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Authors: Anthony, Janet M., Allcock, Richard J. N., Dobrowolski, Mark P., and Krauss, Siegfried L.

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Primer Note

Isolation and characterization of microsatellite primers for the critically endangered shrub *Styphelia longissima* (Ericaceae)[1]

Janet M. Anthony[2,3,6], Richard J. N. Allcock[4], Mark P. Dobrowolski[5], and Siegfried L. Krauss[2,3]

[2]Kings Park and Botanic Garden, Botanic Garden and Parks Authority, Fraser Avenue, West Perth, 6005 Western Australia, Australia; [3]School of Biological Sciences, The University of Western Australia, Crawley, 6009 Western Australia, Australia; [4]Lotterywest State Biomedical Facility: Genomics, School of Pathology and Laboratory Medicine, The University of Western Australia, Crawley, Western Australia 6009, Australia; and [5]Iluka Resources Limited, Level 23, 140 St. Georges Terrace, Perth, Western Australia 6000, Australia

- *Premise of the study:* Microsatellite markers were developed for population genetic analysis in the rare shrub *Styphelia longissima* (Ericaceae).
- *Methods and Results:* We generated ca. 2.5 million sequence reads using a Personal Genome Machine semiconductor sequencer. Using the QDD pipeline, we designed primers for >12,000 sequences with PCR product lengths of 80–480 bp. From these, 30 primer pairs were selected and screened using PCR; of these, 16 loci were found to be polymorphic, four loci were monomorphic, and 10 loci did not amplify reliably for *S. longissima*. For a sample of 57 plants from the only known population, the number of alleles observed for these 16 loci ranged from two to 21 and expected heterozygosity ranged from 0.49 to 0.91. These markers were also amplified in *Astroloma xerophyllum*, a closely related species.
- *Conclusions:* These markers will be used to characterize population genetic variation, spatial genetic structure, mating system parameters, and dispersal to aid in the management and conservation of the rare shrub *S. longissima.*

Key words: *Astroloma xerophyllum*; Ericaceae; microsatellite primers; shotgun sequencing; *Styphelia longissima.*

Styphelia longissima Hislop & Puente-Lel. (Ericaceae) is a newly described species (Hislop and Puente-Lelièvre, 2017) and found only in a single population on sand within remnant kwongan vegetation near Eneabba, in the South West Australian Floristic Region (SWAFR), an international biodiversity hotspot (Hopper and Gioia, 2004). Until very recently, this taxon was assigned to the genus *Leucopogon* R. Br. (Ericaceae) with the temporary name of *Leucopogon* sp. ciliate Eneabba (F. Obbens & C. Godden s.n. 3/7/2003). Recent taxonomic revision has placed this and some other *Leucopogon* species within the Styphelieae, with sister taxa *Leucopogon* sp. Ongerup and *Astroloma* sp. sessile leaf (Puente-Lelièvre et al., 2015; Hislop and Puente-Lelièvre, 2017). In 2007, the population consisted of just 1993 individuals (Woodman Environmental Consulting Pty. Ltd., 2008). Since then, however, mortality has substantially exceeded recruitment (Harris, 2013), and the species is currently listed as Rare Flora under the Wildlife Conservation Act 1950 (Western Australian Minister for the Environment, 2015). *Styphelia longissima* is a spindly to dense shrub, to 0.5(–0.8) m high, with cream-white colored flowers in July that are most likely insect pollinated, and seed dispersal is myrmecochorous (Harris, 2013). Microsatellite markers were developed for *S. longissima* to enable an assessment of population genetic variation, spatial genetic structure, mating system parameters, and dispersal for management and conservation. *Astroloma xerophyllum* (DC.) Sond., a sister taxon, was chosen for cross-amplification based on molecular phylogeny of the Styphelieae (Puente-Lelièvre et al., 2015).

