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PRIMER NOTE

Isolation, characterization, and cross-amplification of 20 microsatellite markers for *Conospermum undulatum* (Proteaceae)

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PREMISE: Recent habitat fragmentation is posing a risk to the wavy-leaved smokebush, *Conospermum undulatum* (Proteaceae), a rare plant species endemic to southwestern Western Australia. Microsatellite markers are required to characterize the genetic diversity and structure of the species for conservation purposes and to facilitate ecological studies.

METHODS AND RESULTS: Illumina MiSeq high-throughput sequencing was used to develop 20 novel microsatellite markers for *C. undulatum*. Polymorphism at each locus was assessed using 72 individuals from three natural populations. Nineteen markers were polymorphic, with the number of alleles per locus ranging from two to 21, and observed and expected heterozygosity ranging from 0.000 to 1.000 and 0.117 to 0.919, respectively. All markers successfully amplified in three congeneric species (*C. stoechadis*, *C. canaliculatum* and *C. triplinervium*).

CONCLUSIONS: The microsatellite markers will be useful for revealing patterns of genetic diversity, dispersal dynamics, and hybridization events for *C. undulatum* to inform future conservation efforts.

KEY WORDS Australia; *Conospermum undulatum*; conservation; hybridization; microsatellite primers; Proteaceae.

The genus *Conospermum* Sm. (Proteaceae) represents an important component of the heathlands and woodlands of Western Australian sandplains. The genus has 53 species endemic to Australia, with its center of distribution in southwestern Western Australia (Bennett, 1995). Within the South West Australian Floristic Region, a global biodiversity hotspot (Myers et al., 2000; Hopper and Gioia, 2004), many *Conospermum* species are of increasing conservation concern, with four taxa already declared rare by the Western Australia government (Government Gazette, 2018). Moreover, as for many proteaceous species, various *Conospermum* species are widely utilized in floriculture (Bennett, 1995; Stone et al., 2006). *Conospermum undulatum* Lindl. is a diploid shrub with its range restricted to ca. 55 km^2 in a rapidly expanding urban zone in the metropolitan area of Perth (Close et al., 2006; Wardell-Johnson et al., 2016). This species is listed as Vulnerable under the Environment Protection and Biodiversity Conservation Act 1999. Habitat fragmentation and hybridization with sympatric *Conospermum* species are likely to pose a risk to the future persistence of *C. undulatum*.

In *Conospermum*, studies of population genetics and reproductive biology have been undertaken using amplified fragment length polymorphism (AFLP) and random-amplified polymorphic DNA (RAPD) markers for only a few species (Stone et al., 2006; Sinclair et al., 2008). To our knowledge, no microsatellite resources have been developed for this genus to date. Considering the growing concern about this endemic genus and the number of species within it, we expect that microsatellite markers will have broad applicability for conservation and population genetic analyses. Here, we report the development and characterization of 20 microsatellite markers for *C. undulatum* that will be useful for the study of its genetic structure, spatial patterns of genetic diversity, and dispersal dynamics. Additionally, we tested for cross-amplification of these loci in three related *Conospermum* species to evaluate the utility of the marker set more broadly and specifically to allow assessment of hybridization between *C. undulatum* and neighboring species.

METHODS AND RESULTS

Genomic DNA was extracted from freeze-dried leaf material (ca. 50 mg) using a modified 2% cetyltrimethylammonium bromide (CTAB) method, with 1% polyvinylpyrrolidone and 0.1% sodium

TABLE 1. Characteristics of 20 microsatellite loci in *Conospermum undulatum*.

