ISOLATION AND CHARACTERIZATION OF MICROSATELLITE PRIMERS FOR THE CRITICALLY ENDANGERED SHRUB STYPHELIA LONGISSIMA (ERICACEAE)

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• 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 Government, 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 (D.C.) 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), 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.
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 Meglész (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 0.25 μL of 2X Multimix and 2.25 μL of 5X Q-Solution (Type-H 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 denaturation at 95°C, 35 cycles of 94°C for 10 s, 62°C (PM 1, 2, 3) or 55°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

| Locus | Primer sequences (5′–3′) | Repeat motif | S. longissima (N = 57) A. xerophyllum (N = 5) Tα (°C) | Fluorescent label | GenBank accession no. |
|-------|--------------------------|--------------|-----------------------------------------------|----------------|----------------------|
| SI01  | TGTTTTAATCTAGGCTTTATGATG | (CT)14       | 203–223                                       | 201–205        | 56                   | 6-FAM KY559296       |
| R1    | AAGATTATCTAGGCTTTATGATG  |              |                                               |                |                      |                     |
| SI06  | CACCAAGTTGAGTGAAGATCCC   | (AG)14       | 87–111                                        | 187–228        | 56                   | VIC KY559297         |
| R1    | GCCGGCCCTGGCTCCTGTTG     |              |                                               |                |                      |                     |
| SI16  | GGTCCCAACCACTATGCTCAT   | (AG)13       | —                                             | 326b           | 62                   | NED MF405897         |
| R1    | CACCTGGTATTGCTCTGATTTG   |              |                                               |                |                      |                     |
| SI17  | CGCTGCTTTACATTCTGTG     | (CA)13       | 113–133                                       | 159–233        | 62                   | VIC KY559298         |
| R1    | GCTGATTTCTGGAATCTCCA    |              |                                               |                |                      |                     |
| SI18  | CGAAGGCTTCCTCTCTCTCT    | (AG)13       | 98–112                                        | 187–194        | 62                   | PET KY559299         |
| R1    | AACCCAGTTCACATGCTGAGG   |              |                                               |                |                      |                     |
| SI22  | AGCCGCGGACACATATACAG    | (AG)12       | —                                             | 163b           | 56                   | 6-FAM MF405898       |
| R1    | GCGTGAGGCGGACATATACAG   |              |                                               |                |                      |                     |
| SI29  | GAAACACATGAGGCTGAGGA    | (GA)12       | —                                             | 274–276        | 62                   | VIC KY559300         |
| R1    | ACCAGCCGAACACATGAGGA    |              |                                               |                |                      |                     |
| SI32  | CGTAGCATCAGAATCTAGGAG    | (GA)11       | 207b                                          | —              | 56                   | VIC MF405901         |
| R1    | GACCAAGAAGACAAGAGAGGAGCA|              |                                               |                |                      |                     |
| SI36  | CCACCAATGCTCTAGAGGTC    | (GAA)11      | 136–185                                       | 136–148        | 62                   | VIC KY559301         |
| R1    | GGTTACATGCTAGTACAGG     |              |                                               |                |                      |                     |
| SI45  | TTGTTGTGATCTGCTGCTG     | (AG)11       | 153b                                          | —              | 56                   | NED MF405902         |
| R1    | TTGTTGTGATCTGCTGCTG     |              |                                               |                |                      |                     |
| SI47  | TTTTTTCTACAGAAGATCTAGGCGG| (GA)10       | 238–246                                       | 233–250        | 56                   | VIC KY559302         |
| R1    | TTTTTTCTACAGAAGATCTAGGCGG|              |                                               |                |                      |                     |
| SI51  | AAATGGACCTGAGCTGATGCC   | (TG)10       | —                                             | 138–150        | 56                   | 6-FAM MF405903       |
| R1    | GAGCTGATCTAATCTATCTT    |              |                                               |                |                      |                     |
| SI53  | GGAAATCACAATCTAGGCA     | (CA)10       | 115–141                                       | 114b           | 62                   | PET KY559303         |
| R1    | CTACAGACCTGAGTCCAGGA    |              |                                               |                |                      |                     |
| SI57  | ACCAACCAACCTAGAAGAGGAC  | (AG)10       | 89–107                                        | —              | 62                   | VIC KY559304         |
| R1    | ATCCAGAATACAGGACCTGCC   |              |                                               |                |                      |                     |
| SI60  | AATTGAGTGATCTGAGATCC    | (GA)10       | 172–262                                       | —              | 62                   | NED KY559305         |
| R1    | ATGAACGTAGCTGACATCTCC   |              |                                               |                |                      |                     |
| SI62  | GAGAGGAGCTCTGAGAAGAAA   | (AG)10       | 180, 182b                                     | —              | 56                   | VIC MF405904         |
| R1    | GAGACTGCTGCTGCTGCTGCT   |              |                                               |                |                      |                     |
| SI65  | ACTGTCGAGCTGCTGATCC    | (CT)9        | 290–338                                       | —              | 62                   | NED KY559306         |
| R1    | CGCACAGTGAATCTAGGACAG   |              |                                               |                |                      |                     |
| SI67  | TCCCAAAGATAAATCATATACACA| (TTG)9       | 143–176                                       | 133–153        | 56                   | VIC KY559307         |
| R1    | TCGATGTTGATGATGATGATG   |              |                                               |                |                      |                     |
| SI68  | CTTAGGCACTCGACATCGCGA   | (AAG)9       | 120–240                                       | —              | 56                   | PET MF405905         |
| R1    | CAAGCTGCTGCTGCTGCTGCTC |              |                                               |                |                      |                     |
| SI70  | TCGGAGGTCAGTTCTTCTTCC   | (AC)9        | 164, 168b                                     | —              | 62                   | PET MF405906         |
| R1    | TTTTTTTTTTTTTTTTTTTTTTT|              |                                               |                |                      |                     |
| SI71  | ACCTCCAAACCAATGAGCC     | (CA)9        | 315–349                                       | 314–329        | 56                   | VIC KY559308         |
| R1    | ACCTCCAAACCAATGAGCC     |              |                                               |                |                      |                     |
| SI75  | TTTCAGCTTGGAGCTGCTGCT   | (AGT)9       | 125, 129b                                     | 133b           | 56                   | 6-FAM MF405907       |
| R1    | TTGTCTTTTTTTTTTTTTTTTTT|              |                                               |                |                      |                     |

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

*a Voucher and locality information are provided in Appendix 1.

*b Monomorphic loci.

*c Fixed heterozygotes.

http://www.bioone.org/loi/apps

Table 1. Characteristics of microsatellite loci developed for *Styphelia longissima* and cross-amplified in *Astroloma xerophyllum*. a
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$^b$ | 8 | 0.614 | 0.771 | * |
| Sl60$^b$ | 18 | 0.386 | 0.821 | *** |
| Sl162 | 2 | 1.00 | 0.500 | *** |
| Sl65$^b$ | 12 | 0.386 | 0.711 | *** |
| Sl67 | 7 | 0.737 | 0.776 | ns |
| Sl70 | 2 | 1.00 | 0.500 | *** |
| Sl71$^b$ | 9 | 0.474 | 0.690 | *** |
| Sl75 | 2 | 1.00 | 0.500 | *** |

Note: $A$ = number of alleles; $H_o$ = expected heterozygosity; $H_e$ = 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.

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.