Characterization of Microsatellite Markers for the Vulnerable Grassland Forb Senecio macrocarpus (Asteraceae)

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PRIMER NOTE

CHARACTERIZATION OF MICROSATELLITE MARKERS FOR THE VULNERABLE GRASSLAND FORB *SENECIO MACROCARPUS* (ASTERACEAE)[1]

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- *Premise of the study:* Development of microsatellite markers for the vulnerable forb *Senecio macrocarpus* was performed to begin an assessment of its population structure and breeding method to aid in the conservation of the species in Victoria, Australia.
- *Methods and Results:* Fifteen microsatellite markers were developed for *S. macrocarpus* from 454 pyrosequencing. The markers were tested on 104 individuals from four populations. The markers produced between two and seven alleles per locus while the expected heterozygosity ranged from 0.20 to 0.67 and the observed heterozygosity ranged from 0.00 to 1.00. The observed heterozygosity is suggestive that the populations may be apomictic.
- *Conclusions:* The microsatellite markers developed for *S. macrocarpus* are intended to be used on future studies that aim to assess the population genetics and local breeding dynamics of the species with an emphasis on conservation.

Key words: 454 sequencing; apomixis; Asteraceae; conservation; microsatellites; polyploidy; *Senecio macrocarpus.*

The Natural Temperate Grassland of the Victorian Volcanic Plain currently occupies less than 1% of its former range (McDougall and Kirkpatrick, 1994). Consequently, many of the plant species that are endemic to the Volcanic Plain are in decline due to habitat loss. *Senecio macrocarpus* F. Muell. ex Belcher (Asteraceae) is no exception. It is a perennial forb that is found in southeastern Australia. It is listed as vulnerable under the Australian *Environment Protection and Biodiversity Conservation Act 1999* (EPBC Act). Currently, *S. macrocarpus* populations are highly fragmented due to agricultural and industrial development and occupy areas that can be categorized as urban fringe, roadside patches, and native remnant vegetation. It has been used with some success for revegetating native grasslands in Victoria (McDougall and Morgan, 2005). To aid grassland revegetation efforts and secure the conservation of *S. macrocarpus*, understanding the genetic variability on the population level is crucial to create viable populations that will persist. Genomic microsatellite markers would be valuable for uncovering the variation across the entire population. Microsatellite markers are available for related species, including *S. madagascariensis* Poir. (Le Roux and Wieczorek, 2006) and *S. inaequidens* DC. (Justy and Maurice, 2012). In Australia, the genus *Senecio* L. consists of a diverse group of native species with both self-compatible and self-incompatible species that are thought to be wind dispersed (Belcher, 1994); the genus also includes differing ploidy levels, with the majority of species being tetraploid or hexaploid (Lawrence, 1985). We characterize microsatellite markers for *S. macrocarpus* to identify the genetic variation within and among populations and to understand how Victorian species are related. We included two other Victorian species to test for possible cross-amplification. The developed markers will be used to identify proper conservation strategies by assessing the level of genetic variability population wide.

METHODS AND RESULTS

Voucher specimens from each population were deposited in the herbarium at the Royal Botanic Gardens, Melbourne (voucher no.: Messent Conservation Park-23705226A, Deep Lead Flora Reserve-2370528A, Bulban Road-2370530A, Derrimut Grassland Reserve-2370532A). Leaf samples were dried and kept in silica gel until DNA extraction could be performed. Genomic DNA was extracted at the Australian Genome Research Facility (AGRF, Adelaide, South Australia, Australia) from 20 mg of plant material using the cetyltrimethylammonium bromide (CTAB) method. The extracted DNA was quantified using the QuantiFluor system (Promega Corporation, Madison, Wisconsin, USA).

