Applications in Plant Sciences 2016 4(11): 1600083

PRIMER NOTE

MICROSATELLITE PRIMERS FOR THE RARE SEDGE *LEPIDOSPERMA BUNGALBIN* (CYPERACEAE)[1]

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- *Premise of the study:* Microsatellite markers were developed for the rare sedge *Lepidosperma bungalbin* (Cyperaceae) to assess genetic variation and its spatial structuring.
- *Methods and Results:* We conducted shotgun sequencing on an Illumina MiSeq and produced 6,215,872 sequence reads. The QDD pipeline was used to design 60 primer pairs that were screened using PCR. We developed 17 loci, of which 12 loci were identified that were polymorphic, amplified reliably, and could be consistently scored. We then screened these loci for variation in individuals from three populations. The number of alleles observed for these 12 loci across the three populations ranged from nine to 19 and expected heterozygosity ranged from 0.41 to 0.89.
- *Conclusions:* These markers will enable the quantification of the potential impact of mining on genetic variation within *L. bungalbin* and establish a baseline for future management of genetic variation of the rare sedge.

Key words: Cyperaceae; *Lepidosperma bungalbin*; microsatellite primers; shotgun sequencing; South West Australian Floristic Region (SWAFR).

Lepidosperma bungalbin R. L. Barrett (Cyperaceae) is a sedge species of conservation priority (Conservation status P1, *Wildlife Conservation Act 1950*, Western Australia), restricted to steep midslopes on the Helena and Aurora Range, an ancient banded iron formation in southern Western Australia (Barrett, 2007). Banded iron formation ranges form a small proportion of the total land area of the region and provide rare and fragmented habitat for endemic flora in comparison to the surrounding matrix (Gibson et al., 2012). They are also the focus of exploration and mining activity, and many short-range endemic species confined to the Helena and Aurora Range are of conservation priority because they are potentially threatened by proposed mining activities. Here we report the isolation and characterization of 12 polymorphic microsatellite loci from *L. bungalbin*, which will be used to examine spatial genetic structure across the species range and to quantify the genetic impact of proposed mining.

METHODS AND RESULTS

Genomic DNA was extracted from fresh leaf material of one individual using the NucleoSpin Plant II method (Macherey-Nagel GmbH and Co., Düren, Germany) (Universal Transverse Mercator [UTM] coordinates 755768E, 6636451N; collector no. Nevill 101; voucher held at the University of Western Australia Herbarium [UWA], Crawley, Western Australia, Australia). We sheared genomic DNA in a volume of 50 µL using a Covaris E220 Focused-ultrasonicator (Covaris, Woburn, Massachusetts, USA). Then, sequencing libraries were prepared following the manufacturer's protocol using Illumina's TruSeq Nano DNA Library Preparation Kit (Life Technologies, San Diego, California, USA).

[1]Manuscript received 12 July 2016; revision accepted 2 September 2016.

The authors thank Polaris Metals Pty Ltd for funding this work and Melissa Hay from Ecologia Environment for assistance with sample collection.

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doi:10.3732/apps.1600083

Libraries were assessed by gel electrophoresis (Agilent D1000 ScreenTape Assay; Agilent, Santa Clara, California, USA) and quantified by qPCR using KAPA Library Quantification Kits for Illumina (KAPA Biosystems, Wilmington, Massachusetts, USA). Sequencing was conducted on an Illumina MiSeq (Life Technologies) with 2 × 250-bp paired-end reads and the MiSeq Reagent Kit version 2. We used the PEAR assembler (Zhang et al., 2013) to stitch FASTAQ sequences from the MiSeq sequencing run and the QDD version 3.1.2 pipeline (Meglécz et al., 2014) with default parameters to screen the raw sequences and design primers. Shotgun sequencing produced 6,215,872 reads, and we excluded loci that contained imperfect repeats, where the primer was overlapping the repeat sequence, and where there were poly-'A' or poly-'T' runs for more than seven base pairs within the sequence.

