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PRIMER NOTE

MICROSATELLITE PRIMERS IN THE FOUNDATION TREE SPECIES *PINUS EDULIS* AND *P. MONOPHYLLA* (PINACEAE)[1]

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- *Premise of the study:* Microsatellite primers were developed in the foundational tree species *Pinus edulis* to investigate population differentiation of *P. edulis* and hybridization among closely related species.
- *Methods and Results:* Using a hybridization protocol, primer sets for 11 microsatellite loci were developed using megagametophyte tissue from *P. edulis* and scored for polymorphism in three populations of *P. edulis* and a single *P. monophylla* population. The primers amplified simple and compound di-, tri-, and pentanucleotide repeats with two to 18 alleles per locus.
- *Conclusions:* These results demonstrate the utility of the described primers for studies of population differentiation within and among *P. edulis* populations as well as across putative hybrid zones where *P. edulis* may coexist with sister species.

Key words: microsatellite; Pinaceae; *Pinus edulis*; *Pinus monophylla*; population genetics.

Pinus edulis Engelm. (Colorado pinyon) and *P. monophylla* Torr. & Frém. (singleleaf pinyon) coexist with *Juniperus* L. spp. as dominant species in the pinyon-juniper woodlands of the southwestern United States. Needle number has been used to distinguish between the species, with *P. monophylla* having one needle per fascicle and *P. edulis* having two needles per fascicle. *Pinus monophylla* occurs mainly in California and Nevada, while the *P. edulis* distribution covers parts of Colorado, New Mexico, Arizona, and Utah. Even though their distributions are largely disjunct, it has been suggested the two species may hybridize to form *P. edulis* var. *fallax* Little, a single-needle variety found where the ranges of *P. monophylla* and *P. edulis* overlap (Cole et al., 2008). Whether *P. edulis* var. *fallax* represents a distinct taxon or results from hybridization requires genetic approaches, but currently available markers (Lesser et al., 2012) exhibit low levels of polymorphism in our samples (3.13 alleles per locus for eight successfully amplified loci) and 38% of loci tested present more than two alleles per primer set (data not shown).

Populations of *P. edulis* have experienced large-scale mortality during recent drought in the southwestern United States (Breshears et al., 2005), with studies in northern Arizona indicating that this mortality is not random (Mueller et al., 2005). Pinyon mortality is clearly associated with environmental gradients and bark beetle infestation (Breshears et al., 2005), but variation in mortality at finer spatial scales can have a genetic basis (Sthultz et al., 2009). Sthultz et al. (2009) observed that trees genetically susceptible to herbivory by the pinyon tip moth (*Dioryctria albovittella*) were three times more likely to survive prolonged drought stress than moth-resistant trees. However, genetic tools for investigating pinyon pine population structure in the context of such effects are not currently available. We report 11 microsatellite markers exhibiting variable levels of polymorphism. We briefly demonstrate their utility in distinguishing *P. edulis* populations from a population of *P. monophylla*.

METHODS AND RESULTS

We enriched *P. edulis* DNA for microsatellite sequences by a modified method of Glenn and Schable (2005). Briefly, 10 µg of DNA extracted from *P. edulis* megagametophyte tissue by the method of Doyle and Doyle (1987) was digested with the restriction endonuclease *Csp*I (Fermentas, Glen Burnie, Maryland, USA). Fragment ends were blunted with mung bean nuclease (New England Biolabs, Beverly, Massachusetts, USA) and dephosphorylated with shrimp alkaline phosphatase (USB Corporation, Cleveland, Ohio, USA), and the blunted dephosphorylated fragments were then purified with QIAquick PCR Purification Kit (QIAGEN, Valencia, California, USA). Polynucleotide linkers (ScaF: 5′-CAGTGCTCTAGACGTGCTAGT-3′; ScaR: 5′-ACTAGCACGTCTAGAGCACTGAAAA-3′; Eurofins MWG Operon, Huntsville, Alabama, USA) were ligated to 3.3 µg of prepared fragments with T4 DNA ligase (Invitrogen, Carlsbad, California, USA) in the presence of the restriction endonuclease *Sca*I (New England Biolabs) to prevent fragment concatenation. One microliter exonuclease I (USB Corporation) was added to the resultant ligation mixture and incubated for 15 min at 37°C to digest remaining single-stranded linker DNA that may interfere with subsequent PCR amplification. Exonuclease I was inactivated by incubation at 80°C for 20 min. Linker-ligated DNA was PCR amplified in a 25-µL reaction containing 12.5 µL JumpStart REDTaq ReadyMix (Sigma-Aldrich, St. Louis, Missouri, USA), 800 nM ScaF linker, and 1 µL linker-ligated DNA. Thermal cycling parameters were 95°C for 2 min; 30 cycles of 95°C for 45 s, 56°C for 60 s, 72°C for 120 s; and 72°C for 10 min. PCR products were purified with QIAquick PCR Purification Kit (QIAGEN). The 825 ng purified PCR product was hybridized with 500 pmol of each probe in separate reactions using the following thermal cycler protocol: 95°C for 5 min, decreasing to 20°C at the rate of 0.1°C every 5 s. Sequences of synthetic oligonucleotide probes used for enrichment were 5′-$(CAT)_{11}$-3′, 5′-$(GCA)_6$-3′, 5′-$(GATA)_{11}$-3′, 5′-$(AAC)_{12}$-3′, 5′-$(ATTT)_{10}(GC)_8$-3′, 5′-$(GCGA)_5$-3′, 5′-$(TTC)_{15}$-3′, and 5′-$(GGT)_7$-3′. All probes were 3′-biotinylated to prevent nonspecific extension during amplification steps. DNA bound to probes was captured with streptavidin-coated magnetic beads (Promega Corporation,

