Microsatellite Primers Identified by 454 Sequencing in the Floodplain Tree Species Eucalyptus victrix (Myrtaceae)

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PRIMER NOTE

MICROSATELLITE PRIMERS IDENTIFIED BY 454 SEQUENCING IN THE FLOODPLAIN TREE SPECIES *EUCALYPTUS VICTRIX* (MYRTACEAE)[1]

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- *Premise of the study:* Microsatellite primers were developed for *Eucalyptus victrix* (Myrtaceae) to evaluate the population and spatial genetic structure of this widespread northwestern Australian riparian tree species, which may be impacted by hydrological changes associated with mining activity.
- *Methods and Results:* 454 GS-FLX shotgun sequencing was used to obtain 1895 sequences containing putative microsatellite motifs. Ten polymorphic microsatellite loci were identified and screened for variation in individuals from two populations in the Pilbara region. Observed heterozygosities ranged from 0.44 to 0.91 (mean: 0.66) and the number of alleles per locus ranged from five to 25 (average: 11).
- *Conclusions:* These microsatellite loci will be useful in future studies of population and spatial genetic structure in *E. victrix*, and inform the development of seed sourcing strategies for the species.

Key words: 454 GS-FLX; *Eucalyptus victrix*; microsatellite primers; northwestern Australia; riparian; shotgun sequencing.

The genus *Eucalyptus* L'Hér. (Myrtaceae) is one of the most diverse of the temperate trees with more than 700 species recognized (Brooker, 2000), many with a high commercial or conservation significance. *Eucalyptus victrix* L. A. S. Johnson & K. D. Hill is a small tree (<15 m tall) that is widely but patchily distributed in northwestern Australia. The species is dependent on groundwater and is confined to floodplains and river-flats north of the Murchison River to Port Hedland and eastward into the central Northern Territory (Hill and Johnson, 1994). In the Pilbara region, mining activity and associated hydrological changes may impact some populations of this riparian tree species. To understand what impact the possible loss of populations may have on overall genetic diversity of the species and to inform seed sourcing for rehabilitation of mine sites, knowledge is needed on the level and structure of genetic diversity within the species. Here, we report the isolation and characterization of 10 polymorphic microsatellite loci that will be used to examine the spatial genetic structure and levels of gene flow within and among the *E. victrix* populations in riparian communities, in the Pilbara region of northwestern Western Australia.

METHODS AND RESULTS

We isolated genomic DNA (5 µg) from the leaf tissue of one individual of *E. victrix* following the protocol of Glaubitz et al. (2001). Shotgun sequencing was performed at the Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Sydney, Australia) on a Roche 454 GS-FLX sequencer with titanium chemistry (Roche Applied Science, Indianapolis, Indiana, USA) following Gardner et al. (2011). The sample occupied 12.5% of a plate and produced 94,885 individual sequences, with an average read length of 344 bp, of which 1895 contained microsatellites. We used the program QDD version 1 (Meglécz et al., 2010) to screen the raw sequences for eight or more di-, tri-, tetra-, or pentabase repeats, remove redundant sequences, and design primers (automated in QDD using Primer3 [Rozen and Skaletsky, 2000]). Default values were used for running parameters except PCR product lengths set to 90–450 bp. Primer pairs were designed for 239 different loci. We excluded all loci that contained imperfect repeats or short repeat motifs within the flanking region or primer sequence, had a greater than 2°C difference between the forward and reverse primer annealing temperature, and polynucleotide runs of four or more in the flanking regions. We selected 30 loci for further development (GenBank accession no.: JX423973–JX424002) and, initially, the loci were amplified using the cost-effective approach of Schuelke (2000) and a QIAGEN Multiplex PCR Kit (QIAGEN, Hilden, Germany). Loci were individually amplified in 20-µL reactions

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TABLE 1. Characteristics of 10 nuclear microsatellite primers developed in *Eucalyptus victrix*.[a,b]

