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Applications in Plant Sciences

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PRIMER NOTE

MICROSATELLITE MARKERS FOR THE NEW ZEALAND ENDEMIC TREE *FUCHSIA EXCORTICATA* (ONAGRACEAE)[1]

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- *Premise of the study:* Microsatellite markers were developed from a New Zealand endemic understory tree, *Fuchsia excorticata*, to investigate factors affecting the mating system.
- *Methods and Results:* Using 454 pyrosequencing, 48 microsatellite markers were developed and tested for polymorphism within populations. Twelve of these microsatellite loci were identified as being polymorphic within at least three populations and consistently amplified in the four populations tested. These primers amplified di-, tri-, and tetranucleotide repeats with 1–10 alleles per population.
- *Conclusions:* These results indicate the utility of microsatellite loci for future mating system and population genetic studies in *F. excorticata*.

Key words: *Fuchsia excorticata*; microsatellite markers; Onagraceae; shotgun 454 pyrosequencing.

Bird abundance in New Zealand, and worldwide, has undergone drastic reductions in the recent past. Because birds play a major role in pollination for many New Zealand plant species (Kelly et al., 2010), this decline may have important repercussions on plant population dynamics. While studies have investigated how lower pollinator abundance has led to reduced fruit or seed production (reviewed in Robertson et al., 2008; Kelly et al., 2010), the impact on the quality of the seeds being produced has not been determined. In self-compatible species, lower pollinator abundance may increase the selfing rate, which could decrease the average fitness of offspring if inbreeding depression occurs.

Fuchsia excorticata L. f. (Onagraceae) is a self-compatible, understory tree species endemic to New Zealand. The National Pollination Survey has shown that many *F. excorticata* populations are pollen-limited, especially where pollinators are less abundant (Robertson et al., 2008). Because self-pollinated offspring have high mortality rates and slower growth (Robertson et al., 2011), an increase in selfing rates could greatly impact population-level processes. Determining how pollinator abundance affects selfing rates is best achieved using mating system analyses, which require genetic markers that are polymorphic within populations. Next-generation sequencing offers a lower-cost option to generate a large quantity of sequence data for nonmodel species without the use of cloning, which is restricted in New Zealand for native species such as *F. excorticata*. Using a shotgun 454 pyrosequencing approach, we developed microsatellite markers that are polymorphic within populations of *F. excorticata*.

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METHODS AND RESULTS

Leaf tissue from a hermaphrodite and a female adult was collected from Karori Sanctuary (Wellington, New Zealand) to be used for sequencing. We chose to sequence the combination of two individuals to detect polymorphic microsatellite regions prior to primer design. To obtain the high-quality DNA required for 454 pyrosequencing, genomic DNA was extracted using either: (1) a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) or (2) a modified cetyltrimethylammonium bromide (CTAB) protocol beginning with a sucrose wash (Shepherd and McLay, 2011), depending on which gave the highest quality DNA for each individual. Extracted DNA was pooled to obtain equal concentrations of the two samples. The library was prepared using the 454 Roche Rapid Library Kit (454 Life Sciences, a Roche Company, Branford, Connecticut, USA), which consisted of DNA fragmentation by nebulization and ligation of adapters. The library was sequenced using a Roche 454 GS Junior according to the manufacturer's instructions. The library preparation and sequencing were completed by New Zealand Genomics Limited (Dunedin, New Zealand).

We obtained 80,773 sequences of an average length of 533.5 bp for a total of >33 Mbp. Sequences were assembled into 11,538 contigs using Geneious 6.0 (Biomatters, Auckland, New Zealand). Putative plastid sequences were removed by performing a local BLAST search of the phylogenetically closest relatives with the most complete mitochondrial and chloroplast sequences obtained from GenBank (*Arabidopsis thaliana* (L.) Heynh. [Brassicaceae] and *Eucalyptus globulus* subsp. *globulus* St.-Lag. [Myrtaceae], respectively) against the *Fuchsia* sequences. The remaining sequences were analyzed to identify perfect di- to hexanucleotide microsatellite repeats with a minimum of five repeat units using a tandem repeat search tool in Geneious (Phobos plugin; Mayer, 2010), which found a total of 3554 repeats. Sequences were removed if regions near the microsatellite contained other perfect microsatellites, single base pair repeats longer than five base pairs, or long, imperfect microsatellites. We also searched for potentially heterozygous microsatellites in the assembled contigs using Phobos. After removing unsuitable loci and including potentially heterozygous loci, a total of 1277 microsatellite regions remained, for which we attempted to design primers using Primer3 within Geneious (Rozen and Skaletsky, 2000). The default settings were used except for: product size = 100–300 bp; primer size = 17 (minimum)–19 (optimal)–21 (maximum); melting temperature (T_m) = 52–55–58°C; GC content = 40–50–60%; maximum T_m difference = 5°C;

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TABLE 1. Characteristics of microsatellite loci developed in *Fuchsia excorticata*.