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TABLE 1. Characteristics of 11 microsatellite loci developed in *Pinus edulis*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Label[b]	Fluor	T_a (°C)	*I*	GenBank accession no.
AAC21	F: AGACGTGCTAGTCCAAATCGAG R: GCCCCTGGTATTTTGTAGATCC	$(AC)_{12}(AT)_7$	183–189	Direct	FAM	63	0.747	JX856174
CAT15	F: TAGGTGCTGTGACTGTGATTGT R: TTTTCTTTGTCTTTGGGGAGAG	$(ATG)_5(N)_{30}(ATG)_5$	309–319	Tail	FAM	63	1.026	JQ821393
CAT39	F: TGGGGAGCAAAAGAAAAAGA R: AAAGATGCTTTTTGGCATGG	$(WGA)_7$	289–292	Tail	TAMRA	63	0.642	JQ821394
GGT19	F: GGAGCCTCATTTGGTTTGTG R: ACTCTACATTTGAAAGCCAAGAA	$(GT)_{24}$	100–122	Direct	FAM	63	1.628	JQ821395
GGT37	F: TAATGGGTTCGCATCCACA R: CTTATGATTGATGCAAGCTTAGG	$(CA)_{12}(TA)_5$	190–206	Tail	FAM	63	1.349	JQ821396
GGT54	F: ATGCCGGTCGAAACTTGTAA R: GCGATGGCACATATGTGTGTG	$(AC)_7AT(AC)_{14}$	78–118	Direct	FAM	63	1.552	JQ821397
TTC41	F: TGCACCAGTGCAAGATGAAT R: TGTGCTTTAGCAACGAGGTG	$(GAA)_4(N)_6(GAA)_6(N)_6(GAA)_{10}$	258–300	Tail	FAM	63	0.967	JQ821398
G448	F: CCATTTCACCACACTAGAAGCA R: CGTGAGCAGGTAGAAGGAAGAG	$(CTT)_4(N)_3(CTT)_3$	107–110	Direct	HEX	63	0.458	JX856176
G504	F: AGAGAAACAACTGAAACCACCG R: ATCATCACCTTGCTAGAGGAGC	$(TGC)_4(T)(CTGCA)_5(CTG)_3(CT)$ $(CTGCA)_5(N)_{11}(CTGCA)_3$	327–334	Direct	FAM	63	0.645	JX856177
G609	F: TTCTCTGCACTGATCTGCATT R: CTCGTAGAAGAGGAGCAGCAA	$(TGC)_3(N)_5(TGC)_6$	263–300	Direct	TAMRA	63	0.884	JX856178
G646	F: GAAGCACTGAACCTTGGATAGG R: TACCTTGGGCATAGGACAACTC	$(CTT)_4(N)_{14}(CTT)_3$	221–231	Direct	TAMRA	63	0.965	JX856179

Note: Fluor = fluorophore used; *I* = information index; T_a = annealing temperature.

[a] Size range and allele number for each locus is based on amplification in both *P. edulis* and *P. monophylla*.

[b] "Label" describes whether the forward primer was tailed or directly labeled.

Madison, Wisconsin, USA). Resulting enriched DNA was PCR amplified for cloning in a 50-µL reaction containing 25 µL JumpStart REDTaq ReadyMix (Sigma-Aldrich), 800 nM ScaF linker, and 10 µL enriched DNA fragments. Thermal cycling parameters were 95°C for 2 min; 30 cycles of 95°C for 45 s, 58°C for 60 s, 72°C for 120 s; and 72°C for 10 min. PCR products were cloned using a QIAGEN PCR Cloning Kit (QIAGEN) and 478 inserts sequenced on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Sequences were analyzed in Staden Package (Staden, 1996) and screened for repeat sequences using Imperfect SSR Finder (Stieneke and Eujayl, 2007). Primer pairs were designed for 71 sequences containing repeat motifs and were screened for polymorphism in four *P. edulis* individuals using either fluorescently labeled or 5′ tailed primers with fluorescently labeled universal primers (Missiaggia and Grattapaglia, 2006) and separated on an ABI 3730xl Genetic Analyzer (Applied Biosystems). Fragment analysis was conducted in GeneMarker version 2.20 (SoftGenetics, State College, Pennsylvania, USA).

