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.

*Meth ods and R esults:* 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*.

**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 cetyl-trimethylammonium 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, 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°e (°C) | GenBank accession no. |
|-------|--------------------------|--------------|-----------------|----------|---------------------|
| FE-10 | F: ATATGCGTCCAGACACCTG   | (TAA)13       | 216–270         | 63/53*   | KF027215            |
|       | R: GGAACAATTCACCTTGTGG   |              |                 |          |                     |
| FE-15 | F: TGTTCAGCTTGGATGCTG    | (GTA)10       | 260–284         | 53       | KF027216            |
|       | R: ACACGAAGTACCTTGTGG    |              |                 |          |                     |
| FE-19 | F: TTGGTGGTTTTAACCTGGAG | (TCT)4        | 238–268         | 63/53*   | KF027217            |
|       | R: GTCCGTCCTTCTGACCTTG   |              |                 |          |                     |
| FE-20 | F: TCTCTGATGCTCTTGGC     | (ATC)10       | 163–178         | 53       | KF027218            |
|       | R: CAGCTCCCATCCTAGATATT  |              |                 |          |                     |
| FE-22 | F: GTGCGGAAACACAAAGCTCC  | (AT)7         | 200–228         | 63/53*   | KF027219            |
|       | R: TAAACCTCCTTATTATCTTC  |              |                 |          |                     |
| FE-28 | F: TGAGGACAGCATATCTCTAC  | (AT)h         | 251–279         | 53       | KF027221            |
|       | R: TGTCGGCAACAGTATATCATC |              |                 |          |                     |
| FE-29 | F: TGGATGCAAAGTACCTGTTG  | (AT)10        | 186–208         | 63/53*   | KF027220            |
|       | R: ACAAGGCTTCCACCTAGGAG  |              |                 |          |                     |
| FE-34 | F: GTGGTTTAAATAGCTGTTGG  | (CT)12        | 243–261         | 53       | KF027222            |
|       | R: CCGTCGAAGTAGGAAccc    |              |                 |          |                     |
| FE-38 | F: GTGAAAGTCCGCCAAGCTCG  | (AT)h         | 308–342         | 63/53*   | KF027223            |
|       | R: GTGGAGGCTTGAACCTGTTG  |              |                 |          |                     |
| FE-40 | F: TAGGGTGAGGAGTACCTGTC  | (AGTT)14      | 192–224         | 53       | KF027224            |
|       | R: TCTCAATGATCTGCGTCG    |              |                 |          |                     |
| FE-43 | F: ACTAAGCTGGAGTAGGACAG  | (AT)14        | 191–225         | 63/53*   | KF027225            |
|       | R: TGGCAATGATGAGAAAGGAC  |              |                 |          |                     |
| FE-48 | F: GGTGTTGCGGATTAGCACAC | (GCT)10       | 275–299         | 53       | KF027226            |
|       | R: GAATCCCTTCTGATGAGCAC  |              |                 |          |                     |

Note: T°e = annealing temperature.

*An M13 tail (CAGACGTTGTAAAACGAC) was added to the 5′ end of the forward primer (CAC-GACCCTTGAAAACGAC). To promote nontemplate (A) addition, a PIG tail (GTTTCTT) was added to the 5′ end of the reverse primer (GTTTCTT; Brownstein et al., 1996).

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 (CAGACCGTTGTAAAACGAC). To promote nontemplate (A) addition, a PIG tail (GTTTCTT) 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: H2O (5–50 ng), 0.45 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

| Locus | Hinewai (n = 25) | Rotoroa (n = 22) | Rotokura (n = 21) | North Island | Total |
|-------|-----------------|-----------------|------------------|--------------|-------|
|       | A    | H°e | H°s | A    | H°e | H°s | A    | H°e | H°s | A    | H°e | H°s | A    | H°e | H°s | A    | H°e | H°s | 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 | 7    | 0.739 | 0.665 | 4    |
| FE-22 | 3    | 0.488 | 0.507 | 2    | 0.160 | 0.211 | 3    | 0.150 | 0.141 | 3    | 0.654 | 0.636 | 5    |
| FE-24 | 5    | 0.462 | 0.64  | 8    | 0.636 | 0.808 | 5    | 0.571 | 0.563 | 5    | 0.704 | 0.665 | 12   |
| FE-28 | 9    | 0.654 | 0.803 | 6    | 0.417 | 0.502 | 7    | 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°s = mean observed heterozygosity; n = sample size.

aSee Appendix 1 for population and voucher information.

bBold values indicate a significant deviation from Hardy–Weinberg expectations after Bonferroni correction.
and FE-28 in Rotokura (on the population. The ability of excluding a parent pair ranged from 99.95% to 99.997%, depending on the population. Significant linkage disequilibrium was only detected between FE-40 and FE-38 in the Karori population. Most loci did not deviate from Hardy–Weinberg equilibrium, except for FE-38 in the populations tested, with a range of 1–10 alleles per population (Table 2). Most loci deviated 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 pair 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 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

**Table 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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