Isolated DNA from one *S. macrocarpus* individual was sequenced by a Roche GS FLX sequencing platform (454 Life Sciences, a Roche Company, Branford, Connecticut, USA) for 1/8 coverage. The 454 results were processed in the program QDD (Meglécz et al., 2009) for selection and design of microsatellite primers. The QDD output isolated a total of 22,909 possible microsatellite primer pairs. Paring down the number of possible markers was done with selection criteria that consisted of penalty score (lowest score indicates 'best' primer pair at the locus), size of PCR product (100–400 bp), primer designs (ranked by primer length, melting temperature, percentage of G and C nucleotides, and mononucleotide repeats), and motif repeat size. The 96 selected loci were manufactured (Integrated DNA Technologies, Coralville, Iowa, USA) and screened for amplification and variability in seven *S. macrocarpus* individuals using the three-primer approach described in Blacket et al. (2012). This method enabled efficient amplification of loci in multiplex PCRs using all four fluorochromes (FAM, VIC, NED, PET). Each fluorochrome was attached to

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TABLE 1. Characteristics of 15 microsatellite primers developed for *Senecio macrocarpus* ($2n = 60$).

Locus	GenBank accession no.	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Size range (bp)	Label
sm003*	KF018218	F: TGAACCACCCTCTCAACCAC	$(ACC)_6$	60	106–128	NED
		R: GTGAGCTTGGACTCATGGGA				
sm008*	KF018219	F: GGTGAGATAAGAGCATACGTACCA	$(AAAC)_5$	60	136–140	PET
		R: GGAAGGAATTGATTGCGAGTT				
sm010*	KF018220	F: TTGGACGAGTTGGTGGAGTC	$(ACC)_7$	60	119–148	VIC
		R: TGATCTAAGGAAGTCCGGCA				
sm015*	KF018221	F: GTCTAGGCGGTTCCATGTTG	$(ACC)_5$	60	149–158	NED
		R: CACTGCGGAGCTTACCTTGT				
sm020*	KF018222	F: GGGAGCTAAATTCCTGCGTT	$(AAC)_8$	60	131–160	PET
		R: TCCCGGTCTATATTGGCCTT				
sm023*	KF018223	F: CTGCCCAATTTGTGACCTCT	$(AC)_{10}$	60	148–160	FAM
		R: GCATCGATTTGAAAGTTGCC				
sm026*	KF018224	F: CCTTCAACCACCGAGGATAA	$(AC)_6$	60	174–180	PET
		R: CACCATATCAATCGCATCCA				
sm036*	KF018225	F: TTCTTCGAATCGGAAACAGC	$(ACT)_5$	60	183–191	FAM
		R: CCAACCGTACACATCTCCGT				
sm040*	KF018226	F: TCATCCTCAGACTCGTCGGT	$(AGT)_5$	60	195–260	VIC