METHODS AND RESULTS

Genomic DNA was extracted from a single tissue-cultured plant, sampled from the only known population (Appendix 1), using a Carlsons method (Carlson et al., 1991) with modifications outlined in Anthony et al. (2016). Next-generation sequencing was performed on a Personal Genome Machine (PGM) semiconductor sequencer (Life Technologies, Carlsbad, California, USA) at the Lotterywest State Biomedical Facility Genomics Node in Perth, Western Australia, as described previously (Anthony et al., 2016). After sequencing, signal processing, base-calling, and quality trimming were performed using the default settings of TorrentSuite 4.0 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and library-specific FASTQ files were also generated. Sequencing resulted in >2.5 million reads, with a modal read length of 344 bp and a total data output of 766 Mb (National Center for Biotechnology Information [NCBI] Sequence Read Archive Bioproject no. PRJNA397350).

The raw sequences were screened using QDD version 3.1 pipeline (Meglécz et al., 2014) to remove redundant sequences and design primers for >12,000 sequences with PCR product lengths of 80–480 bp. The default parameters of the program were used both for the screening steps and for primer design. The

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[6]Author for correspondence: janet.anthony@dbca.wa.gov.au

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resultant sequences were filtered to ensure that the primer was not overlapping the repeat sequence, there were no poly-'A' or poly-'T' runs for more than seven base pairs within the sequence, and there was only one repeat motif between the primers. Subsequently, 30 primer pairs were selected based on the suggestions of Meglécz (2014).

Initial screening was performed with CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA) using 5 µL of SsOAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories), 0.2 µM each of forward and reverse primers, and 5–10 ng of genomic DNA in a 10-µL reaction volume. Initially, screening included reliable amplification of a single sample across a range of temperatures to determine the most appropriate annealing temperature, followed by evidence of polymorphism among eight individuals. Consequently, 16 polymorphic loci (including three loci that were fixed heterozygotes) and four monomorphic loci were selected to complete the study (Table 1), and the remaining 10 loci did not amplify reliably for *S. longissima*. Amplification of 57 individuals using 16 polymorphic loci was performed using a Veriti thermocycler (Life Technologies) within four multiplex mixes containing 6.25 µL of 2× Multimix and 2.25 µL of 5× Q-Solution (Type-It Microsatellite PCR kit; QIAGEN, Hilden, Germany), 1.25 µL of primer mix, and 2.25 µL of 5–100 ng DNA in a 12-µL reaction. Primer Mix (PM) 1 contained the primers Sl36, Sl65, Sl53; PM 2 contained Sl17, Sl18, Sl57, Sl60; PM 3 contained Sl6, Sl26; and PM 4 contained Sl01, Sl47, Sl67, Sl71 using the following PCR conditions: an initial 1-min denaturation at 95°C; 35 cycles of 94°C for 10 s, 62°C (PM 1, 2, 3) or 56°C (PM 4) for 30 s, and 72°C for 45 s; followed by a final extension of 15 min at 72°C. Electrophoresis was performed using the ABI 3500 sequencer (Life Technologies), and allele sizes were determined using Geneious version 7.1 (Biomatters Ltd., Auckland, New Zealand). Multiple replicate runs were performed to ensure the accuracy of the final data set. Genetic diversity parameters were

