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Primer Note

Isolation and characterization of microsatellite loci for the large-seeded tree *Protorhus deflexa* (Anacardiaceae)[1]

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- *Premise of the study: Protorhus deflexa* is an endemic large-seeded tree in Madagascar that depends heavily on insects for cross-pollination and on large-bodied frugivores for seed dispersal. Because such mutualistic relationships are vulnerable to human disturbance, the development of microsatellite markers will enhance analyses of gene flow in this tree species in degraded forests.
- *Methods and Results:* Nineteen microsatellite markers were developed for *P. deflexa* using 454 pyrosequencing. The number of alleles ranged from two to nine, and the ranges of observed and expected heterozygosities were 0.200–0.800 and 0.303–0.821, respectively. The parentage exclusion probability by the 19 loci reached 0.98583 for the first parent and 0.99971 for the second parent.
- *Conclusions:* These markers will be useful for studying gene flow via pollination and seed dispersal by animals and the genetic structure of *P. deflexa* in protected and degraded forests in Madagascar.

Key words: 454 pyrosequencing; Anacardiaceae; large-seeded plants; microsatellite; *Protorhus deflexa.*

Protorhus deflexa H. Perrier (Anacardiaceae) is a dioecious tree that can reach 10–20 m in height (Schatz, 2001) and is found in tropical dry forests in western Madagascar (Sato, 2012). Based on the phylogenetic analysis of Randrianasolo (2003), Malagasy species of *Protorhus* Engl. will be formally validated as a new endemic genus, *Abrahamia* Randrian. & Lowry. From the end of the dry season to the beginning of the rainy season, *P. deflexa* blossoms and insects including bees visit the flowers (H. Sato, personal observation). Due to its breeding system without self-fertilization, this plant depends on insects for cross-pollination. During the middle of the rainy season, *P. deflexa* bears reddish fruits containing a large seed (Sato, 2012). Given this large seed size, large-bodied frugivorous lemurs of the genus *Eulemur* are the only effective seed dispersers of *P. deflexa* (Sato, 2012).

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The vulnerability of animal-mediated gene flow in plants to human-induced disturbance has been pointed out because habitat destruction and hunting can decrease the densities of pollinators and seed dispersers (Corlett, 2007). Given the extinction crisis for Malagasy primates, seed dispersal of large-seeded plants including *P. deflexa* seems to be one of the most vulnerable systems. Although most of the forested areas and fauna in the Malagasy forest are threatened by human activity, we have a poor understanding of the negative impacts on gene flow via the failed services of animals for each plant. In recent years, genetic analyses using microsatellite markers have successfully demonstrated the critical roles of pollinators and seed dispersers in gene flow in plant populations (Ashley, 2010). However, because such efficient markers have not been available in *P. deflexa* and even in congeneric species, it is necessary to isolate a large number of microsatellite loci in this plant species. In this study, we applied shotgun 454 pyrosequencing, which is a more efficient approach for isolation of microsatellites at a fraction of the cost and effort compared to traditional Sanger methods (Zalapa et al., 2012), to develop 19 nuclear microsatellite markers for *P. deflexa*.

METHODS AND RESULTS

Leaf samples were collected from 20 adult trees (>5 cm dbh) of *P. deflexa* (identified using specimen accession numbers 3505 and 14728, Parc de Tsimbazaza [TAN] herbarium, Madagascar) in the primary dry deciduous forest of Ampijoroa Forestry Station (16°31′S, 46°82′E) in Ankarafantsika National Park. Genomic DNA was extracted from the dried leaf tissues of each individual

TABLE 1. Characteristics of 19 microsatellite markers developed in *Protorhus deflexa*.

