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MICROSATELLITE MARKERS IN THE WESTERN PRAIRIE FRINGED ORCHID, *PLATANHERA PRAECLARA* (ORCHIDACEAE)¹

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- *Premise of the study:* Primers for 31 microsatellite-containing loci were developed for the threatened orchid *Platanthera praeclara* to enable characterization of the population genetics of this tallgrass prairie native.
- *Methods and Results:* Sixteen polymorphic microsatellite loci were identified from four populations. Six of these loci were not in linkage disequilibrium. The average number of alleles per locus per population ranged from 6.4 to 8.9.
- *Conclusions:* The results indicate that six of the polymorphic loci will be useful in future studies of population structure, gene flow, and genetic diversity.

Key words: microsatellites; Orchidaceae; orchids; PCR primers; *Platanthera*; simple sequence repeat markers (SSRs).

The western prairie fringed orchid (*Platanthera praeclara* Sheviak & M. L. Bowles) is one of 37 North American orchid species in the *Platanthera* Rich. genus. It is also one of the rarest, earning it federally threatened status. The historical range of *P. praeclara* is throughout the upper Great Plains in close association with tallgrass prairie. Large-scale conversion of the original tallgrass prairie to agriculture is believed to be the leading cause of local extirpation and widespread reductions in the numbers of *P. praeclara* (Sheviak and Bowles, 1986).

This particular species of *Platanthera* is diploid and self-compatible with a flower morphology typical of plants adapted to hawk moth pollination (Sheviak and Bowles, 1986). Here we present the results of the development of microsatellite markers for *P. praeclara* in the hopes of providing a tool for further study of this species and genus.

METHODS AND RESULTS

To detect genomic regions with microsatellites in *P. praeclara*, leaf material was collected from eight individuals from a population in the Sheyenne grasslands of eastern North Dakota (Ransom County) and stored at -20°C. The leaf material was combined and ground in liquid nitrogen, and DNA was extracted following the manufacturer's protocol using a PowerPlant DNA

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Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA). DNA was then serially enriched twice for microsatellites using three probe mixes (mix 2 = (AG)₁₂, (TG)₁₂, (AAC)₆, (AAG)₈, (AAT)₁₂, (ACT)₁₂, (ATC)₈; mix 3 = (AAAC)₆, (AAAG)₆, (AATC)₆, (AATG)₆, (ACAG)₆, (ACCT)₆, (ACTC)₆, (ACTG)₆; mix 4 = (AAAT)₈, (AACT)₈, (AAGT)₈, (ACAT)₈, (AGAT)₈) following Glenn and Schable (2005). Briefly, DNA was digested with restriction enzymes *RsaI* (New England Biolabs, Ipswich, Massachusetts, USA) and then ligated to double-stranded SuperSNX linkers (SuperSNX24 Forward 5'-GTTTAAGGC-CTAGCTAGCAGCAGAATC-3' and SuperSNX24 Reverse 5'-GATCTGCT-AGCTAGGCCTTAAACAAAA-3'). Linker-ligated DNA was denatured and hybridized to biotinylated microsatellite oligonucleotide mixes, which were then captured on magnetic streptavidin beads (Life Technologies, Grand Island, New York, USA). Unhybridized DNA was washed away, and the remaining DNA was eluted from the beads, amplified in PCR using the forward SuperSNX24 as a primer, and cloned with TOPO-TA Cloning Kits (Invitrogen, Grand Island, New York, USA). Inserts were PCR amplified and sequenced with M13 forward and reverse primers using the BigDye Terminator version 3.1 (Applied Biosystems, Grand Island, New York, USA) and ABI 3130xl capillary sequencer. Sequences from both strands were assembled and edited in Sequencher 4.6 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Microsatellites were identified using MSATCOMMANDER version 0.8.1 (Faircloth, 2008) and primers designed with Primer3 (Invitrogen). One primer from each pair was modified on the 5' end with an engineered sequence (CAG tag 5'-CAGTC-GGGCGTCATCA-3') to enable use of a third primer in the PCR (identical to the CAG tag) that was fluorescently labeled for detection.

To determine which primer sets would produce consistent amplification products and the degree of polymorphism for each set, leaf material was collected from a total of 115 plants in four populations. Two of the populations (A-annex and Viking) were located in Ransom County, North Dakota, and were approximately 25 km apart. The other two populations (Bluestem and Ulen) were located approximately 55 km away in Clay County, Minnesota, and were separated by 30 km. The GPS coordinates of the four study populations are withheld due to the threatened status of this species. Leaves were stored on ice in the field and at -20°C upon return to the laboratory. Leaves were ground in liquid nitrogen and genomic DNA was extracted using a DNeasy Miniprep Kit following the manufacturer's protocol (QIAGEN, Valencia, California, USA).

