High-Throughput Microsatellite Marker Development for the Distylous Herb Primula mistassinica (Primulaceae)

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Primer Note

High-throughput microsatellite marker development for the distylous herb *Primula mistassinica* (Primulaceae)[1]

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- *Premise of the study:* Twelve microsatellite markers were developed for *Primula mistassinica*, a distylous, diploid arctic-alpine plant. The markers will be used to investigate the landscape genetics of a disjunct population on Isle Royale, Michigan, and the phylogeographic patterns of the species.
- *Methods and Results:* We used Roche/454 high-throughput technology to sequence microsatellite-enriched regions in the *P. mistassinica* genome. We developed 12 polymorphic microsatellite primer sets. These loci contained di-, tri-, and tetranucleotide repeats with two to nine alleles per locus when assessed in 23 individuals.
- *Conclusions:* Understanding the historical movements of *P. mistassinica* will provide insight to the survival prospects of current Arctic plant populations, which face the pressures of global, anthropogenic climate change.

Key words: 454 sequencing; arctic-alpine plant; microsatellite enrichment; polymorphism; *Primula mistassinica*; Primulaceae.

Primula mistassinica Michx. (Primulaceae) is a small, distylous herb native to boreal Alaska and Canada, with scattered populations in Minnesota, Illinois, Michigan, Wisconsin, Maine, Vermont, and New York. It is listed as endangered in Illinois, threatened in New York and Vermont, and of special concern in Maine (USDA, NRCS, 2012). Primarily an arctic plant, *P. mistassinica* tends to grow in cool moist places (Voss and Reznicek, 2012) and thus may be impacted by changes in temperature and moisture. In general, climate change is predicted to have major impacts on biodiversity, forcing species to adapt or migrate if they are to persist (Dawson et al., 2011; Bellard et al., 2012). Understanding the population and landscape genetics of *P. mistassinica* will help determine the conservation priorities for this species in the face of climate change, and will be beneficial, more generally, for understanding how plants respond to a changing climate.

We developed 12 polymorphic loci that will be used to investigate the landscape genetics of the disjunct population on Isle Royale, Michigan, and to study the phylogeographic history of the species. *Primula mistassinica* is distylous and self-incompatible, making it an obligate out-crosser (Richards, 2003). Thus, the polymorphic microsatellite markers described here will also provide a window onto the effects of these life history characteristics on the plant's genetic diversity and genetic structure.

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METHODS AND RESULTS

Leaf samples of *P. mistassinica* were collected from 24 individuals from seven islands at the northeastern end of the Isle Royale archipelago (Isle Royale National Park, Keeweenaw Co., Michigan, USA) (South Government Island: 48.16988°N, 88.42137°W; North Government Island: 48.17832°N, 88.42283°W; Split Island: 48.17597°N, 88.42867°W; Edwards Island: 48.17047°N, 88.43870°W; Third Island: 48.18235°N, 88.42545°W; Long Island: 48.17653°N, 88.43627°W; and Main Island: 48.18632°N, 88.42692°W), where it grows on the basaltic rocky shoreline. A voucher specimen was deposited at the University of Michigan herbarium (MICH). The specimen is labeled "*Primula mistassinica*, Michigan, Keweenaw Co., Isle Royale National Park, Split Island, Edwards, 31 Jul 2012" (MICH-1474909).