Locus	Primer sequences (5′–3′)[a]	Repeat motif	Size range (bp)	T_a (°C)	GenBank accession no.
FE-10	F: ATAATGGCTCAGACACGTG	$(TAA)_{13}$	216–270	63/53*	KF027215
	R: GGAACAAATCCCCTGATGG				
FE-15	F: TTGTCAGCTTGAAGTCCAG	$(GTA)_{10}$	260–284	53	KF027216
	R: ACACACAAGCATAGCTACC				
FE-19	F: TTGGGTTGTTTAGCTCGAG	$(TCT)_9$	238–268	63/53*	KF027217
	R: GTCCGTCTTCTGACTTCTG				
FE-20	F: TCTCTGCATTCCTTTCGAC	$(ATC)_{10}$	163–178	53	KF027218
	R: CAGTCCTCCACCTACAATG				
FE-22	F: GGTCGGAAAACAAAATCCC	$(AT)_7$	200–228	63/53*	KF027219
	R: TAACGCTCGCTTTAAATGC				
FE-28	F: TGGAGCACACAGATTCATC	$(AT)_8$	251–279	53	KF027221
	R: TGGTGGCAGCATATTCATC				
FE-29	F: TGCATGCAAGTACTCTGTG	$(AT)_{10}$	186–208	63/53*	KF027220
	R: ACAAAGCTCCTTATGCGAG				
FE-34	F: GGTGGTTAATAGTGTTGCG	$(CT)_{12}$	243–261	53	KF027222
	R: CCCTCGAAATAGAAACCCC				
FE-38	F: GTGAAAGTGTCCCAAATGC	$(AT)_6$	308–342	63/53*	KF027223
	R: GGTGAGGCTGATCAGTTG				
FE-40	F: TTAAGTGGGTAGTTAGCTGC	$(AGTT)_9$	192–224	53	KF027224
	R: TCTACAATGTACTGCCTGC				
FE-43	F: ACTAGTCTGAGTGGACAGG	$(AT)_{14}$	191–225	63/53*	KF027225
	R: TGGCATGATGATAGAAGAGC				
FE-48	F: GGTTTTGGCGGATTTACAC	$(GCT)_{10}$	275–299	53	KF027226
	R: GAATCCTCTCATGGACGAC				

Note: T_a = annealing temperature.

[a]An M13 tail (CACGACGTTGTAAAACGAC) was added to the 5′ end of each forward primer and a PIG tail (GTTTCTT) was added to the 5′ end of each reverse primer.

*Touchdown PCR.

GC clamp = 1; maximum poly x = 4. Primers were successfully designed for 1138 microsatellite regions. To avoid fluorescently labeling individual primers, an M13 tag was added to the 5′ end of the forward primer (CACGACGTTGTAAAACGAC). To promote nontemplate (A) addition, a PIG tail was added to the 5′ end of the reverse primer (GTTTCTT; Brownstein et al., 1996).

Forty-eight primer pairs were chosen, based on potential heterozygosity and number of repeats, for initial testing on seven individuals from different populations from which DNA was extracted using the modified CTAB protocol described earlier (Shepherd and McLay, 2011). The 10-μL PCR cocktail contained 1 μL of 1 : 10 dilution DNA : H_2O (5–50 ng), 0.02 μM forward primer, 0.45 μM reverse primer, 0.45 μM M13 primer (labeled with FAM, NED, or VIC), 1.5 mM $MgCl_2$, 1× buffer BD (Solis BioDyne, Tartu, Estonia), 250 μM of each dNTP, and 0.4–0.5 U Firepol *Taq* polymerase (Solis BioDyne). PCRs were carried out following one of two cycling programs (see Table 1). For most loci, the PCR cycling program had an initial denaturation of 95°C for 3 min; 35 cycles of 95°C for 30 s, annealing temperature (Table 1) for 40 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. For loci with nonspecific amplification, a touchdown PCR program was used instead, which consisted of an initial denaturation of 95°C for 3 min; 10 cycles of 95°C for 30 s, annealing temperature decreasing by 1°C each cycle starting at 63°C for 40 s, and 72°C for 1 min; 25 cycles of 95°C for 30 s, 53°C for 40 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products (0.14–1.25 μL) of 2–3 loci with differing fluorophores were coloaded and added to 9 μL Hi-Di formamide (Applied