Resulting microsatellite loci were individually amplified in samples from *P. edulis* populations at Sunset Crater National Monument (SCNM), Arizona (*N* = 30); Red Mountain, Arizona (*N* = 18); Anvil Rock Road, Seligman, Arizona (*N* = 8); and from a *P. monophylla* population near Mono Lake, California (*N* = 8) (Appendix 1). One specimen from each *P. edulis* population has been deposited at the Deaver Herbarium at Northern Arizona University, Flagstaff, Arizona. Although no voucher specimen was collected from the Mono Lake population, pinyons from this site are well known to consist of only *P. monophylla*. PCR was performed in 4-µL reaction volumes containing 0.01 U/µL Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1× HF Buffer, and 400 nM each primer when using directly labeled fluorophores or 40 nM tailed forward primer, 400 nM FAM-labeled universal fluorescent primer, and 400 nM reverse primer when using universal tailed primers. Thermal cycling parameters for all loci were as follows: 90°C for 2 min; 35 cycles of 90°C for 30 s, 63°C for 2 min, 72°C for 15 s; 72°C

TABLE 2. Amplification in both *Pinus edulis* and *P. monophylla* of primers developed in *P. edulis*.[a]

	SCNM (*N* = 30)				RM (*N* = 18)				ARR (*N* = 8)				ML (*N* = 8)			
Locus	*A*	H_o	H_e	*P*[b]	*A*	H_o	H_e	*P*[b]	*A*	H_o	H_e	*P*[b]	*A*	H_o	H_e	*P*[b]
AAC21	4	0.828	0.549	**0.039**	2	0.857	0.490	**0.005**	2	1.000	0.500	**0.005**	2	1.000	0.500	**0.014**
CAT15	4	0.611	0.702	0.096	5	0.727	0.715	0.657	5	0.286	0.745	0.065	0	0.000	0.000	monomorphic
CAT39	2	0.536	0.448	0.302	2	0.444	0.494	0.671	2	0.500	0.375	0.505	2	0.167	0.486	0.107
GGT19	10	0.682	0.818	0.252	7	0.583	0.833	0.179	6	0.625	0.734	0.615	4	1.000	0.660	0.466
GGT37	5	0.650	0.716	0.363	5	0.700	0.735	0.740	5	0.875	0.750	0.796	5	0.500	0.500	0.084
GGT54	12	0.444	0.828	**0.000**	9	0.357	0.847	**0.001**	9	0.714	0.867	0.325	0	0.000	0.000	monomorphic
TTC41	4	0.536	0.471	0.958	3	0.400	0.451	0.788	3	0.750	0.617	0.081	5	0.571	0.673	0.838
G448	5	0.500	0.437	0.639	5	0.375	0.328	1.000	1	0.000	0.000	monomorphic	2	0.125	0.117	0.850
G504	2	0.037	0.036	0.922	3	0.111	0.106	0.996	4	0.429	0.694	0.177	3	0.000	0.571	**0.003**
G609	3	0.448	0.600	0.321	2	0.278	0.461	0.091	5	0.750	0.688	0.318	2	0.250	0.375	0.346
G646	4	0.500	0.695	**0.000**	3	0.400	0.531	0.517	4	0.625	0.602	0.081	2	0.875	0.492	**0.028**

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; *N* = sample size for each population.

[a] Population localities: SCNM = Sunset Crater National Monument, Arizona, USA; RM = Red Mountain, Arizona, USA; ARR = Anvil Rock Road, Arizona, USA; ML = Mono Lake, California, USA. For more information on localities, see Appendix 1.

[b] Results of χ^2 tests for Hardy–Weinberg equilibrium for each population (*P* value). Boldfaced values indicate a significant deviation from Hardy–Weinberg equilibrium.

