened, 13 had distinct polymorphic banding patterns and are developed further here; 12 were monomorphic and not developed any further (Appendix S1). The repeat motifs for the 25 loci included 17 dinucleotides and eight trinucleotides. The 13 polymorphic microsatellite markers were tested for locus variability and marker consistency on four separate populations of *Diuris* species (Table 1). The four populations used in this study were Greenhouse (accession no.: 2370569A), Chatsworth-Woorndoo Road (37.88832°S, 142.77430°E; accession no.: 2370561A and 2370554A), Inverleigh Common (38.08045°S, 144.02983°E; accession no.: 2323619A), and Dunkeld (37.58558°S, 142.18885°E; accession no.: 2370562A). Vouchers are lodged at the National Herbarium of Victoria, Melbourne. Once PCR was performed on all individuals, capillary electrophoresis was used to determine fragment size on an ABI 3130xl (Applied Biosystems, Foster City, California, USA) by Macrogen (Seoul, South Korea). GeneScan

TABLE 2. Genetic properties of the 13 newly developed microsatellites of *Diuris* species.[a]

	Greenhouse *D. basaltica* (n = 18)			Chat-Woorndoo *D. gregaria* (n = 20)			Inverleigh Common *D. chryseopsis* (n = 20)			Dunkeld *D. chryseopsis* (n =20)			Total (n = 78)		
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
db003	3	0.33	0.44	3	0.50	0.50	2	0.37	0.37	2	0.30	0.49	4	0.38	0.49
db012	6	0.71	0.74	5	0.75	0.76	5	0.40	0.57	3	0.65	0.64	7	0.63	0.74
db025	3	0.44	0.43	4	0.45	0.46	4	0.50	0.56	2	0.50	0.39	7	0.47	0.48
db028	2	0.06	0.17	4	0.25	0.46	5	0.56	0.57	1	0.00	0.00	5	0.22	0.32
db047	5	0.41	0.45	3	0.47	0.40	3	0.40	0.45	1	0.00	0.00	6	0.32	0.34
db051	7	0.83	0.72	5	0.30	0.67	6	0.39	0.47	5	0.20	0.49	10	0.43	0.63
db052	3	0.33	0.55	2	0.75	0.51	2	0.30	0.49	2	0.45	0.35	3	0.46	0.51
db053	4	0.56	0.68	4	0.30	0.54	4	0.15	0.47	3	0.75	0.53	7	0.44	0.69
db066	10	0.94	0.84	6	0.60	0.76	8	0.55	0.77	4	0.80	0.72	15	0.72	0.82
db080	1	0.00	0.00	4	0.30	0.27	3	0.25	0.37	1	0.00	0.00	4	0.14	0.18
db081	2	0.61	0.48	8	0.65	0.74	6	0.65	0.63	3	0.85	0.64	9	0.69	0.63
db086	10	1.00	0.88	9	0.75	0.84	18	0.80	0.93	5	0.95	0.77	29	0.86	0.95
db094	5	0.38	0.76	2	0.19	0.35	6	0.14	0.71	3	0.89	0.60	8	0.40	0.68

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

[a]Geographic and voucher information for populations: Greenhouse (accession no.: 2370569A), Chatsworth-Woorndoo Road (37.88832°S, 142.77430°E; accession no.: 2370561A and 2370554A), Inverleigh Common (38.08045°S, 144.02983°E; accession no.: 2323619A), and Dunkeld (37.58558°S, 142.18885°E; accession no.: 2370562A).

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500 LIZ (Applied Biosystems) was used as the internal size standard to size alleles, which were scored with the microsatellite plugin in Geneious software (version 5.6.2; Drummond et al., 2012). The microsatellite profiles obtained from some loci suggest that these *Diuris* species may be tetraploid. Seven of the 13 loci show a diploid pattern, while the remaining six loci display higher ploidy patterns. The polyploid pattern shown with these microsatellite markers may limit future gene flow analysis due to the possible inaccuracy in calculating allele frequencies from ambiguous alleles within the polyploidy loci.

For the polymorphic microsatellite markers, the number of alleles per locus (A = 3–29), observed heterozygosity (H_o = 0.18–0.95), and expected heterozygosity (H_e = 0.14–0.86) were calculated in GenoDive version 2.0b24 (Meirmans and van Tienderen, 2004) for each locus within and among the four populations (Table 2). The number of alleles per species range from one to 10, two to nine, and two to 18 for *D. basaltica*, *D. gregaria*, and *D. chryseopsis*, respectively. Total allele numbers for each individual across all loci ranged between 13 and 23. Heterozygosity was confirmed with SPAGeDi (Hardy and Vekemans, 2002) because it is unknown whether these species are auto- or allotetraploids and GenoDive was developed for autotetraploids. Departure from HWE could not be detected due to polyploidy. To distinguish between the three species, loci heterozygosity was compared among species in SPAGeDi and four of 39 locus comparisons were significantly different ($P < 0.001$).

CONCLUSIONS

The 13 microsatellite markers characterized are intended to facilitate the assessment of population differentiation and the delineation of closely related species. We plan to analyze the population structure, gene flow, and population viability throughout the geographical range of *Diuris* in the VVP.

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