Sixty potentially suitable microsatellite loci were identified and selected for screening using DNA from six individuals, each from a different population. Microsatellite loci were amplified in 6-µL reaction volumes that contained PCR buffer, Bioline IMMOLASE DNA polymerase and dNTPs provided by Bioline (all Bioline Reagents Ltd., London, United Kingdom), 1.5 mM $MgCl_2$, 0.06 µM of M13-labeled forward locus–specific primer, 0.13 µM of reverse locus–specific primer, 0.13 µM of fluorescently labeled (FAM [Sigma-Aldrich, St. Louis, Missouri, USA]; NED, VIC, and PET [Invitrogen/Thermo Fisher Scientific, Waltham, Massachusetts, USA]) M13 primer, and 15 ng gDNA. Thermocycling was performed with an Applied Biosystems 384-well Veriti Thermal Cycler (Life Technologies), and conditions were as follows: 94°C for 5 min; followed by 11 cycles at 94°C for 30 s, 60°C for 45 s (decreasing 0.5°C per cycle), and 72°C for 45 s; followed by 30 cycles at 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s; followed by 15 cycles at 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s; and a final elongation step at 72°C for 10 min. Markers were pooled together and 1 µL of pooled sample was then applied to a 10-µL mixture of Applied Biosystems Hi-Di Formamide and GeneScan 500 LIZ Size Standard (Life Technologies) and heated at 95°C for 5 min. An Applied Biosystems 3730 DNA Analyzer (Life Technologies) was used to conduct capillary electrophoresis of the product. Run time for a 96-well plate was approximately 1 h (230 V, 32 A). We determined allele sizes using Geneious version 7.1 (Biomatters Ltd., Auckland, New Zealand). Seventeen loci produced readable electropherograms, and five were excluded from further analyses because they amplified inconsistently or were difficult to score accurately (Table 1). Subsequently, 12 loci were selected to complete the study using the conditions described above. Linkage disequilibrium among loci was tested using FSTAT version 2.9.3.2 (Goudet, 1995), and sequential Bonferroni corrections were applied to alpha values to correct for

TABLE 1. Characteristics of 17 microsatellite loci developed in *Lepidosperma bungalbin*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Fluorescent label[b]	GenBank accession no.
lbcur6	F: GGGTTTAGCCTGGCTACTCT	$(GA)_{19}$	144–192	NED	Pr032816349
	R: ACGAAGCACACAACATTTCTCA				
lbcur14	F: GGGCAATCAAGCTTAAGTGGT	$(AG)_{15}$	172–210	NED	Pr032816338
	R: CACAACATCGCACGACCTTC				
lbcur16	F: ACTCATCAAGGAGGCCCAAC	$(TG)_{18}$	164–236	VIC	Pr032816339
	R: CTTGAGCTGGCCTAGGGAAG				
lbcur21	F: GGACTTGAGACCGACTATGCT	$(GA)_{17}$	196–214	FAM	Pr032816340
	R: TCCTCACGTACCCTAGCACT				
lbcur27	F: TGGACACAAAGGAGACCATGA	$(AG)_{17}$	209–237	PET	Pr032816341
	R: ACGCCTCTTCATATGCATCCA				
lbcur33	F: CCTTCGGTGTGCAGTTAGGT	$(TC)_{22}$	216–248	FAM	Pr032816342
	R: GGTTGTTAATCTCAGAATTGGGAG				
lbcur38	F: TCACTTTCAAAGCCTGCCTCT	$(CA)_{16}$	236–256	NED	Pr032816343
	R: CGCAGAACATAACTGTTTCCCA				
lbcur40	F: TCCAAAGTCGGAAGTCGTCG	$(TC)_{14}$	274–294	VIC	Pr032816344
	R: AGAGGAGCTACTTGCCAAGC				
lbcur41	F: CAGCCTCGCCATTACCCTTT	$(AG)_{19}$	236–284	FAM	Pr032816345
	R: TGAGCAGGAAGGTCTTGTTGA				
lbcur50	F: AATTGGATGGCTGCAGCTTT	$(AG)_{34}$	360–435	NED	Pr032816346
	R: ACTGTTGTTCTCTGTGTTCTGG				
lbcur53	F: TGCAAGGTGCCATCACTTCT	$(TC)_{15}$	282–326	FAM	Pr032816347
	R: CTACGGAAAGTACACGCCGT				
lbcur57	F: AGATCCAAGTACACAGGCCC	$(AG)_{15}$	287–316	FAM	Pr032816348
	R: GCACATTCAACTTACCCGCC				
lbcur13[c]	F: CCCTCCCTCTGATTCAATCCC	$(TC)_{20}$	161–197	FAM	Pr032816399
	R: GGGAGGAAGAAAGCCTGAGC				
lbcur35[c]	F: GGCAACTGGTAATAGGGCACA	$(GA)_{20}$	226–270	NED	Pr032816400
	R: ACGATGCGGCCATACTTTGA				
lbcur43[c]	F: GATTTGCTTACCCTGCACCTC	$(ATA)_{23}$	223–363	PET	Pr032816401
	R: ATGGTCGGATCGGCTTGAAA				
lbcur45[c]	F: TGACACTTGTTTAGCCCAGCA	$(CT)_{14}$	246–292	FAM	Pr032816402
	R: TGGTAACTGCAGCCCTTGAT				
lbcur59[c]	F: CGACATGCAACTTGAGTCGG	$(AT)_{30}$	298–340	PET	Pr032816403
	R: ACGCAGTCAAGCAGAGTTGT				