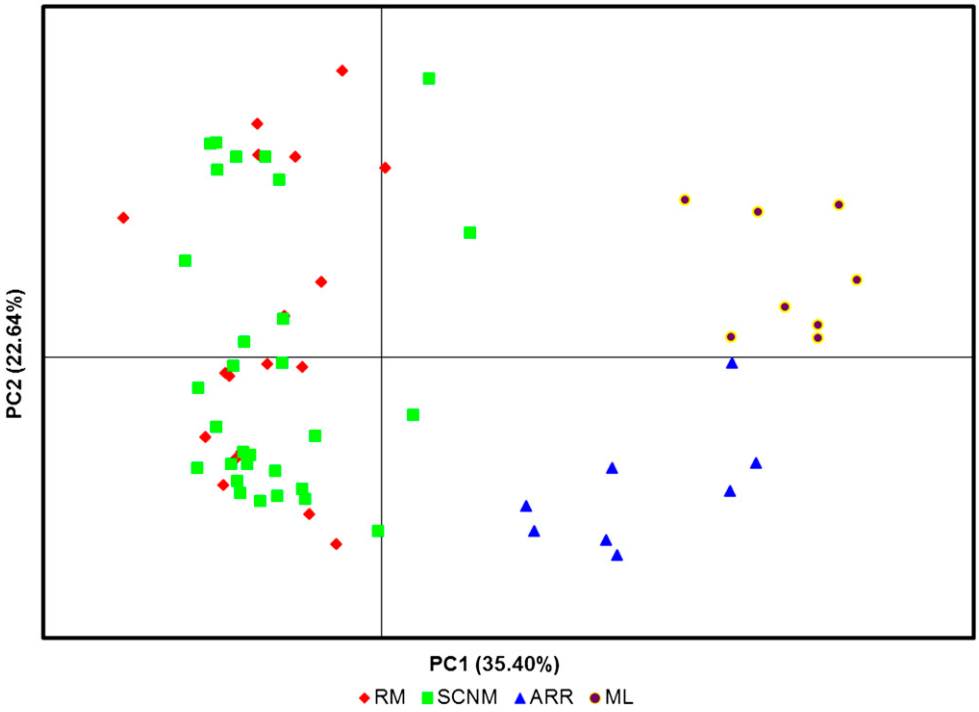

Fig. 1. Principal coordinates analysis (PCA) illustrating population differentiation of three *Pinus edulis* populations (RM, SCNM, and ARR) and one *P. monophylla* population (ML) using the 11 microsatellite loci presented here. ARR = Anvil Rock Road, near Seligman, Arizona; ML = Mono Lake, near Lee Vining, California; RM = Red Mountain, near Flagstaff, Arizona; SCNM = Sunset Crater National Monument, near Flagstaff, Arizona.

for 10 min. Calculation of summary statistics and principal coordinates analysis was performed in GenAlEx 6 (Peakall and Smouse, 2006), and pairwise tests of composite linkage disequilibrium (LD) were calculated in GENEPOP (Rousset, 2008).

The 11 polymorphic primer sets consistently amplified microsatellite loci containing di-, tri-, and pentanucleotide repeats in *P. edulis* (Table 1). Shannon's information index for all loci ranged from 0.458 to 1.628 (Table 1). Within each population, the total number of alleles for these microsatellites ranged from 0 to 12 per marker with all markers exhibiting variable levels of heterozygosity (Table 2). To test the broad applicability of these primers in related taxa, each locus was also amplified in *P. monophylla*. Although we used only a small number of *P. monophylla* individuals, cross-species amplification was successful in most cases (Table 2). Chi-square (χ^2) tests for Hardy–Weinberg equilibrium (HWE) showed that most loci are neutrally evolving within each population (Table 2). Locus AAC21 was the exception, observed to not be in HWE for all populations tested. After removing rare alleles (frequency < 0.05), *G*-tests for LD revealed no statistical associations between any loci presented here. Principal coordinates analyses using these data show population differentiation across the populations sampled for this study (Fig. 1).

CONCLUSIONS

The microsatellite primer pairs presented here represent the first available non-EST-derived polymorphic nuclear markers for the study of *P. edulis* and closely related taxa. The demonstration here of the amplification of 69 alleles from 11 loci among *P. edulis* across northern Arizona and *P. monophylla* in eastern California emphasizes the potential power of these markers to resolve species delineations and putative hybridization zones between these two species as well as for investigating populations that show genetic tolerance to periods of drought stress. Because these markers are apparently suitable for use in other closely related taxa, they may be especially useful in the study of various soft pines that range from the American Southwest to Mexico.

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Appendix 1. Taxa used in this study, population sampled, GPS coordinates of sampled population, and herbarium information.

Taxon	Source population	GPS coordinates	Herbarium
Pinus edulis Engelm.	Sunset Crater, near Flagstaff, Arizona, USA	35.391689°N, 111.429686°W	ASC
Pinus edulis Engelm.	Red Mountain, near Flagstaff, Arizona, USA	35.536778°N, 111.858127°W	ASC
Pinus edulis Engelm.	Anvil Rock Road, near Seligman, Arizona, USA	35.274349°N, 113.087192°W	ASC
Pinus monophylla Torr. & Frem.	Mono Lake, near Lee Vining, California, USA	37.980568°N, 119.138618°W	no voucher specimen collected

Note: ASC = Deaver Herbarium, Northern Arizona University.