		R: GAGCGTTCCGGATCAGAGTA				
sm043*	KF018227	F: ATGCAAGGTTCTTCGTTGGA	$(AAG)_5$	60	199–211	FAM
		R: TGTTTGCATCAACATTTGGG				
sm049*	KF018228	F: ATGCAAATTCACCATGGACC	$(ACC)_5$	60	196–228	VIC
		R: CCTCACGAAATTGTCCTCTTG				
sm051*	KF018229	F: CGCCTCCTTGAATTTAGCAA	$(AT)_7$	60	221–268	PET
		R: CAGGCCCAATAATGACCAAA				
sm052*	KF018230	F: AAGCTTGCTTACGAGGCTCA	$(AG)_8$	60	216–244	NED
		R: CACAGCAACTGAACACCCAA				
sm057*	KF018231	F: GGGCTGGAACAACAAGAGAA	$(AC)_7$	60	236–246	VIC
		R: TGGCTGGACTTGAAGGCTAT				
sm064*	KF018232	F: ACCTCACTTTGGTGGGATTG	$(AAC)_8$	60	252–264	FAM
		R: CCCAACGGAATTAACTTGGAC				
sm002		F: TACCGCAACCTCTCACTTCC	$(AGT)_5$	60	113	VIC
		R: GGTTTGAGGATGGTGGATTG				
sm005		F: AAGTCAAGGCGATGAAGCAG	$(AG)_7$	60	119	FAM
		R: AAGTGGATTCCCTTTCTGGG				
sm011		F: GGCAAATCATTTACAGGCCA	$(AAC)_5$	60	135	NED
		R: GGACTTGAACCCTTGCTTGA				
sm012		F: ATGCCACCGTTAGAAGCAGA	$(AAG)_5$	60	138	PET
		R: CAATGTTGACAGCCATTTCG				
sm034		F: ACGTGAAAGAATGCAGCCAT	$(ACT)_5$	60	183	PET
		R: TCTCTGCATCACGAAACGAA				
sm039		F: CCATGTGGAGAGGTACCAGC	$(AAG)_5$	60	190	FAM
		R: CATGGAGCTCAATCCTCCTC				
sm046		F: GCAGAATCAGCAAAGGGAAA	$(ACT)_{12}$	60	189	PET
		R: GGAACATGGACATTGCTTTGA				
sm047		F: GGAATAGTTGCCTATATTATGTGTTCG	$(AAT)_8$	60	211	FAM
		R: AATCCAATGGGTTGGCTTCT				
sm053		F: CACAATCCAGACCAACCCAT	$(AATC)_5$	60	222	VIC
		R: GGTACATACGGCAGCGAGAG				
sm061		F: TTCCCAGTCAATTCAGTTCCA	$(AGT)_6$	60	242	VIC
		R: TGGATTGCTGATGATCTTGG				
sm068		F: TTTCACCTTCTCCTTGACCA	$(AGT)_6$	60	244	NED
		R: ATCGCTCATTGCATCTTTGC				
sm069		F: TTGGGCCACTTTGCTAGAGA	$(AAC)_5$	60	262	VIC
		R: TGTTTCATGCATGCTGGAGT				
sm070		F: ATCCACCTGCCATCTGAGAA	$(AGT)_5$	60	277	FAM
		R: ACGCTGGGTCGTATGCTAAA				
sm071		F: CAATGTTTGCGGCGATAGAT	$(AAG)_5$	60	281	PET
		R: CGTGTGATATAAATGAAACCGAA				
sm077		F: CCAAACATTAATTGGGCGTG	$(AT)_5$	60	300	PET
		R: TGTTAATTGAGTGCCCGGTT				
sm082		F: TCGGCACTCGTATGGAGTTT	$(AG)_5$	60	311	NED
		R: GCTAAACAGGAGGGTTGAGAA				
sm083		F: GCGTAGCCAACCTTTGAGAG	$(AG)_5$	60	313, 317	VIC
		R: GGAGTGGTTAACGGACATGC				
sm088		F: TTCATCTACAACGGGATGGG	$(AT)_5$	60	334	FAM
		R: GGAGATCACTCCATTCGTGA				
sm090		F: GTTACCTTGAGGGAAGCGGT	$(AGG)_5$	60	354	NED
		R: TGAAGCGCGTAAGGTCTAGG				
sm091		F: TCTGCTTGAACCAACCCAGT	$(AT)_5$	60	349	VIC
		R: CAACACTAAGACTGAGCAAATGGA				

