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PRIMER NOTE

MICROSATELLITE MARKER DEVELOPMENT FOR THE COASTAL DUNE SHRUB *PRUNUS MARITIMA* (ROSACEAE)[1]

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- *Premise of the study:* Microsatellite primers were developed in the beach plum, *Prunus maritima*, to investigate the genetic composition of remaining populations in need of conservation and, in future studies, to determine its relation to *P. maritima* var. *gravesii.*
- *Methods and Results:* Fourteen primer pairs were identified and tested in four populations throughout the species' geographic range. Of these 14 loci, 12 were shown to be polymorphic among a total of 60 *P. maritima* individuals sampled (15 individuals sampled from four populations). Among the polymorphic loci, the number of alleles ranged from two to 10 and observed heterozygosity of loci ranged from 0.07 to 0.93 among specimens tested.
- *Conclusions:* These microsatellites will be useful in evaluating the population genetic composition of *P. maritima* and in developing approaches for further conservation and management of this species within the endangered coastal dune ecosystem of the northeastern United States.

Key words: coastal dune ecosystem; conservation genetics; microsatellites; plum; population genetics; *Prunus maritima*; Rosaceae.

The endangered coastal dune ecosystem of the northeastern United States consists of extreme abiotic conditions including frequent exposure to high levels of salinity, wind, erosion, and broad temperature fluctuations (McLachlan, 1991). This unique ecosystem provides niches for highly specialized organisms such as *Prunus maritima* Marshall (beach plum; Rosaceae), which have adapted to thrive in this harsh environment. Throughout the past century, human-mediated habitat destruction and fragmentation of coastal lands has resulted in a significant decline of highly endemic species, such as the beach plum (Feagin et al., 2005). Today, *P. maritima* is listed as endangered in three states within its limited geographic range including Maine, Maryland, and Pennsylvania (USDA, NRCS, 2015). The beach plum is a long-lived shrub 3–4 m tall, typically possessing lanceolate leaves, although the shrub varies greatly in habit, fruit color, and size. Reproduction occurs in mid-May, at which time white, five-petaled, generalist-pollinated flowers are produced. The subsequent fruits develop over the summer months, ripening in late August and September and functioning as an important food resource for migrating bird species (Uva, 2003).

Previous research has revealed that *P. maritima* is a sister taxon to *P. geniculata* R. M. Harper (Shaw and Small, 2005), a federally listed species endemic to the central Florida scrublands. In light of this established evolutionary relationship, Germain-Aubrey et al. (2011) developed eight microsatellite loci in *P. geniculata* as a tool for investigating the conservation genetics of this rare lineage. These loci were further tested on samples of *P. maritima* collected from Massachusetts and Delaware to assess preliminary levels of polymorphism. All loci were polymorphic at all locations sampled, rendering these loci potentially useful for future conservation genetics research of both taxa.

The goal of this study was to generate an additional suite of microsatellite markers specifically developed for *P. maritima* and tailored to generate a robust evaluation of the genetic composition of remaining populations. To this end, 14 microsatellite markers were developed to assess levels of genetic variation and the genetic structure of populations of *P. maritima* along the northeastern coast of the United States. In future studies, we will also use these microsatellite markers to determine the relatedness of *P. maritima* and *P. maritima* var. *gravesii* (Grave's beach plum), which is now considered to be extinct in the wild (Anderson, 1980).

METHODS AND RESULTS

Leaf samples of 15 *P. maritima* plants were collected from each of the following populations in the summer and fall of 2011 and 2012: Rachel Carson National Wildlife Refuge, Biddeford, Maine (43.4469, –70.3741); Milford