Locus[a]	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Fluorescent label[b]	Primer mix (μL)[c]	GenBank accession no.
Multiplex 1						
Cu4	F: GGAGACGGGAGAACTGTGGT	$(AG)_{10}$	88–112	VIC	3	MH917262
	R: TACTAAACCCTACCACCCTACCC					
Cu16	F: AGGATCCATATAGCCGACCC	$(AAC)_7$	100–127	PET	3	MH917265
	R: AGCAGTTGCAGTTTCTGTGG					
Cu31	F: GCAAGACAGACGCCCTAAGT	$(AG)_{18}$	131–207	6-FAM	3	MH917272
	R: GGCATTGTGGGTCATCTCCA					
Cu41	F: ATGTCCCACCGGTATTCAGA	$(AG)_6$	242–276	VIC	3	MH917277
	R: CTGAAGAGGAAGCAGGCCTT					
Multiplex 2						
Cu8	F: GCATATGGCCCTCATTGTCT	$(AG)_6$	95	VIC	3	MH917263
	R: GACCTCCCAAAGATATGAGGCT					
Cu17	F: AGCACTCACAAGTCTGACCC	$(AG)_6$	103–111	PET	6	MH917266
	R: GCTCAGTAGCTGCCTTTGTC					
Cu24	F: TGAGACCACAAACCAGACCC	$(AAG)_7$	140–146	NED	5	MH917269
	R: TGTGTCTTCTGTGGCAGTAGT					
Cu32	F: ACACAACAAGCCCTCATCAGT	$(AG)_9$	159–189	6-FAM	4	MH917273
	R: GGTCTGGCAAGTCCACTCTT					
Multiplex 3						
Cu9	F: GACTCTACAGAAGTCTCGCCC	$(AG)_{12}$	86–132	VIC	3	MH917264
	R: GGCAAAGCAAGAGCATGGTT					
Cu18	F: AACCCGCCAACAGAATCGAT	$(AG)_8$	105–111	PET	6	MH917267
	R: TGATCACATGAGGGTAGTAAGC					
Cu28	F: GTTCCTCCATTTCGAAACCCT	$(AG)_{17}$	124–160	NED	4	MH917270
	R: ACCGTTTCGTTTCGTCTCAGT					
Cu33	F: AAGAAATGAAGCAAGGCGTG	$(AG)_{22}$	144–198	6-FAM	3	MH917274
	R: GTAGGAGTCCAAGACCCGTTG					
Multiplex 4						
Cu20	F: TCTCCATCAGTACCGCTACCT	$(AG)_7$	122–128	VIC	2	MH917268
	R: GCTTCCAGTTCCCACCAAAC					
Cu29	F: GACCAGTGAACTCTCAAGGACT	$(AG)_7$	142–186	PET	6	MH917271
	R: CGCAGCTAGCCGACTTAGAA					
Cu36	F: TGCTTCCTTTCAACGCTTGG	$(AGC)_8$	186–201	NED	4	MH917275
	R: TGTAAGTGTACAAGGGTCGCC					
Cu39	F: ACACCAAAGCAAGGCATGAA	$(AG)_{10}$	201–255	6-FAM	4	MH917276
	R: TGCAAACAAGTGGCCTACCA					
Multiplex 5						
Cu15	F: TCGTGATTTCAACCTTGACCA	$(AG)_9$	93–131	VIC	3	MK570861
	R: TGGAACTGGTCATCCCTCCA					
Cu22	F: TGCACAAAGAAGATGGAAGCTG	$(AG)_{11}$	122–158	NED	1	MK570862
	R: CCGTCCACGTATTGCAGAGA					
Cu38	F: AGTTCATATGCCAGCGTAATCG	$(AG)_9$	196–232	6-FAM	3	MK570863
	R: AACGTCCCAGACCAACGATC					
Cu45	F: CTCCAATGGCTACCGTCGAG	$(AG)_{10}$	249–281	PET	5	MK570864
	R: TGACAATTCACATGCATGATGC					

[a]An annealing temperature of 60°C was used for all loci.
[b]Fluorescent label refers to Applied Biosystems fluorescent dyes used in sequencing reactions.
[c]Microliters of primer working solution (2 μM) in 100 μL.

sulphite added to the extraction buffer (Byrne et al., 2001). High-quality DNA extracted from a single *C. undulatum* individual was used by the Monash University Malaysia genomics facility (Petaling Jaya, Selangor, Malaysia) for microsatellite development. Briefly, the extracted DNA was sheared to 500 bp using a Covaris M220 Focused-Ultrasonicator (Covaris, Woburn, Massachusetts, USA), and a NEBNext Ultra DNA preparation kit for Illumina (New England Biolabs, Ipswich, Massachusetts, USA) was used for library preparation after sequencing on the Illumina MiSeq desktop sequencer (Illumina, San Diego, California, USA). Sequencing resulted in a total of 313,174 reads and a total data output of 78 Mbp (data available from the Dryad Repository: https://doi.org/10.5061/dryad.f81k3q7). The obtained reads were searched for microsatellite loci having a minimum of five repeats using the QDDv3.1 pipeline (Meglécz et al., 2014).