Table 1. Characteristics of microsatellite loci developed for *Styphelia longissima* and cross-amplified in *Astroloma xerophyllum*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)		T_a (°C)	Fluorescent label	GenBank accession no.
			S. longissima (N = 57)	*A. xerophyllum* (N = 5)			
Sl01	F: TGGTTATACTCAGCTTATGTATTGG	$(CT)_{14}$	203–223	201–205	56	6-FAM	KY559296
	R: AAGATTTACTTGCATTCTGCCA						
Sl06	F: CACAAGTGGTTGATGAGTCCC	$(AG)_{14}$	87–111	187–228	56	VIC	KY559297
	R: CATCCTTCTCCCTTCCCTTC						
Sl16	F: GGCTCACCAATTGGCTACAT	$(AG)_{13}$	—	326[b]	62	NED	MF405897
	R: CACTTGTGTACTTGTGACTTCATTTG						
Sl17	F: CGCTGCTTTACATCTTTGCTC	$(CA)_{13}$	113–133	159–233	62	VIC	KY559298
	R: CCTGATTTCCCTGAACTCCA						
Sl18	F: CCAGAAAGTCTTCCTCTCCCT	$(AC)_{13}$	98–112	187–194	62	PET	KY559299
	R: ATCGGGAAGTGGAAGGTCAG						
Sl22	F: AGCCGCGGAACATATACAAG	$(AAG)_{12}$	—	163[b]	56	6-FAM	MF405898
	R: GGACCAATTCATTCACTTGGAG						
Sl24	F: TCTCGACAGGAAGTGGAGGT	$(AG)_{12}$	345[b]	254–262	56	NED	MF405899
	R: GGTTACCAATATGTACACGGCA						
Sl26	F: AGCGTTCAAGTAAGGCGAAA	$(AG)_{12}$	155–213	153–161	62	NED	KY559300
	R: GGCGATGGCCACTTTACTAC						
Sl29	F: GGAACACATCGTAACGTGGA	$(GA)_{12}$	—	274–276	56	6-FAM	MF405900
	R: ACCACGCAAACACTGTGAAA						
Sl32	F: CGATCGATCAAATCTCAGCA	$(AG)_{11}$	207[b]	—	56	6-FAM	MF405901
	R: GACCAAGAAGACAAAGAAGACGA						
Sl36	F: CCCACGATAGCTCAGAGGTC	$(GAA)_{11}$	136–185	136–148	62	VIC	KY559301
	R: GGGATACAGCAGGTAAGTTCA						
Sl45	F: TTGGTCGTAATCACTTGGGAG	$(AG)_{11}$	153[b]	—	56	NED	MF405902
	R: GGCATTGGCACAACTTCAG						
Sl47	F: TTTGTTTACAGAACTTAGGCGG	$(GA)_{10}$	238–246	233–250	56	VIC	KY559302
	R: TTTGTAGTTCTTTCAGAATTGAGGA						
Sl51	F: AAATGAGCTGAGCTGAATGC	$(TG)_{10}$	—	138–150	56	VIC	MF405903
	R: GGGTGTTTCCACTTAACCTGC						
Sl53	F: GGAAATCACAATCACGACCA	$(CA)_{10}$	115–141	114[b]	62	PET	KY559303
	R: TAGACATCCCTTCCGGATCA						
Sl57	F: AACACCAAACTAGGAAAGCCG	$(AG)_{10}$	89–107	—	62	VIC	KY559304
	R: ATTCAGAATCAGGACCTGCC						
Sl60	F: AATTGGGTACGTGACATCGG	$(GA)_{10}$	172–262	—	62	NED	KY559305
	R: ATGAACGTCAGCCCATTTCC						
Sl62	F: GAGAGGGACTTCGGAACAAA	$(AG)_{10}$	180, 182[c]	—	56	VIC	MF405904
	R: CCCACTCTCCAAATCCTTCA						
Sl65	F: ACTGGTCAGCCTGTTAGCGT	$(CT)_9$	290–338	—	62	NED	KY559306
	R: CGCAAGTGAAATCAAGTACGA						
Sl67	F: TTCCAAGATAACTAAATCATATACACA	$(TTG)_9$	143–176	133–153	56	PET	KY559307
	R: TGAATGTTGTCATGGTTCTG						
Sl68	F: TGCAATCAAATTATACTTACAGCCA	$(AAG)_9$	240[b]	—	56	PET	MF405905
	R: CAACCCTGTTCAATTCCACC						
Sl70	F: TGTGTAGGTTCGATTCTTATTCG	$(AC)_9$	164, 168[c]	—	56	PET	MF405906
	R: CTCTGGACGCAAGGTACTGA						
Sl71	F: ACCTCCAACACCAATGAACC	$(CA)_9$	315–349	314–329	56	VIC	KY559308
	R: ACCTCCAACACCAATGAACC						
Sl75	F: TTGCACTTTGAGCCTCTCATT	$(AGT)_8$	125, 129[c]	133[b]	56	6-FAM	MF405907
	R: TTTGCCTTCAATAAGTGGTAATTT						

