American Journal of Botany

American Journal of Botany: e465–e467. 2012.

AJB Primer Notes & Protocols in the Plant Sciences

Microsatellite primers for an Amazonian lowland tropical tree, *Protium subserratum* (Burseraceae)[1]

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- *Premise of the study:* The first microsatellite primers were developed for *Protium subserratum*, a widespread Amazonian tree, to investigate genetic differentiation between populations found on clay, brown-sand, and white-sand soils.
- *Methods and Results:* Seventeen primer pairs were identified from two individuals of *P. subserratum* found on white-sand and brown-sand soil types. Polymorphism was analyzed in 63 individuals from a total of three populations, each found on a different soil type. The primers amplified tetra-, tri-, and dinucleotide repeats with three to 24 alleles per locus. Excluding monomorphic loci, observed and expected heterozygosities ranged from 0 to 0.852 and 0.036 to 0.901, respectively.
- *Conclusions:* These new microsatellite markers will be useful in studies of genetic diversity, population differentiation, and gene flow across habitat types in *P. subserratum*.

Key words: Amazon; Burseraceae; microsatellite; *Protium subserratum.*

Protium subserratum (Engl.) Engl. (Burseraceae) is a widespread neotropical tree found across the lowland Amazon Basin (Daly and Fine, 2011). It is dioecious with flowers that are small, white, nectar-producing, and odiferous, indicating generalist pollinator affinities (Daly, 1987). *Protium subserratum* represents one of the few soil generalist species in the genus (Fine et al., 2005), with two morphologically differentiated subpopulations endemic to the widespread and relatively fertile clay and brown-sand forests, as well as to the comparatively rare patches of nutrient-poor white-sand forest habitats (Daly and Fine, 2011). A recent phylogeographic study comparing measurements of leaf traits in individuals from all three habitat types demonstrated that *P. subserratum* found in white-sand habitats differed morphologically in vegetative traits from populations found on terrace and clay soil types. Nuclear sequence data from the same study also showed that populations of *P. subserratum* from geographically distant clay and terrace soils were more closely related to each other than they were to nearby white-sand populations (Fine et al., 2012). While these results are consistent with the idea that adaptations to different soil types may be playing an important role in population divergence, microsatellite markers will provide a more powerful tool to examine fine-scale population differentiation and gene flow.

[1]Manuscript received 7 May 2012; revision accepted 12 June 2012.

The authors acknowledge support from the National Science Foundation (DEB 0919567) and the Vincent Coates Fund at the University of California, Berkeley.

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doi:10.3732/ajb.1200229

METHODS AND RESULTS

Genomic DNA from two individuals of *P. subserratum* from a brown-sand population and a white-sand population (Appendix 1) was sent to the Savannah River Ecological Laboratories (SREL) at the University of Georgia for microsatellite marker development and primer design. Microsatellite markers were developed according to the protocol developed by Glenn and Schable (2005). At SREL, genomic DNA was combined, digested, ligated with linkers SimpleXL12_U (5′-AAAGCTGGCGTCGAAGT-3′) and SimpleXL12_Lp (5′-pACTTCGACGCCAGC-3′), enriched with biotinylated probes, and recovered via PCR. The enriched library was then sequenced on a 454 using titanium chemistry (454 Life Sciences, a Roche company, Branford, Connecticut, USA). A total of 6123 sequences were obtained. A total of 2201 reads, identified using MSATCOMMANDER version 0.8.1 (Faircloth, 2008), contained microsatellite repeats suitable for primer design. Primers were designed using Primer3 (Rozen and Skaletsky, 2000) to use a three-primer PCR protocol (Schuelke, 2000). One primer from each pair was modified with the addition of an M13R tag (5′-GGAAACAGCTATGACCAT-3′) to enable the use of a third universal primer (identical to the M13R tag), fluorescently labeled for detection. The sequence GTTT was added to primers without the M13R tag to promote adenylation.

One hundred and thirteen primer pairs were tested for amplification and polymorphism using DNA obtained from 10 individuals of *P. subserratum*; five from a brown-sand population and five from a white-sand population. PCR amplifications were performed in a total reaction volume of 12.5 μL containing 6.5 μL 2× GoTaq Green Master Mix (400 μM dNTPs, 3 mM $MgCl_2$, and 1 unit of *Taq* DNA polymerase; Promega Corporation, Madison, Wisconsin, USA), 0.6 μM untagged primer, 0.3 μM tag-modified primer, 0.3 μM M13R primer fluorescently labeled with either 6-FAM or HEX, 1 μL of undiluted DNA template, and DNase free water. Amplifications for all loci were conducted using a touchdown PCR protocol beginning with an initial denaturation step of 2 min 30 s at 95°C; followed by 30 cycles at 95°C for 30 s, annealing at a temperature of 60°C for 30 s (decreased by 0.5°C per cycle), 72°C for 1 min; and 30 cycles at 95°C for 30 s, 45°C for 30 s, and 72°C for 1 min. A final extension was done at 72°C for 10 min. Amplification products were co-loaded on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, California, USA) with 0.3 μL GS-500 LIZ size standard (Applied Biosystems) to allow allele length sizing. Electrophoretic results were initially scored using Peak Scanner version 1.0 (Applied Biosystems) followed by visual confirmation. Seventeen of the tested primer pairs amplified high-quality PCR product that exhibited polymorphism (Table 1).