Silica-dried leaves were ground in a TissueLyser II (QIAGEN, Valencia, California, USA), and total genomic DNA was extracted using the DNeasy Plant Minikit (QIAGEN). Genomic DNA (300 ng) from one individual was sent to the Evolutionary Genetics Core Facility at Cornell University (Ithaca, New York, USA) for Roche/454 sequencing. At the Evolutionary Genetics Core Facility, sequencing techniques included library construction that followed a modified protocol based on Hamilton et al. (1999). The DNA was enriched for microsatellite regions by hybridization to biotinylated oligonucleotide repeat probes (GT, TC, TTC, GTA, GTG, TCC, GTT, TTTC, GATA, TTAC, GATG, and TTTG), and the resulting libraries were submitted for Titanium 454 sequencing. The 454 sequencing returned 116,442 total reads with an average length of 323 bp. These reads were assembled with SeqMan Pro (Lasergene version 8.1.1; DNASTAR Inc., Madison, Wisconsin, USA) into 52,938 contigs of microsatellite-enriched regions (average coverage: 2.2 sequences), of which 7503 had sufficiently large flanking regions for primer design. We designed primers for 42 contigs with the online software Primer3 version 4.0 (Rozen and Skaletsky, 2000). These contigs were selected from the 7503 candidate contigs, ensuring that they had at least two times coverage, and displayed a range of microsatellite repeat types (19 di-, 17 tri-, four tetra-, and two hexanucleotide repeats). For each primer pair, a long tag (5′-CGAGTTTTCCCAGTCACGAC-3′) was added to the 5′ end of the shorter primer (Schuelke, 2000) and a short tag (5′-GTTTCTT-3′) was added to the longer primer (Brownstein et al., 1996; Schuelke, 2000). The long tag is necessary for genotyping with a three-primer PCR protocol, as its product anneals with a third, fluorescently marked primer (6-FAM; Integrated DNA Technologies, Coralville, Iowa, USA) of identical

TABLE 1. Characteristics of 12 microsatellite loci developed in *Primula mistassinica.*

Locus	Primer sequences (5′–3′)	Fragment size range (bp)	Repeat motif	T_a (°C)	GenBank accession no.
2145	F: ATCATTGGCCACCAGTTCA	175–179	$(AC)_8$	55	JX982246
	R: GGAGATGACTACTTGCCTTTCA				
6027	F: CACAAAGTCTCTCTCTGCAGATTC	173–193	$(AG)_{11}$	55	JX982247
	R: CAGTTTtGACAGGGAAGCAA				
7978	F: CAAGTCGCCAAATTTTAAGCA	191–205	$(AG)_9$	55	JX982251
	R: GGCGTCTAAGCCACACATCT				
8198	F: TTTCCTTTTTGGCCAGTTATTT	184–196	$(AAC)_6$	55	JX982242
	R: GAGAACGTTGGACAAATTGGTAT				
8586	F: TCGTGTTAGTGGATCTTTTTCAA	198–207	$(AAC)_8$	55	JX982248
	R: ACCGAATCACAGATCCCAAA				
11180	F: TGAATGGCATGTATTGCTCA	244–259	$(CAA)_7$	55	JX982252
	R: CGGTTCTTTCCTTTGGCATA				
11295	F: CCACTGGAATTCGGAAGAAG	273–279	$(AAC)_6$	55	JX982241
	R: GCACAAAGGCCCTCCTATTT				
12001	F: TGTGCGCAACTTGAGATTTT	202–210	$(GA)_{10}$	55	JX982244
	R: TGCTGCTTGTTTCGACTCTG				
13912	F: TTTATTATCCACCTCTAGGGCTTA	172–174	$(AG)_{12}$	55	JX982249
	R: TCAAGTTGATCCCACGAAAT				
20936	F: ATCAACACCTTGGCTGGAAT	187–199	$(ATAG)_6$	55	JX982250
	R: ATAGTGGCCCCTTCCAATTT				
28564	F: CCCGATTTACAGAATCGTAGAAG	184–192	$(AAAG)_7$	55	JX982245
	R: TCCAATTTGATTGAAACCCATA				
42126	F: ACAACGGGGATGGAGTTG	168–183	$(AGAT)_6$	55	JX982243
	R: CAACAAACTACATTTTGGTAATCG				

Note: T_a = annealing temperature.