Note: — = no amplification; N = number of individuals used in this study; T_a = annealing temperature.

[a] Voucher and locality information are provided in Appendix 1.

[b] Monomorphic loci.

[c] Fixed heterozygotes.

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TABLE 2. Results of primer screening with 16 polymorphic primers for *Styphelia longissima.*

Locus	*A*	H_o	H_e	HWE[a]
Sl01[b]	7	0.421	0.727	***
Sl06	11	0.825	0.825	ns
Sl17	12	0.825	0.857	ns
Sl18[b]	7	0.368	0.692	***
Sl26[b]	21	0.691	0.914	***
Sl36[b]	8	0.456	0.591	***
Sl47	5	0.439	0.491	ns
Sl53[b]	5	0.246	0.647	***
Sl57[b,c]	8	0.614	0.771	*
Sl60[b]	18	0.386	0.821	***
S162	2	1.00	0.500	***
Sl65[b]	12	0.386	0.711	***
Sl67	7	0.737	0.776	ns
S170	2	1.00	0.500	***
Sl71[b,c]	9	0.474	0.690	***
S175	2	1.00	0.500	***

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium.

[a] Significant departures from HWE are indicated as $*P \leq 0.05$, $***P \leq 0.001$, ns = not significant.

[b] Evidence suggesting null alleles.

[c] Evidence of stuttering.

calculated using GenAlEx version 6.4 (Peakall and Smouse, 2006). Departure from Hardy–Weinberg equilibrium (HWE) was assessed for each locus and population by χ^2 tests, and the possibility of null alleles was checked using MICRO-CHECKER version 2.2.3 (van Oosterhout et al., 2004).

The number of alleles observed for the 16 polymorphic loci ranged from two to 21, and the observed and expected heterozygosities ranged from 0.25 to 1.00 and 0.49 to 0.91, respectively (Table 2). There were significant departures from HWE for 12 of the 16 loci, with four loci showing no evidence of stuttering, large allele dropout, or null alleles (Table 2). Two loci showed evidence of stuttering indicated as a deficit of heterozygote genotypes with alleles of one base pair repeat difference. These loci have been thoroughly checked by repeat PCR and analysis scoring, and the one base pair difference shown to be real. These departures from HWE are most likely due to inbreeding within a small isolated population.

Using the same extraction method, five individuals were cross-amplified for the sister taxon *A. xerophyllum.* Amplification was performed using the same initial screening method outlined above and resulted in 12 polymorphic and four monomorphic loci (Table 1).

CONCLUSIONS

These 16 polymorphic microsatellites will be used for conservation genetic studies in the rare *S. longissima* to underpin management and conservation. These microsatellites are likely to be useful for genetic studies in other related species given the initial success in cross-amplification for *A. xerophyllum.*

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APPENDIX 1. Locality and voucher information for species used in this study.[a]

Species	Voucher specimen accession no.	Collection locality (Population ID)	Geographic coordinates	*N*
Styphelia longissima Hislop & Puente-Lel.	PERTH 0709170	Eneabba	Rare flora	57
Astroloma xerophyllum (DC.) Sond.	PERTH 8448108	16 km N of Eneabba	–29.683333, 115.483333	5

Note: *N* = number of individuals used in this study.

[a] Vouchers are stored in the Western Australian Herbarium (PERTH), Perth, Western Australia, Australia.

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