TABLE 2. Genetic properties of the newly developed microsatellite loci from *Fuchsia excorticata*.[a,b]

	South Island						North Island						
	Hinewai (n = 25)			Rotoroa (n = 22)			Rotokura (n = 21)			Karori (n = 27)			Total
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A
FE-10	8	0.542	0.556	8	0.826	0.797	5	0.571	0.619	4	0.692	0.739	15
FE-15	3	0.462	0.584	7	0.833	0.773	4	0.500	0.469	7	0.852	0.836	9
FE-19	2	0.077	0.074	3	0.458	0.510	3	0.619	0.523	3	0.739	0.665	4
FE-20	3	0.458	0.497	2	0.160	0.211	3	0.150	0.141	3	0.654	0.636	5
FE-22	5	0.462	0.64	8	0.636	0.808	3	0.571	0.563	5	0.704	0.665	12
FE-28	9	0.654	0.803	6	0.417	0.502	5	0.762	0.709	7	0.815	0.669	13
FE-29	7	0.542	0.616	6	0.773	0.707	7	0.857	0.791	5	0.808	0.730	11
FE-34	4	0.200	0.334	2	0.136	0.268	2	0.286	0.363	3	0.296	0.308	6
FE-38	4	0.600	0.662	4	0.391	0.664	2	0.200	0.180	4	**0.200**	**0.254**	5
FE-40	6	0.458	0.657	1	0.000	0.000	3	0.381	0.544	4	0.720	0.636	6
FE-43	2	0.269	0.286	2	0.080	0.147	7	0.762	0.746	10	0.704	0.636	14
FE-48	5	0.750	0.665	4	0.682	0.739	3	0.524	0.534	4	0.444	0.629	7

Note: A = number of alleles; H_e = mean expected heterozygosity; H_o = mean observed heterozygosity; n = sample size.

[a]See Appendix 1 for population and voucher information.

[b]Bold values indicate a significant deviation from Hardy–Weinberg expectations after Bonferroni correction.

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Biosystems, Carlsbad, California, USA) and 1 μL CASS ladder (Symonds and Lloyd, 2004) for subsequent fragment sizing on an ABI 3730 Genetic Analyzer (Applied Biosystems) by Massey Genome Service at Massey University (Palmerston North, New Zealand). Alleles were visualized and scored using GeneMapper version 3.7 (Applied Biosystems). Of the 48 primer pairs tested, 19 were polymorphic across the samples, 17 were monomorphic, 10 were unscorable, and seven did not amplify. Polymorphic loci were further tested using the above PCR conditions on 95 individuals from four geographically distant populations (Table 2, Appendix 1). The number of alleles, observed and expected heterozygosities, departure from Hardy–Weinberg equilibrium, and the cumulative exclusion probability were determined using GenAlEx (Peakall and Smouse, 2006). We tested for linkage disequilibrium within populations and overall using GENEPOP 4.2 (Rousset, 2008). Voucher specimens were deposited in the Dame Ella Campbell herbarium (MPN) at Massey University (Appendix 1).

Twelve of the 19 polymorphic loci amplified consistently across the four populations tested, with a range of 1–10 alleles per population (Table 2). Most loci did not deviate from Hardy–Weinberg equilibrium, except for FE-38 in the Karori population ($P = 0.0002$). After applying the Bonferroni correction ($P = 0.00075$), significant linkage disequilibrium was only detected between FE-40 and FE-28 in Rotokura ($P = 0.0005$). The cumulative probability of excluding a parent when one parent is known ranged from 90.7% to 96.4% and the probability of excluding a parent pair ranged from 99.95% to 99.997%, depending on the population.

CONCLUSIONS

Using 454 sequencing, we developed 12 primer sets for microsatellite loci that were polymorphic within and/or among populations of *F. excorticata*. The high polymorphism and high exclusion probabilities indicate these markers, or a subset of them, should be suitable for use in mating system studies, as well as other population genetic analyses.

Appendix 1. Voucher information for *Fuchsia excorticata* populations used in this study. All vouchers are deposited in the Dame Ella Campbell herbarium, Massey University, Palmerston North, New Zealand.

Population	Geographic coordinates	Voucher specimen accession no.
Hinewai	43°48′42.00″S, 173°1′22.80″E	MPN 47482, 47483
Rotoroa	41°47′41.52″S, 172°35′56.96″E	MPN 47484, 47485
Rotokura	39°26′15.60″S, 175°31′10.32″E	MPN 47486, 47487
Karori	41°17′47.88″S, 174°44′50.64″E	MPN 47488

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