sequence (Schuelke, 2000). The short tag prevents excessive stutter (Brownstein et al., 1996). Before genotyping, each primer pair was tested on two individuals. PCRs were performed in 10-μL volume containing 1× buffer, 200 μM dNTPs, 0.2 μM of each primer, 1 μL template, and 0.05 μL HotMaster *Taq* polymerase (5 Prime Inc., Gaithersburg, Maryland, USA) under the following conditions: 94°C for 2 min, then 35 cycles of 94°C for 20 s, 55°C for 30 s, and 65°C for 30 s. Primer pairs that successfully amplified a microsatellite region, as determined by the presence of one or two distinct bands on a 1% agarose gel stained with ethidium bromide, were then used for three-primer PCR. These reactions were also carried out in 10-μL volume containing 1× buffer, 200 μM dNTPs, 0.15 μM fluorescent primer, 0.05 μM long-tail-tagged (forward) primer, 0.2 μM reverse primer, 1 μL template, and 0.05 μL HotMaster *Taq* polymerase (5 Prime Inc.). The same PCR conditions as before were used, except that the annealing time at 55°C was increased to 1 to 2 min for robust amplification (due to low concentration of forward primer). Products with a strong band on ethidium bromide–stained agarose gels were diluted five times, whereas PCR products with a weak band were not diluted. A total of 1.5 μL PCR product was added to 18 μL Hi-Di Formamide (Applied Biosystems, Foster City, California, USA) and 0.15 μL GeneScan 500 LIZ Size Standard (Applied Biosystems) and sent to Cornell Life Sciences Core Laboratory Center for fragment analysis on an Applied Biosystems 3730xl DNA Analyzer. Electropherograms were visualized using GeneMapper version 4.0 (Applied Biosystems). The Excel Microsatellite Toolkit (Park, 2008) was used for initial calculations of allele frequencies and heterozygosity. Further analyses (estimates of Hardy–Weinberg equilibrium [HWE] and linkage disequilibrium) were performed with GENEPOP version 4.0.10 (Raymond and Rousset, 1995) and FSTAT (Goudet, 1995).

Out of the 42 primer pairs tested, 37 successfully amplified a unique DNA fragment and were used in three-primer PCR genotyping. Of these 37, only 12 were polymorphic (Table 1). Among the 23 individuals tested, the number of alleles per locus in the population varied from two to nine with a mean of 4.0 (±2.13). We were unable to amplify locus 11295 in two individuals and locus 42126 in five individuals, suggesting the existence of null alleles at these loci.

In the Isle Royale population, expected heterozygosity (H_e) ranged from 0.125 to 0.817, and observed heterozygosity (H_o) ranged from 0.130 to 0.783 (Table 2). Only locus 42126 deviated significantly from HWE ($P = 0.003 < 0.05$). Microsatellite scoring for all loci was run through MICRO-CHECKER version 2.2.0.3 (van Oosterhout et al., 2004), which determined that there was evidence for null alleles in locus 42126, but not in any of the other loci, explaining why locus 42126 deviated from HWE. Two of the loci, 7978 and 8198, were found to be in gametic disequilibrium ($P = 0.002$). The average expected heterozygosity for the population was $H_e = 0.548 \pm 0.057$, implying relatively high genetic diversity within the population, consistent with the out-crossing, self-incompatible mating system of this plant.

CONCLUSIONS

The microsatellite markers we described here are the first for *P. mistassinica.* They display high polymorphism and will be useful for calculating population genetics parameters such as genetic diversity, inbreeding, and population differentiation. *Primula mistassinica* is an arctic-alpine species with disjunct populations located in southern, warmer climatic zones (i.e., Isle Royale). These microsatellite markers will help to determine the species' phylogeographic history, shedding light on

TABLE 2. Results of initial primer screening for *Primula mistassinica* among 23 individuals sampled.

Locus	*A*	H_o	H_e	HWE[a]
2145	3	0.391	0.569	0.059
6027	9	0.783	0.817	0.580 (±0.02)
7978	7	0.696	0.733	0.295 (±0.016)
8198	3	0.435	0.608	0.153
8586	5	0.478	0.670	0.025 (±0.004)
11180	4	0.478	0.561	0.427
11295	2	0.286	0.251	1.000
12001	3	0.364	0.458	0.531
13912	2	0.304	0.507	0.090
20936	4	0.522	0.708	0.185
28564	2	0.130	0.125	1.000
42126	4	0.222	0.567	0.003
All loci	4.0 (±2.13)	0.424 (±0.03)	0.548 (±0.057)	

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium.

[a] *P* value for deviation from HWE (calculated in GENEPOP [Raymond and Rousset, 1995]).

the future performance of these plants in the face of anthropogenic climate change.

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