The resulting 9848 loci were sorted based on PCR product size, repeat class, repeat length, and multiplexing potential. Of these, 48 candidate loci characterized by perfect repeat motifs and different expected product sizes within the 90–300-bp interval were tested for amplification on a total of six individuals from different populations. Initial screening was performed with Eppendorf Mastercycler ep (Eppendorf, Hamburg, Germany) using 15-μL

TABLE 2. Genetic characterization of 20 newly developed microsatellite loci across three populations of *Conospermum undulatum*.[a]

		Population 1 (n = 24)				Population 2 (n = 24)				Population 3 (n = 24)			
Locus	A_T	A	H_o	H_e	**Null**	A	H_o	H_e	**Null**	A	H_o	H_e	**Null**
Multiplex 1													
Cu4	11	9	0.667	0.811		10	0.750	0.822		9	0.833	0.824	
Cu16	8	5	0.667	0.745		8	0.625	0.694		6	0.333	0.681	0.173
Cu31	21	16	0.833	0.894		17	0.750	0.884		15	1.000	0.891	
Cu41	3	3	0.083	0.424	0.239	2	0.167	0.500		2	0.000	0.486	0.303
Multiplex 2													
Cu8	1	1	ND	ND		1	ND	ND		1	ND	ND	
Cu17	5	5	0.444	0.656		2	0.000	0.413	0.280	2	0.087	0.499	0.311
Cu24	3	3	0.391	0.322		2	0.333	0.278		2	0.125	0.117	
Cu32	9	8	0.667	0.637		5	0.625	0.648		5	0.542	0.582	
Multiplex 3													
Cu9	21	14	0.875	0.904		13	0.750	0.882		16	0.958	0.919	
Cu18	4	4	0.542	0.674		4	0.667	0.702		4	0.500	0.672	
Cu28	18	15	1.000	0.914		14	0.917	0.888		11	0.875	0.846	
Cu33	21	14	0.792	0.878		15	0.958	0.886		11	0.833	0.861	
Multiplex 4													
Cu20	4	4	0.250	0.463		2	0.435	0.499	0.155	2	0.250	0.413	
Cu29	16	10	0.417	0.800	0.176	9	0.174	0.696	0.297	8	0.375	0.643	
Cu36	6	5	0.375	0.419		5	0.417	0.436		4	0.417	0.355	
Cu39	17	12	0.652	0.863	0.138	14	0.875	0.888		10	0.917	0.852	
Multiplex 5													
Cu15	15	11	0.417	0.792	0.177	9	0.500	0.485		13	0.583	0.828	0.108
Cu22	15	10	0.708	0.822		10	0.833	0.841		12	0.792	0.823	
Cu38	15	10	0.917	0.800		10	0.833	0.808		8	0.833	0.742	
Cu45	16	14	0.708	0.839		11	0.625	0.870		12	0.750	0.877	
Mean	11.45	8.650	0.570	0.683		8.150	0.562	0.656		7.650	0.550	0.646	
SE	1.521	1.037	0.060	0.054		1.120	0.067	0.055		1.062	0.075	0.059	

Note: A = number of alleles; A_T = overall number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled; Null = estimated frequency of null alleles where different from zero; ND = not determined.