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	GenBank accession no.
Adf01	F: CGCCATCAGCTGTTCAACTC	$(CT)_7$	203–209	57	AB819872
	R: GCTGAAGAAGGGAGATCTTGG				
Adf02	F: GTTCTGTTCTGATAGTGACATGG	$(AG)_7$	344–364	55	AB819873
	R: AGAGATTGATGCAACCACAGG				
Adf03	F: AGCCAATCATCGACTGTTTCC	$(AG)_{11}$	185–191	55	AB819874
	R: AGCGTCGGAGAAAGACCTC				
Adf04	F: GCCAAGCCGAGCTCATTTC	$(CT)_9$	320–326	55	AB819875
	R: CGCGGTAAAGCAGACAACC				
Adf05	F: GTCTGCGTCGCCTACTTAC	$(GT)_9$	253–261	55	AB819876
	R: GTGTCATTCTATAAGTTCGGGTG				
Adf06	F: ATGCACTAGGAAACGGGTG	$(GT)_{10}$	347–369	57	AB819877
	R: AGAGCAGCGTAGGACTGAC				
Adf07	F: ATCATGGTATAACGATTGGAAGAC	$(ATT)_{22}$	339–378	55	AB819878
	R: GTTCGAATCATATTGTATTGGGAAG				
Adf08	F: AAGTCCTAGCGCCTCACAG	$(AC)_{14}$	288–300	55	AB819879
	R: TGAGCTCCTTGTGACTATGC				
Adf09	F: AGCTAGCATGACAAGACCC	$(GT)_{10}$	319–329	55	AB819880
	R: TCTCCAATATTAGGCTTTGGCG				
Adf10	F: CAAAGAATCAAATCGCCCAGC	$(AG)_{12}$	374–380	55	AB819881
	R: GCTGATGGCATTTCTGGTG				
Adf11	F: AGTTGCTTGTGCAATAATGTGATAG	$(AC)_{10}$	220–248	55	AB819882
	R: TTACCCAACAACCCTAATTATACAG				
Adf12	F: TTCGGCATCAGCGTAAGTG	$(AC)_{10}$	408–410	55	AB819883
	R: GCCATCTCGAGGATTTCCC				
Adf13	F: GTTTGGTACAACTTTCACCGC	$(AC)_8$	335–339	57	AB819884
	R: CATGGGCTCCAGTGCTTTG				
Adf14	F: ATCGAGCCGATGAAAGCC	$(AG)_{10}$	173–191	60	AB819885
	R: GCCCACGAGTAGGCTAGATTG				
Adf15	F: GGGAGTACAATGGATCAGCAC	$(CT)_9$	178–184	57	AB819886
	R: AGGTCCAGTTACAGCTATGAG				
Adf16	F: GGCATTGTTAGTAAAGCCTTCG	$(AG)_{10}$	193–195	57	AB819887
	R: GTGCCAACCAAAGCTCCAG				
Adf17	F: TCACACGGTGGATGTAGGC	$(AC)_8$	266–268	57	AB819888
	R: ACGTTGAAGGATCCAAGGTC				
Adf18	F: GATCACACTGAGAATGTTGTTTAAG	$(AAT)_{15}$	356–389	55	AB819889
	R: GGTTTCAGACATTGGACAGGG				
Adf19	F: GAACTTATACGATTCACCCTTGG	$(AC)_9$	291–303	57	AB819890
	R: GCAAAGGAGTGGTAAGGAGC				

Note: T_a = annealing temperature.

using the modified cetyltrimethylammonium bromide (CTAB) extraction protocol of Murray and Thompson (1980). A DNA library was prepared with one individual sample of *P. deflexa* using a GS Junior Titanium Series Kit (Roche Diagnostics, Mannheim, Germany). A 500-ng aliquot of genomic DNA was nebulized at 0.24 MPa for 1 min. The DNA fragments were end-repaired, A-tailed, ligated to the Rapid Library Adapter (Roche Diagnostics), and suitably sized by removing short fragments (<350 bp) using an SPRIworks Fragment Library System II Kit (Beckman Coulter, Brea, California, USA). The quality and quantity of the DNA fragments were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany, USA). The fragments were then mixed with capture beads and amplified through emulsion polymerase chain reaction (emPCR) using a GS Junior Titanium emPCR Kit (Roche Diagnostics). After emPCR, the beads were collected, and those capturing the DNA library fragments were enriched before annealing with sequencing primers. The amplified fragments were sequenced using the GS Junior benchtop system (Roche Diagnostics). Because the first round of sequencing failed to yield sufficient reads, sequencing was conducted twice. A total of 112,363 DNA sequence reads were obtained (first: 22,814 reads; second: 89,549 reads).