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Point, Milford, Connecticut (41.1742, −73.1027); West Meadow Beach, Long Island, New York (40.9334, −73.1454); and Island Beach State Park, Seaside Park, New Jersey (39.8199, −74.0896). *Prunus maritima* is a clonal shrub so care was taken to avoid sampling from ramets of the same genet. Therefore, samples were collected at random throughout the population from plants that appeared to be physically separated by a minimum of 3 m from the nearest shrub, with greater distances between plants sampled from more expansive populations. A representative voucher specimen for *P. maritima* is located at the Eastern Kentucky University Herbarium (voucher sine numero; collected by Bryan A. Connolly in New London Co., Groton, Bluff Point Reservation, Connecticut, USA; 28 July 2013). Samples for DNA extraction were stored in silica gel. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA) following the standard protocol. Microsatellites were isolated following the protocol of Glenn and Schable (2005), in which DNA is cut by the *Rsa*I restriction enzyme, ligated to SuperSNX linkers, amplified by PCR, and enriched for simple sequence repeat regions using biotinylated oligonucleotides and Dynabeads (Invitrogen, Carlsbad, California, USA). The enriched DNA was then amplified using PCR, inserted into plasmids, and cloned using the TOPO TA Cloning Kit (Invitrogen). Of the hundreds of bacterial colonies obtained, 112 colonies were randomly selected and amplified by PCR utilizing the M13F and M13R primers, with 94 (83.9%) of these PCR products within the desired size range of 500–1200 bp. The PCR products of desired size were cleaned using the QIAquick PCR Purification Kit (QIAGEN) and sequenced on an ABI 3730xl capillary electrophoresis instrument (Applied Biosystems, Carlsbad, California, USA) at the Biotechnology Resource Center, Cornell University, Ithaca, New York, USA. DNA sequences were visualized with Geneious 6.1 software (Biomatters Ltd., Auckland, New Zealand; http://www.geneious.com/) to ensure quality sequences and were examined for microsatellite regions using the online Tandem Repeats Finder (Benson, 1999). Primers were designed using the program Primer3 (Rozen and Skaletsky, 1999) for 24 sequenced fragments containing appropriate microsatellite regions and were amplified using unlabeled reverse and forward primers, using gradient PCR to assess ideal primer annealing temperatures between 48°C and 60°C. Gel electrophoresis was used to survey putative primer pairs for an ideal annealing temperature that produced consistent amplification of a single nuclear fragment.

Fluorescently labeled primers (6-FAM, VIC, PET, NED; Applied Biosystems) were ordered for 18 of the forward primers that consistently amplified single-banded PCR products. Fluorescent primers were tested both alone and in 10-μL multiplex mixtures consisting of 5 μL of Multiplex PCR Master Mix (QIAGEN), 3.5 μL of water, 1 μL of 2 μM forward and reverse primers, and 0.5 μL of DNA template. PCR was performed using the optimal conditions of 10 min at 95°C; followed by 28 cycles of 30 s at 95°C, 45 s at 55°C, and 45 s at 72°C; with a final elongation step of 10 min at 72°C. PCR products were stored at 4°C. GeneScan 500 LIZ Size Standard (Applied Biosystems) was mixed with the PCR product, separated on an ABI 3730xl capillary electrophoresis instrument, then analyzed using GeneMapper version 4.0 software (Applied Biosystems). Based on the results of the initial fragment analysis, 14 primer pairs produced easily discernable, amplified products that could be accurately and consistently genotyped using electropherograms. For the remainder of genotyping analyses, these 14 microsatellites were amplified in four sets based on variation in fragment size produced by each primer pair and the results of different multiplex mixtures tested: (1) PM1, PM9, PM14, PM21; (2) PM2, PM8, PM13, PM18; (3) PM7, PM11, PM16, PM22; (4) PM3, PM20. All 14 of the primer pairs in these sets consistently amplified *P. maritima* DNA in four populations sampled from across the species range, and 12 primer pairs revealed polymorphic loci. Only PM8 and PM22 were determined to be monomorphic across the four populations and the 60 samples analyzed for this study. Primer sequence, repeat type, fragment size, ideal annealing temperature (calculated as 5°C below the lowest melting temperature of the primer pair), fluorescent label, and GenBank accession number are shown in Table 1. Ten of the 14 loci investigated amplified perfect repeat units.

Characteristics of each microsatellite region, such as the number of alleles and observed and expected heterozygosity values, were calculated for *P. maritima* samples using Genetic Data Analysis (GDA; Lewis and Zaykin, 1999) (Table 2). Polymorphic loci ranged from two to 10 total alleles, with the highest average number of alleles (4.07) found within the Island Beach State Park population. Shortly after the Island Beach State Park samples were collected, this population was nearly decimated by Hurricane Sandy (October 2012), which has likely resulted in a genetic bottleneck. Observed heterozygosity levels of individual polymorphic loci ranged from 0.07–0.93 across populations, with mean population values across all loci determined to be moderately high and remarkably similar, ranging from 0.41–0.49.