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	Label (Multiplex)[c]	GenBank accession no.
KPEV02	F: CTTCGTCTTTGAGGGTGAGG	$(ATA)_{14}$	129–205	FAM (1)	JX423974
	R: TGGCACTTTTCCCTTCAACT				
KPEV03	F: TTCACTGCGGATGTTGAGAA	$(CT)_{12}$	80–155	PET (2)	JX423975
	R: TTGACTGTTCGCAGTTGCAG				
KPEV04	F: ACAAGCGGAAAAGGTCTTCA	$(CT)_7$	154–168	PET (1)	JX423976
	R: CGAGGCCAAGTAGAGCAAAC				
KPEV09	F: GGTTTCCGATCGATTCTTCA	$(AGA)_8$	120–138	NED (1)	JX423981
	R: CGACGTCTGATATGCTTCACA				
KPEV10	F: AGTCCCACGCAACCTCATAC	$(AG)_{16}$	154–191	NED (2)	JX423982
	R: ACGTTGCTTGTCGAGGAGAT				
KPEV13	F: CAGGTTAGGGTTGGAGACGA	$(CGC)_5$	120–135	FAM (2)	JX423985
	R: GAAGCTGCGGTCCTTCATC				
KPEV20	F: TTCTTCTCCCAGCCTTTTCA	$(TTC)_{10}$	210–216	VIC (1)	JX423992
	R: TGGCTAGCCTATTGCGGTAT				
KPEV22	F: AGGGTCGTGCCCTAGAATTT	$(GA)_{17}$	196–256	FAM (S)	JX423994
	R: ATCGAGTCGTCATCGCTCTT				
KPEV28	F: CCACATTCCAACACAACTCG	$(TCT)_{12}$	200–227	FAM (2)	JX424000
	R: GTCGTGGCACTGGAGAATTT				
KPEV30	F: CCAGAACAGCATGCGATAGA	$(AG)_8$	235–241	FAM (1)	JX424002
	R: CGTACGAGAAGGAACCGAAA				

[a] Values are based on samples from two populations in the Pilbara region of Western Australia (WW-LP: UTM coordinates 725022E 7462878N, collection no. PN101; WW-MP: UTM coordinates 722880E 7462179N, collection no. PN102).

[b] An annealing temperature of 56°C was used for all primers.

[c] Multiplex marker sets are identified as (multiplex 1, 2, or single [S]).

containing 10 µL QIAGEN Multiplex PCR Master Mix; 2.5 µL Q-solution; forward primer (with M13 tag at 5′ end, unlabeled) 0.05 µM, reverse primer (unlabeled) 0.2 µM, and M13 tag (labeled; unique to primer) 0.2 µM; 10–50 ng DNA; plus sterile H_2O to 20 µL. PCR cycling was performed in a Corbett Gradient Palm-Cycler (Corbett Life Science, Sydney, Australia) according to the manufacturer's protocol as follows: *Taq* activation 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 90 s; followed by a final extension at 60°C for 30 min. PCR products were visualized on a 2% agarose gel stained with SYBR Safe (Invitrogen, Carlsbad, California, USA). Twenty-four loci amplified an unambiguous product of the expected size and these loci were then screened on eight individuals to test for polymorphism using an ABI 3730 genetic analyzer (Applied Biosystems, Foster City, California, USA) and GENEMAPPER version 4.0 software (Applied Biosystems).