TABLE 1. Characteristics of 17 microsatellite loci developed in *Protium subserratum*.

Locus	Primer sequences (5′–3′)[a]	Repeat motif	T_a (°C)	T_m (°C)	Size range (bp)	GenBank accession no.
Prot02	F: *ATAAACCCTCTTACGGTGAG	$(ACAT)_6$	60/45	59.2	174–224	JX014415
	R: ‡GGGATTTGTTGACTTTGAAC			59.6		
Prot08	F: ‡TATGTCCCACAATGATCCTC	$(ACAT)_7$	60/45	59.9	184–239	JX014416
	R: *TTTATAGGAGCGCTCTGATC			60.4		
Prot13	F: *TGATTTCTTGTCCCAAAGAG	$(AAAG)_7$	60/45	60.2	305–313	JX014417
	R: ‡AGCCACATACCGATAAACTC			59.7		
Prot22	F: *TAACCCTTGACAAGCATTTC	$(AGAT)_9$	60/45	60.7	336–364	JX014418
	R: ‡AAATTACGGCTTCAGAATTG			60.7		
Prot28	F: ‡CGCAGTTTCAGAAATATCAG	$(ACAT)_8$	60/45	58.7	230–251	JX014419
	R: *GCATGATTCGATGTTATAGG			58.4		
Prot29	F: *TGAACTGACCTTTGCATGAC	$(AAAG)_{15}$	60/45	61.1	121–168	JX014420
	R: ‡AAGAGGGTGGTCTGAACTG			59.7		
Prot67	F: ‡TCATGCTGTAATTCCCTGTC	$(ACAT)_7$	60/45	60.6	198–234	JX014421
	R: *GAGAAGAGCAAAGATTCGATAG			60.0		
Prot70	F: *CCATTATTAAGCATGCAAAC	$(AAG)_9$	60/45	59.4	113–262	JX014422
	R: ‡CAATGGCCTGTTCATATAAAG			60.3		
Prot71	F: *CCATCCTCAGCTCTTACTTTC	$(AAG)_8$	60/45	60.7	402–414	JX014423
	R: ‡GATCGGTCACAGATTCAATG			60.3		
Prot78	F: ‡CACACCAGGAAAGACTCAAG	$(AAG)_8$	60/45	59.9	137–143	JX014424
	R: *TTGGAAGGAGGATTTATAGG			59.5		
Prot83	F: ‡CGTCTGGATGGAAGATAAAG	$(AAC)_{10}$	60/45	60.0	168–186	JX014425
	R: *TCCTCGTTCTCCACTACAAC			59.6		
Prot97	F: *ATTCCGATTAAACCTCATTC	$(AG)_{13}$	60/45	59.2	150–163	JX014426
	R: ‡GGGTATGAGCTTGAATTAGG			59.7		
Prot99	F: *ATGCTATGATAATCGGTTCC	$(AG)_{12}$	60/45	59.8	159–201	JX014427
	R: ‡GAAATGGTTGCACTTCACTC			60.1		
Prot100	F: *ATCTCTCGTTCCAACTCAAC	$(AC)_{10}$	60/45	58.7	158–184	JX014428
	R: ‡CGTCGAAGTACTCACCACTC			59.9		
Prot101	F: ‡CATTTAGGGACCACGTTTAC	$(AG)_{11}$	60/45	60.3	274–290	JX014429
	R: *ATTGTTCCAGGATCTAGGTG			59.0		
Prot102	F: ‡GTCGACCAAATAATGTCACC	$(AG)_{15}$	60/45	60.0	350–378	JX014430
	R: *ATGGACACACAGGACCTATC			58.7		
Prot104	F: *TAACCGCAATATCAACTCTC	$(AC)_{11}$	60/45	58.2	271–289	JX014431
	R: ‡ACACCACGACTAAAGACTGG			59.7		

Note: T_a = annealing temperatures used for touchdown cycling; T_m = calculated melting temperatures.
[a] *Indicates M13R tag (5′-GGAAACAGCTATGACCAT-3′); ‡indicates GTTT tag.