[a]Localities and voucher information are provided in Appendix 1.

reaction volumes containing 10 ng of genomic DNA, 50 mM KCl, 20 mM Tris–HCl (pH 8.4), 0.2 mM each dNTP, 2 μM of forward and reverse primers, 2.75 μM $MgCl_2$, and 0.1 μL *Taq* DNA polymerase. PCR reactions were performed with the following conditions: initial denaturation at 96°C for 2 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 5 min. The PCR products were checked on 8% polyacrylamide gels to assess for successful amplification across all tested individuals. Of the markers that amplified successfully based on their multiplexing potential and consistent amplification within the expected size range, 22 were selected for initial testing for polymorphism and labeled with fluorescent dyes (VIC, PET, NED, 6-FAM; Applied Biosystems, Foster City, California, USA). In this step, we used 3.75 μL 2× Master Mix (QIAGEN, Hilden, Germany), 0.75 μL of 2 μM primer mix, 1 μL of 5–20 ng genomic DNA, and 2 μL sterile RNase-free water (QIAGEN) in a 7.5-μL reaction with the following PCR conditions: initial denaturation at 95°C for 15 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 90 s, and extension at 72°C for 60 s; and a final extension at 60°C for 30 min. The PCR products were diluted 10× and 1.0 μL of the dilution was added to a mix of 12.0 μL Hi-Di Formamide (Applied Biosystems) and 0.1 μL GeneScan 500 LIZ Size Standard (Applied Biosystems) for sequencing on a 3730xl DNA analyzer (Applied Biosystems). After testing, 20 markers (17 containing dinucleotide and three containing trinucleotide microsatellites) that consistently amplified with easily scorable peaks were selected and combined into five multiplexes (Table 1). Subsequently, those multiplexes were tested in 72 *C. undulatum* individuals from three populations (Appendix 1) using the same PCR conditions as described above. Plants were selected as evenly spaced as possible throughout the populations by using a grid of 15 × 15-m quadrats where the closest plant to each corner of each quadrat was sampled. Leaves were kept separated per source plant, stored in silica gel after collection, and then freeze-dried. DNA was extracted from 50 mg of freeze-dried leaf material using a modified 2% CTAB method, as outlined above. Multiple runs were performed to ensure both the consistency of scoring and the accuracy of the final data set. In addition, we tested cross-amplification with a total of 45 samples of three congeners (*C. stoechadis* Endl., *C. canaliculatum* Meisn., and *C. triplinervium* R. Br.) sampled within a 30-km radius from the *C. undulatum* populations (Appendix 1).

Allele size was determined using GeneMapper Software v5 (Applied Biosystems). GenAlEx v6.51 (Peakall and Smouse, 2012) was used to calculate number of alleles per locus and levels of expected and observed heterozygosity for loci in three populations (Table 2). Evidence of linkage disequilibrium was assessed by GENEPOP (Rousset, 2008) based on 10,000 permutations. The frequency of possible null alleles, genotyping failure, and inbreeding were estimated using INEST 2.2 (Chybicki and Burczyk, 2009) after 500,000 Markov chain iterations.

Evaluation of loci showed no indication of linkage disequilibrium for any pairwise combination of loci, nor was there significant genotyping failure. Loci Cu15, Cu17, Cu29, and Cu41

TABLE 3. Cross-amplification of 20 microsatellite loci developed for *Conospermum undulatum* in three related species.[a]

	C. stoechadis (*n* = 15)			*C. canaliculatum* (*n* = 15)			*C. triplinervium* (*n* = 15)		
Locus	**Amplification**	***A***	**Allele size (bp)**	**Amplification**	***A***	**Allele size (bp)**	**Amplification**	***A***	**Allele size (bp)**
Multiplex 1									
Cu4	15	7	86–104	14	4	84–92	15	1	92
Cu16	15	5	100–112	15	5	100–112	15	2	103
Cu31	15	12	131–167	15	13	133–165	15	2	139–141
Cu41	15	3	242–246	15	4	242–248	15	2	246–250
Multiplex 2									
Cu8	15	1	95	15	2	75–95	15	2	75–95
Cu17	15	4	107–113	15	4	101–111	15	2	100–111
Cu24	15	3	140–146	15	3	140–146	15	1	140
Cu32	15	4	161–187	15	6	161–175	15	1	163
Multiplex 3									
Cu9	15	8	88–122	15	10	82–120	14	1	102
Cu18	15	4	105–111	15	4	105–111	15	1	109
Cu28	15	10	124–154	15	13	124–168	15	3	136–148
Cu33	15	11	146–178	15	12	152–188	15	4	164–168
Multiplex 4									
Cu20	15	2	124–126	15	4	122–128	15	2	128–132
Cu29	15	6	150–178	14	9	148–172	15	2	150–152
Cu36	15	3	189–201	15	6	186–201	15	2	186–198
Cu39	15	9	201–237	15	10	213–249	15	3	215–223
Multiplex 5									
Cu15	15	10	93–123	14	10	91–129	15	3	105–111
Cu22	14	6	126–152	13	11	116–152	14	4	128–148
Cu38	15	6	196–220	15	7	200–232	15	2	202–204
Cu45	15	11	247–275	15	12	247–281	14	3	259–269