Of the 24 loci, 10 (42%) were polymorphic, alleles at 12 (50%) loci were difficult to score, and two (8%) did not amplify. The 10 polymorphic and scorable loci were then screened for variation in 20 individuals from each of two populations of *E. victrix* from the Pilbara region (Table 1) (WW-LP: Universal Tranverse Mercator [UTM] coordinates 725022E 7462878N, collection no. PN101; WW-MP: UTM coordinates 722880E 7462179N, collection no. PN102). Herbarium material was deposited at the Kings Park and Botanic Garden Herbarium, Perth, Western Australia (KPBG). The forward primer of each locus was 5′ labeled with a fluorescent tag (6-FAM [GeneWorks, Hindmarsh, Australia], NED, PET, or VIC [Applied Biosystems]) (Table 1), and nine loci were amplified in two multiplex PCRs (and KPEV22 singly after persistent amplification failures [see below for conditions]) in 12.5-µL reactions containing 6.25 µL QIAGEN Multiplex PCR Master Mix (QIAGEN); 1.25 µL Q-solution; 0.1 µM of each forward and reverse primer, 10–50 ng DNA; plus sterile H_2O to 12.5 µL. PCR conditions were as per the initial screening; however, the annealing temperature was reduced to 56°C to improve amplification. KPEV22 was amplified in 10-µL reaction volumes containing: 3.5 µL of 5× PCR buffer (Fisher Biotec, Perth, Australia), 3 mM of $MgCl_2$ (Invitrogen), a primer concentration of 0.2 µM for each forward and reverse primer, 40 ng of template DNA, 1 unit of *Taq* DNA polymerase (Invitrogen), plus sterile H_2O to 10 µL. PCR cycling was carried out using the following reaction conditions: 94°C for 3 min; 30 cycles of denaturation at 94°C for 40 s, annealing at 56°C for 40 s, and extensions at 72°C for 30 s; followed by a final extension at 72°C for 15 min. We used GenAlEx version 6.4 (Peakall and Smouse, 2006) to calculate genetic diversity parameters, and GENEPOP 3.4 (Raymond and Rousset, 1995) to calculate deviation from Hardy–Weinberg equilibrium (HWE) (Table 2). The sequential Bonferroni method (Hochberg, 1988) was used to adjust *P* values from HWE tests for multiple tests of significance. The number of alleles observed at the 10 loci ranged from three to 25 with an average of 11 alleles per locus (Table 2). The observed and expected heterozygosities ranged from 0.44 to 0.91 and 0.53 to 0.94, respectively (Table 2). Significant departures from HWE ($P < 0.01$) were detected in the KPEV10 locus at both WW-LP and WW-MP, in the KPEV20 locus at both WW-LP and WW-MP, and in the KPEV22 locus at WW-MP (Table 2). We checked all pairs of loci for linkage disequilibrium in GENEPOP and none were significant after sequential Bonferroni adjustment. The sequences of the 10 microsatellite loci have been deposited in GenBank (see Table 1 for accession numbers).

TABLE 2. Results of primer screening in two populations of *Eucalyptus victrix*.[a]

	WW-LP			WW-MP		
Locus	*A*	H_o[b]	H_e	*A*	H_o[b]	H_e
KPEV02	17	0.91	0.91	18	0.90	0.93
KPEV03	10	0.52	0.72	11	0.55	0.72
KPEV04	6	0.65	0.62	6	0.50	0.48
KPEV09	7	0.72	0.75	6	0.70	0.71
KPEV10	8	0.44*	0.72	5	0.50*	0.58
KPEV13	7	0.85	0.79	9	0.65	0.81
KPEV20	6	0.62*	0.70	6	0.65*	0.75
KPEV22	22	0.75	0.91	25	0.60*	0.93
KPEV28	11	0.83	0.82	9	0.85	0.80
KPEV30	5	0.70	0.75	5	0.80	0.71

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

[a] Values are based on samples from two populations in the Pilbara region of Western Australia (WW-LP: UTM coordinates 725022E 7462878N, collection no. PN101; WW-MP: UTM coordinates 722880E 7462179N, collection no. PN102).

[b] Significant deviations from Hardy–Weinberg equilibrium (*$P < 0.01$) after correction for multiple tests (sequential Bonferroni procedure) are reported.

CONCLUSIONS

The microsatellite loci developed for *E. victrix* in this study will be used to examine levels of past and current gene flow within and between geographically proximate riparian systems and inform seed sourcing for rehabilitation of mine sites.

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