To search for potential microsatellite loci, including dinucleotide and trinucleotide loci of at least seven and four repeats, respectively, the sequences were screened using MSATCOMMANDER (Faircloth, 2008). A total of 972 primer pairs, including 211 dinucleotide repeats and 761 trinucleotide repeats, were designed by the default setting of the Primer3 program embedded in MSATCOMMANDER, using the following settings: primers designed to amplify regions of 100–500 bp, an optimal oligo melting temperature range of 57–62°C, GC content range of 20–80% with an optimum rate of 50%, low levels of self- or pair-complementarity, and a maximum end-stability (ΔG) of 8.0 (Faircloth, 2008). Based on the structure of the repeat, 67 primer pairs (49 dinucleotide and 18 trinucleotide loci) were selected for the initial screening of microsatellites using four individuals of the sampled 20 trees of *P. deflexa*. To avoid labeling individual primers, an M13 tail (5′-GTTGTAAAACGACGGCCAGT-3′) was added to the 5′ end of each forward primer and labeled (Schuelke, 2000). The reaction mixture had a final volume of 5 μL, which included 10 ng of template DNA, 0.05 U of LA-*Taq* DNA polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan), 2.0 μM of GC Buffer I (TaKaRa Bio Inc.), 400 μM of each dNTP, 0.25 μM M13-tailed forward primer, 0.5 μM reverse primer, and 0.5 μM FAM-labeled M13 primer. The amplification profiles included an initial denaturation for 5 min at 94°C; followed by 30 cycles of 30 s at 94°C, annealing for 45 s at 55°C, 57°C, or 60°C (Table 1), and extension for 45 s at 72°C; incorporation of the fluorescent dye into the PCR product followed by eight cycles of 30 s at 94°C, 45 s at 53°C, and 45 s at 72°C; and a final extension for 15 min at 72°C (Schuelke, 2000). The product sizes were measured using an ABI PRISM 3130xl Genetic Analyzer and Peak Scanner software (Applied Biosystems, Foster City, California, USA). Primers with a monomorphic locus (13 pairs) and primers that could not amplify over half of the four samples (35 pairs) were removed from the marker set. A final set of 19 successful polymorphic markers was used to genotype 20 unrelated adult trees of *P. deflexa* (Table 1).

The number of alleles, observed and expected heterozygosities (H_o and H_e), and probability of exclusion (PE) were calculated using GenAlEx version 6.5 (Peakall and Smouse, 2012). The number of alleles per locus ranged from two to nine (mean: 4.6); the ranges of H_o and H_e were 0.200–0.800 (mean: 0.484) and 0.303–0.821 (mean: 0.565), respectively (Table 2). PE over all loci reached 0.98583 for the first parent and 0.99971 for the second parent, whereas PE for excluding a putative parent pair was greater than 0.99999. These values of PE

TABLE 2. Genetic properties of the newly developed 19 microsatellites of *Protorhus deflexa*.

Locus	*A*	H_o	H_e	*NAF*
Adf01	3	0.500	0.460	0.000
Adf02	4	0.350	0.404	0.024
Adf03	4	0.350	0.303	0.000
Adf04	4	0.500	0.636	0.102
Adf05	5	0.421	0.643	0.131
Adf06	4	0.500	0.558	0.020
Adf07	6	0.550	0.605	0.012
Adf08	5	0.450	0.445	0.005
Adf09	4	0.700	0.674	0.000
Adf10	6	0.700	0.711	0.000
Adf11	8	0.474	0.821	0.202
Adf12	2	0.600	0.495	0.000
Adf13	3	0.400	0.571	0.125
Adf14	9	0.550	0.636	0.027
Adf15	4	0.500	0.588	0.034
Adf16	2	0.300	0.455	0.111
Adf17	2	0.350	0.349	0.000
Adf18	8	0.800	0.789	0.014
Adf19	4	0.200	0.588	0.249
Average	4.6	0.484	0.565	

Note: *A* = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; *NAF* = null allele frequency.

reached a level high enough to detect mating system and gene flow of *P. deflexa*. The null allele frequency was determined for all loci using FreeNA (Chapuis and Estoup, 2007). Because the null allele frequency was <0.2 for all loci except Adf11 and Adf19 (Table 2), the results of analyses using those loci may not be changed significantly by null alleles (Latinne et al., 2011). Linkage disequilibrium (LD) between pairs of loci was tested using GENEPOP version 4.0 (Rousset, 2008). There were no pairs with significant LD after Bonferroni correction ($P > 0.00029$).

CONCLUSIONS

We characterized 19 polymorphic microsatellite loci for *P. deflexa*. These microsatellite markers will be useful for investigating gene flow via pollination and seed dispersal by animals and the genetic structure of *P. deflexa* in protected and degraded forests in Madagascar.

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