[a] An annealing temperature of 60°C was used for all loci.
[b] Forward 5′ label.
[c] Marker not selected; size range values based on six individuals (see Methods and Results section).

multiple comparisons of linkage disequilibrium (Rice, 1989). GenAlEx version 6.5 (Peakall and Smouse, 2006) was used to assess departure from Hardy–Weinberg equilibrium by χ^2 tests for each locus, and MICRO-CHECKER version 2.2.3 (van Oosterhout et al., 2004) was used to examine the possibility of null alleles. Finally, GENODIVE (Meirmans and Van Tienderen, 2004) was used to calculate standard measures of genetic variation for each locus including observed and expected heterozygosity and number of alleles.

After Bonferroni corrections there was no evidence of linked loci, consistent departure from Hardy–Weinberg equilibrium, or evidence for null alleles, for any locus, across all sites. The number of alleles observed for these 12 loci across the three populations ranged from nine to 19 and expected heterozygosity from 0.41 to 0.89 (Table 2). Assessment of cross transferral of loci was not possible given project resources, timelines, and the geographic distribution of closely related taxa. The sequences of the microsatellite loci developed have been deposited in GenBank.

CONCLUSIONS

These markers will add to molecular tools currently available to examine genetic patterns in banded iron formation flora (e.g., Nevill et al., 2010; Binks et al., 2014). The microsatellite loci developed for *L. bungalbin* in this study will be used to quantify the potential impact of the removal of plants associated with proposed mining on genetic variation within the species. Should mining be approved, they will also facilitate studies of the longer-term genetic consequences of increasing the geographic isolation of remaining plants and any effects on seed and pollen dispersal.

TABLE 2. Results of primer screening of 12 polymorphic loci identified in three populations (LB 4, LB 5, and LB 10) of *Lepidosperma bungalbin*.[a]

	LB 4			LB 5			LB 10		
Locus	*A*	H_o[b]	H_e	*A*	H_o[b]	H_e	*A*	H_o[b]	H_e
lbcur6	13	0.68	0.84	11	0.81	0.79	8	0.71*	0.82
lbcur14	11	0.76	0.85	9	0.81	0.77	12	0.83	0.89
lbcur16	10	0.68	0.85	10	0.91	0.83	14	0.83	0.88
lbcur21	4	0.52	0.65	7	0.81	0.73	5	0.50	0.52
lbcur27	10	0.80	0.83	12	0.90	0.88	9	0.61**	0.66
lbcur33	10	0.68	0.84	10	0.67	0.86	7	0.72	0.74
lbcur38	8	0.62	0.72	7	0.71	0.81	5	0.67	0.67
lbcur40	8	0.68	0.82	12	0.52***	0.75	8	0.82	0.73
lbcur41	3	0.19*	0.41	7	0.67	0.53	4	0.53	0.43
lbcur50	10	0.68	0.83	9	0.71	0.77	6	0.79	0.66
lbcur53	10	0.71	0.88	12	0.95	0.82	9	0.67	0.84
lbcur57	9	0.65	0.75	8	0.62	0.79	6	0.78	0.75

Note: *A* = number of alleles sampled; H_e = expected heterozygosity; H_o = observed heterozygosity.

[a] Values are based on samples from three populations in the Helena and Aurora Range of Western Australia. Twenty individuals were genotyped from each population (LB 4 UTM coordinates: 755768E, 6636451N; LB 5 UTM coordinates: 758630E, 6638545N; LB 10 UTM coordinates: 761310E, 6642801N).

[b] Significant deviation from Hardy–Weinberg equilibrium: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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