Table 1. Continued.

Locus	GenBank accession no.	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Size range (bp)	Label
sm095		F: GACCAGGCCAACCAAGAGAT R: TCTGTCCATTCCTTCCTCCA	$(AG)_5$	60	374	VIC

Note: T_a = annealing temperature.
*Polymorphic and variable locus; primer pairs for these loci were used in this study, while primer pairs for the other loci presented in the table were not.

one of four unique universal "tails" (Blacket et al., 2012). Forward locus-specific primers were allocated to one of the four tails, which then dictated the fluorochrome labeling locus-specific PCR products. PCRs consisted of 10-µL reactions with approximately 20 ng of DNA, 5 µL Master Mix Type-it Kit (QIAGEN, Hilden, Germany), 0.1 µL of forward primers (10 µM), 0.2 µL of reverse primers (10 µM), and 0.1 µL of each dye (10 µM; FAM, VIC, NED, PET). All PCRs were carried out under the same conditions: 5 min denaturation at 95°C, followed by 32 cycles of 95°C for 30 s, annealing at 60°C for 1 min 30 s and 72°C for 30 s, and a final extension at 60°C for 30 min. Each reaction was multiplexed with four different primer pairs. The initial primer screening left 15 primer pairs that were amplifiable and variable among the seven individuals (Table 1). These primer pairs included five dinucleotide, nine trinucleotide, and one tetranucleotide repeat motifs. The 15 primer pairs were subsequently used on 104 individuals among four *S. macrocarpus* populations: Messent Conservation Park (36.10156°S, 139.74366°E), Deep Lead Flora Reserve (37.01269°S, 142.72166°E), Bulban Road (37.92488°S, 144.59474°E), and Derrimut Grassland Reserve (37.80962°S, 144.71980°E) with the same protocol used in the amplification described above. Two different Victorian species were used to test cross-species amplification. The more common *S. quadridentatus* Labill. (GPS coordinates: 37.62569°S, 144.96216°E; unvouchered specimen determined by Neville Walsh) is closely related to *S. macrocarpus* and is widespread in southern Australia, whereas *S. behrianus* Sond. & F. Muell. ex Sond. (voucher no.: MEL 2296795A) is a rare Victorian endemic known to reproduce both clonally and by seed. Fragment analysis of the PCR product was performed via capillary electrophoresis on an ABI 3130xl (Applied Biosystems, Foster City, California, USA) by Macrogen (Seoul, South Korea). Alleles were sized against the internal size standard GeneScan 500 LIZ (Applied Biosystems) and scored using Geneious Software version 5.6.2 (Biomatters Ltd., Auckland, New Zealand). All 15 microsatellite markers showed a clear consistent amplification pattern; locus sm043 had one or two alleles (three individuals had three alleles), all individuals had three alleles for locus sm052, and all other loci had one or two alleles. Chromosome counts show that *S. macrocarpus* is hexaploid ($2n = 60$; data not shown), while *S. quadridentatus* is tetraploid ($2n = 40$; Lawrence, 1985) and *S. behrianus*' ploidy is unknown. The allele numbers per individual are unexpectedly low for a hexaploid outcrossing species. However, these patterns could be attributed to apomixis and *S. macrocarpus*' evolutionary history.

The number of alleles, observed heterozygosity, and expected heterozygosity were calculated in GenoDive version 2.0b24 (Meirmans and Van Tienderen, 2004) for each locus within and among the four populations (Table 2). The deviations from Hardy–Weinberg equilibrium were extreme and not typical of predominantly outcrossing species. The results show that some alleles are fixed (as homozygotes and heterozygotes) within but not among populations. Apomixis could be contributing to these patterns as it occurs naturally in Asteraceae in at least 22 genera and seven tribes (reviewed in Noyes, 2007). Indeed, our observation of low pollen to ovule ratio and lack of pollen exertion, in addition to the population structure found in *S. macrocarpus*, further support this idea.

In terms of cross-amplification, only two markers failed to amplify in the other two species. Five markers amplified and were polymorphic for *S. behrianus* while a different set of five markers amplified and were polymorphic for *S. quadridentatus*. Three markers were monomorphic for *S. behrianus* while eight markers were monomorphic for *S. quadridentatus* (Table 3).

CONCLUSIONS

The 15 microsatellite loci characterized herein for the vulnerable forb *S. macrocarpus* are being used for research to document the population structure, gene flow, and breeding system, and to assess population viability of current and future populations. They will also be used to investigate interspecific relationships between Australian species. The results of these studies will aid in the conservation of *S. macrocarpus* in a fragmented Australian landscape.

Table 2. Results for primer screening of all samples of 15 microsatellite loci from four populations of *Senecio macrocarpus* ($2n = 60$).[a]

	Messent CP ($N = 54$)			Derrimut Grassland ($N = 20$)			Bulban Road ($N = 15$)			Deep Lead FR ($N = 15$)			Total ($N = 104$)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
sm003	3	0.32	0.28	1	0.00	0.00	2	1.00	0.52	2	1.00	0.52	3	0.58	0.43
sm008	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	0.50
sm010	7	0.31	0.55	0	—	—	1	0.00	0.00	1	0.00	0.00	7	0.10	0.49
sm015	2	0.94	0.50	2	1.00	0.51	1	0.00	0.00	1	0.00	0.00	2	0.49	0.37
sm020	3	0.08	0.56	1	0.00	0.00	2	0.07	0.07	2	0.07	0.07	5	0.05	0.67
sm023	3	0.00	0.52	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	3	0.00	0.47
sm026	2	0.00	0.14	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	0.50
sm036	2	0.87	0.49	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.22	0.20
sm040	2	0.00	0.07	2	0.05	0.05	1	0.00	0.00	1	0.00	0.00	2	0.01	0.50
sm043	5	1.00	0.63	2	1.00	0.51	3	1.00	0.58	2	1.00	0.52	5	1.00	0.61
sm049	2	1.00	0.51	1	0.00	0.00	2	1.00	0.52	2	0.93	0.52	2	0.73	0.48
sm051	0	—	—	1	0.00	0.00	2	0.00	0.40	2	0.07	0.07	3	0.02	0.56
sm052	4	1.00	0.68	3	1.00	0.68	3	1.00	0.68	3	1.00	0.68	4	1.00	0.71
sm057	2	0.04	0.10	2	0.10	0.10	2	1.00	0.52	2	1.00	0.52	3	0.53	0.41
sm064	3	0.07	0.62	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	3	0.02	0.58