TABLE 1. Preliminary characteristics of 14 microsatellite primers developed in *Prunus maritima*.[a]

Locus	Primer sequence (5′–3′)	Repeat motif	Allele size range (bp)	Fluorescent label	GenBank accession no.
PM1	F: AAAGTGCTTTTTACAACTTTGCTT	$(CA)_5$	147–149	6-FAM	KM013816
	R: GACATGAGGGATGAATGCAG				
PM2	F: ATATAGCGGGCAAAATGGAG	$(CA)_8$	189–193	NED	KM013817
	R: TTATGTATTTTCAAACAGAAGATTGC				
PM3	F: CCAAAGGCCAGGTCTCTCTT	$(TC)_5$	236–250	VIC	KM013818
	R: ATGGCTCGCACCAAGTCTAC				
PM7	F: TTTTAGACCAGCCATGCAAA	$(GA)_{10}$	208–264	VIC	KM013819
	R: CTCTGTCCAAGTCCTCAGAGC				
PM8	F: AGAGTTTGGAGCTCGATTGC	$(GA)_4A(GA)_3$	195	PET	KM013820
	R: TTCCCTCTGGAATTGTTTTGA				
PM9	F: TGATTACGTTAACCTCTTCTTTTTCTT	$(AG)_9$	175–177	PET	KM013821
	R: TCCTTCAGCACTCACCAACA				
PM11	F: AACCTGATGCCCTTTTGATG	$(CT)_{11}$	219–248	NED	KM013822
	R: TGGGCAAGAGAAAACAAACC				
PM13	F: AATTGGTTGGAGCATTGGAG	$(GA)_8GG(GA)_{12}$	164–208	VIC	KM013823
	R: GAATGGAGGCCACACGTTAC				
PM14	F: AGGACTTGTGTGGCAGCAAT	$(CT)_{10}$	125–152	VIC	KM013824
	R: GAGGCCTAATTGGCAAAAGC				
PM16	F: GTGGTCCATCCTTCAATTTCA	$(TG)_6(AG)_{13}$	195–226	6-FAM	KM013825
	R: TGCATGAATGAACCAAATGC				
PM18	F: TGTAAATATTTGCCCACTGGAA	$(TC)_8TT(TC)_5$	168–190	6-FAM	KM013826
	R: TCGCAAATGTGGTTGAAAAC				
PM20	F: CCTTCAGCACTCACCAACATT	$(CT)_9$	228–233	6-FAM	KM013827
	R: CTGGCATGGGTTTTGAGAAT				
PM21	F: AATCTGCACATGACCACCAG	$(GT)_{10}$	176–182	NED	KM013828
	R: GGTTGTTGTTTTCAGCAGCA				
PM22	F: CAGAAGCCATTTTCCCTTTTC	$(CT)_6$	216	PET	KM013829
	R: CCCCAATCTGATTTTCCATTT				

[a]The ideal annealing temperature for all primers is 55°C.

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TABLE 2. Results of initial primer screening and genotyping in four populations (15 individuals per population) of *Prunus maritima*.

	Rachel Carson Refuge, Biddeford, ME			Milford Point, Milford, CT			West Meadow Beach, Long Island, NY			Island Beach, Seaside Park, NJ		
Locus	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e
PM1	2	0.07	0.07	2	0.40	0.33	2	0.53	0.50	2	0.53	0.40
PM2	1	0.00	0.00	1	0.00	0.00	2	0.40	0.33	3	0.13	0.13
PM3	2	0.60	0.43	3	0.47	0.66	4	0.53	0.72	6	0.53	0.80
PM7	6	0.60	0.68	7	0.93	0.81	9	0.80	0.84	8	0.67	0.69
PM8	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00
PM9	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.27	0.24
PM11	7	0.93	0.82	7	0.73	0.75	5	0.53	0.65	6	0.93	0.81
PM13	4	0.80	0.69	6	0.93	0.80	10	0.87	0.84	7	0.53	0.57
PM14	4	0.47	0.60	4	0.27	0.70	3	0.33	0.61	3	0.20	0.38
PM16	6	0.60	0.58	4	0.60	0.56	5	0.73	0.72	4	0.47	0.45
PM18	6	0.73	0.78	2	0.53	0.40	4	0.60	0.56	6	0.80	0.80
PM20	2	0.47	0.52	3	0.27	0.25	2	0.67	0.46	3	0.40	0.61
PM21	4	0.80	0.64	4	0.93	0.68	3	0.80	0.57	5	0.40	0.53
PM22	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00
Mean	3.36	0.43	0.41	3.29	0.43	0.42	3.71	0.49	0.49	4.07	0.42	0.46

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity.

CONCLUSIONS

The developed primers were found to successfully amplify 14 microsatellite loci in *P. maritima*. These microsatellites are reliably amplified in populations across the species range and are sufficiently variable for studying the population genetics of this species to evaluate the need for further conservation efforts and population management. In future studies, we will use these loci to assess the genetic composition of the closely related *P. maritima* var. *gravesii*. Using *P. maritima* as a model organism, these microsatellite loci are also helpful in providing a genetic context for interpreting the effects of habitat loss and fragmentation on flora within the coastal dune ecosystem.

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