We assessed the variability of the 17 polymorphic loci in 63 individuals from three different populations. One voucher specimen per population was deposited at the University of California, Berkeley, University Herbarium (Appendix 1). Conditions and characteristics for each of the 17 loci are presented in Tables 1 and 2. The presence of null alleles was tested using MicroChecker version 2.2.3 (van Oosterhout et al., 2004). Number of alleles per locus (A), observed and expected heterozygosities (H_o and H_e), Shannon's information index (I), and deviations from Hardy–Weinberg equilibrium (HWE) were

TABLE 2. Statistical analysis of 17 microsatellite loci for three populations of *Protium subserratum* found on different soil types.

	Lagunas (N = 15)				Porvenir 1 (N = 27)				Porvenir 2 (N = 21)			
Locus	A	H_o	H_e	I	A	H_o	H_e	I	A	H_o	H_e	I
Prot02	7	0.533	0.791	1.717	9	0.250*	0.837	1.965	7	0.636	0.796	1.735
Prot08	10	0.538	0.876	2.172	13	0.565	0.851	2.181	14	0.333*	0.901	2.452
Prot13	1	—	—	—	—	—	—	—	3	0.591	0.483	0.843
Prot22	4	0*	0.625	1.127	3	0.148*	0.427	0.677	5	0.381	0.546	1.093
Prot28	6	0.467	0.709	1.407	2	0.037	0.036	0.092	5	0.810	0.687	1.308
Prot29	6	0.733	0.602	1.280	3	0.519	0.524	0.814	6	0.909	0.731	1.492
Prot67	8	0.733	0.760	1.688	5	0.593	0.676	1.249	6	0.857	0.753	1.563
Prot70	7	0.800	0.698	1.521	4	0.370	0.366	0.690	1	—	—	—
Prot71	3	0.375	0.461	0.777	—	—	—	—	2	0.176	0.251	0.418
Prot78	3	0.583	0.531	0.829	—	—	—	—	2	0.409	0.375	0.562
Prot83	3	0.476	0.438	0.716	4	0.308	0.353	0.677	4	0.286	0.294	0.610
Prot97	5	0.467	0.556	1.112	6	0.778	0.695	1.329	4	0.318	0.350	0.704
Prot99	7	0.467	0.671	1.367	7	0.826	0.750	1.576	8	0.682	0.784	1.738
Prot100	5	0.467	0.673	1.319	5	0.320	0.510	1.054	4	0.333	0.579	1.009
Prot101	4	0.400	0.344	0.703	4	0.444	0.490	0.929	4	0.714	0.695	1.237
Prot102	6	0.533	0.798	1.674	6	0.852	0.694	1.418	8	0.700	0.825	1.866
Prot104	3	0.600	0.518	0.802	2	0.400	0.365	0.551	3	0.545	0.464	0.725

Note: — = monomorphic loci; A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of samples; I = Shannon's information index.
* Indicates that H_o is significantly departed from H_e under Hardy–Weinberg equilibrium after sequential Bonferonni corrections ($P < 0.05$).

estimated using GenAlEx version 6.4 (Peakall and Smouse, 2006). *P* values for tests of deviation from HWE were adjusted using a sequential Bonferroni correction (Rice, 1989).

While each population has at least one monomorphic locus, all loci displayed polymorphism when compared across populations, with the total number of alleles ranging from three to 24 alleles per locus. Loci Prot02, Prot08, Prot102, and Prot22 tested positive for the presence of null alleles across all populations. Observed heterozygosity ranged from 0 to 0.852 and expected heterozygosity ranged from 0.036 to 0.901. Locus Prot22 significantly deviated from HWE in the Lagunas and Porvenir 1 populations, Prot02 significantly deviated from HWE in the Porvenir 1 population, and locus Prot08 showed significant deviation from HWE in the Porvenir 2 population ($P < 0.05$). Genetic diversity as measured by Shannon's information index ranged from 0.319 to 2.172.

CONCLUSIONS

These 17 new microsatellite loci described here will provide useful tools in future studies of genetic diversity, gene flow, and genetic differentiation across habitat types in the Amazon tree, *P. subserratum.*

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APPENDIX 1. Location, soil types, and voucher specimens of three natural populations of *Protium subserratum*. Voucher specimens are deposited in the University Herbarium, University of California, Berkeley.

Population (locale)	Geographical coordinates (degrees decimal)	Soil type	Voucher specimen no.
Lagunas (Loreto, Peru)	Latitude: −3.8303233202193137, Longitude: −73.59467610059791	Clay	TM28
Porvenir 1 (Loreto, Peru)	Latitude: −3.915609635759378, Longitude: −73.55211354953575	White sand	TM29
Porvenir 2 (Loreto, Peru)	Latitude: −3.8966818509317767, Longitude: −73.53723449019981	Brown sand	TM30