Note: A = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity.
[a]Population locality information: Messent Conservation Park (36.10156°S, 139.74366°E), Derrimut Grassland Reserve (37.80962°S, 144.71980°E), Bulban Road (37.92488°S, 144.59474°E), Deep Lead Flora Reserve (37.01269°S, 142.72166°E).

TABLE 3. Cross-amplification of 15 microsatellite markers for two Victorian species related to *Senecio macrocarpus* (2*n* = 60): *S. behrianus* (2*n* = unknown) and *S. quadridentatus* (2*n* = 40).[a]

Species	*N*	sm003	sm008	sm010	sm015	sm020	sm023	sm026	sm036	sm040	sm043	sm049	sm051	sm052	sm057	sm064
S. behrianus	4	P (3)	—	—	NV (2)	—	P (3)	P (3)	M (1)	—	P (3)	—	NV (2)	P (2)	—	—
S. quadridentatus	5	M (1)	—	M (1)	NV (2)	—	P (4)	M (1)	M (1)	M (1)	P (3)	NV (2)	P (2)	NV (2)	P (3)	P (2)

Note: — = failed amplification; M = monomorphic; NV = not variable; P = polymorphic.
[a]Numbers in parentheses indicate the number of alleles amplified at the locus.

LITERATURE CITED

BELCHER, R. O. 1994. *Senecio. In* Flora of Australia, vol. 49. Australian Government Publishing Service, Canberra, Australia.

BLACKET, M. J., C. ROBIN, R. T. GOOD, S. F. LEE, AND A. D. MILLER. 2012. Universal primers for fluorescent labelling of PCR fragments: An efficient and cost-effective approach to genotyping by fluorescence. *Molecular Ecology Resources* 12: 456–463.

JUSTY, F., AND S. MAURICE. 2012. Isolation and characterization of polymorphic microsatellite markers for the invasive *Senecio inaequidens* DC. (Asteraceae). *Conservation Genetics Resources* 4: 703–705.

LAWRENCE, M. E. 1985. *Senecio* L.(Asteraceae) in Australia: Rcombination [*sic*] systems of a polyploid series. *Australian Journal of Botany* 33: 209–219.

LE ROUX, J. J., AND A. M. WIECZOREK. 2006. Isolation and characterization of polymorphic microsatellite markers from fireweed, *Senecio madagascariensis* Poir. (Asteraceae). *Molecular Ecology Notes* 7: 327–329.

MCDOUGALL, K. L., AND J. B. KIRKPATRICK. 1994. Conservation of lowland native grasslands in south-eastern Australia. World Wide Fund for Nature, Sydney, New South Wales, Australia.

MCDOUGALL, K. L., AND J. W. MORGAN. 2005. Establishment of native grassland vegetation at Organ Pipes National Park near Melbourne, Victoria: Vegetation changes from 1989 to 2003. *Ecological Management & Restoration* 6: 34–42.

MEGLÉCZ, E., C. COSTEDOAT, V. DUBUT, A. GILLES, T. MALAUSA, N. PECH, AND J.-F. MARTIN. 2009. QDD: A user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics (Oxford, England)* 26: 403–404.

MEIRMANS, P. G., AND P. H. VAN TIENDEREN. 2004. GENOTYPE and GENODIVE: Two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes* 4: 792–794.

NOYES, R. D. 2007. Apomixis in the Asteraceae: Diamonds in the rough. *Functional Plant Science and Biotechnology* 1: 207–222.