Note: A = number of alleles; Amplification = number of individuals successfully amplified; *n* = number of individuals used.
[a]Localities and voucher information are provided in Appendix 1.

showed evidence of null alleles in two out of three populations and should be used with caution in analyses whose results may be inflated by the occurrence of null alleles. One locus was monomorphic in the three *C. undulatum* populations investigated in this study (i.e., Cu8; Table 2). However, this marker was polymorphic in two of the three other *Conospermum* species considered. Overall, we observed 229 alleles at the 20 microsatellite loci, with an average of 11.45 alleles per locus. Observed and expected heterozygosity levels per locus ranged from 0.000 to 1.000 and from 0.117 to 0.919, respectively (Table 2). Inbreeding was not included as a variable in the most likely INEST model to explain excess of homozygosity and, therefore, average within-population inbreeding was not statistically different from zero in any of the three analyzed populations. All microsatellite loci showed successful cross-amplification in *C. stoechadis*, *C. canaliculatum*, and *C. triplinervium* (Table 3) using the same extraction method and amplification conditions outlined above. Analysis of amplification showed similar number of alleles amplified in *C. stoechadis* and *C. canaliculatum* as in *C. undulatum*, whereas fewer alleles were detected in *C. triplinervium*.

CONCLUSIONS

Twenty microsatellite markers were developed for *C. undulatum*. These markers will be used for investigating population genetic structure, dispersal dynamics, and possible hybridization events of this rare species to underpin its management and conservation. These newly developed markers are likely to be useful for genetic studies on phylogenetically related species given the successful cross-amplification for three different *Conospermum* species.

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AUTHOR CONTRIBUTIONS

N.D. wrote the manuscript, designed the experiment, and collected and analyzed data. A.P. contributed with analyses and interpretation of data and critically revised the manuscript. E.J.v.E. contributed to the design the experiment and collection of data and revised the manuscript. W.D.S. designed the experiment and critically revised the manuscript. M.B. helped improve the design of the experiment and critically revised the manuscript.

DATA ACCESSIBILITY

Raw sequencing data are available from the Dryad Repository (https://doi.org/10.5061/dryad.f81k3q7). Sequence information for the developed primers has been deposited to the National Center

for Biotechnology Information (NCBI); GenBank accession numbers are provided in Table 1.

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APPENDIX 1. Locality information for *Conospermum* species used in this study.

Species	Voucher[a]	Collection locality	*N*	Geographic coordinates
Conospermum undulatum Lindl.	PERTH 06797083	Maida vale	24	Rare flora
	PERTH 09006176	Orange grove	24	Rare flora
	PERTH 09006192	Orange grove	24	Rare flora
Conospermum stoechadis Endl.	PERTH 07795092	Koondoola regional bushland	15	−31.846111, 115.874722
Conospermum canaliculatum Meisn.	PERTH 06256430	7 km NE of Yanchep	15	−31.489641, 115.668538
Conospermum triplinervium R. Br.	PERTH 07800754	Kings Park, Perth	15	−31.966827, 115.836388

Note: N = number of individuals per population.
[a]Vouchers are stored in the Western Australian Herbarium (PERTH), Perth, Western Australia, Australia.