Microsatellites were amplified via PCR. Each 20-μL reaction contained 8.8 μL double-distilled H₂O, 4 μL of 5× GoTaq Flexi Buffer (Promega Corporation, Madison, Wisconsin, USA), 1.2 μL of 25 mM MgCl₂, 0.8 μL of dNTPs (5 mM each), 1 μL of 0.5 μM CAG tag-modified primer, 1 μL of 5 μM non-CAG tag-modified primer, 1 μL of 5 μM fluorescent tag (Integrated DNA Technologies,

TABLE 1. Primer sequences of 31 polymorphic microsatellite loci developed for *Platanthera praeclara*.

| Locus | Primer sequences (5'–3') | Repeat motif | Size (bp) | Probe PUID ^a |
|-------|--|--------------|-----------|-------------------------|
| PP02 | F: ATGAGGGTCTTCACGCATGT R: CCACGGGATCTCCTTCCAAT | CT | 177–199 | 12324941 |
| PP03 | F: TGGAGATCAACCACGCGATA R: ACTTCAGGTAAGCAGGCTTTG | AAAC | 189 | 12324946 |
| PP05 | F: TACCCGAGTTCCTTGCTGAC R: CCTCTCGACAACAACAGT | CT | 202–214 | 12324949 |
| PP07 | F: ACCCTCGTAGATCGTTTCGG R: GTGGATTTTCGTGTGCCTT | AG | 239–245 | 12324950 |
| PP09 | F: CCATCTCTCCGTGGATACG R: GGACATGCACATAATCGGCAC | GTTT | 253–263 | 12324951 |
| PP12 | F: GGTGCGGTCACATAACTTTGA R: GGCGCAACCCACATTGATT | AC | 256–328 | 12324936 |
| PP13 | F: TTGTGGCGCTCGATCATCTT R: TTTCCCTCACCGCCTCTTT | GT | 248–318 | 12324937 |
| PP15 | F: TCCGGGTTTCCTTTGACGTA R: AGGTGCTTCAACGATCCAAAC | GTTT | 293–298 | 12324938 |
| PP17 | F: GCATGTCTCAAGCTCTCACG R: TCGCTCTCATTTCCACCG | GTTT | 311–323 | 12324939 |
| PP19 | F: GCTTCACTGACATTTCTTGGGT R: TTCAGCAATCATTCCGCACA | GTTT | 335–339 | 12324940 |
| PP20 | F: CGATCCGCGAGAGTGTAGAA R: GTCGCCTGTGAGTTTGAGA | AG | 386 | 12324942 |
| PP23 | F: GAAGTGTCCGCAGCTCTTTC R: CATCACGGTTGCGAGGTATC | AG | 368–392 | 12324943 |
| PP27 | F: CAATGGTTGTGCTCTGAATGAC R: CCGGTTCCAACAAGTGC | GT | 435–451 | 12324944 |
| PP29 | F: TTTTCATCAGCGCCAAGAAT R: TCCTACATTGGCCGCTACTC | AGAT | 465 | 12324945 |
| PP30 | F: CTGAGCAAAGAGGCGTAGA R: TGCCACATTCTGAGCTACC | GT | 475–507 | 12324947 |
| PP31 | F: AGAAGGCACGGTACTCAA R: TCTCCATTCTCTGAATCCTTGTTG | AC | 503–517 | 12324948 |
| PP01 | F: TTTATTTCTTCTCTGGGCCG R: TATACCTTCAGCACACACTC | GT | 167 | |
| PP04 | F: GGGCACAATGGAGACAAAGG R: GAGAGGGCAGACCCAAGAAT | GTT | 190 | |
| PP06 | F: GGTCTGTTTCCAACCTCCC R: GACCACCAACCTACATGCTG | GTTT | 202 | |
| PP08 | F: GGTGGGAATCAGGTGGCTAA R: TCTTCGCGGATCTCTCCTTG | CT | 237 | |
| PP10 | F: TGCTGGAGTCAAGTCTCAT R: GACCAAGGAGGAGGGTTTCAG | AC | 248 | |
| PP11 | F: CCATCTCTCCGTGGATACG R: TGGGCATGCACATAATCAACAC | GTTT | 249 | |
| PP14 | F: GAGTGCCAAAGTCCATCGTG R: AGCCTCGTATGGTTCCATCT | ACAG | 275 | |
| PP16 | F: TCGAGGTGCTTCAACGATCC R: TCCGGGTTTCCTTTGACGTA | AAAC | 283 | |
| PP18 | F: GATCGGGACGTGAAACCTCT R: CGAAAGTCATATGTGGCATCCT | GT | 311 | |
| PP21 | F: ATCAATGCCTCATCAGACCA R: GATTTCTCCAACCCGCACT | AG | 352 | |
| PP22 | F: CTGTGGGAGATTCGCGAGAG R: CAGAAGGGCTCAGATGACCG | CTT | 358 | |
| PP24 | F: TGGGTCTTCCGTGGGTTT R: AGGCACCTGTTAGACGTTGT | AG | 366 | |
| PP25 | F: GATCTTGCAACCCTTCAGGC R: GTCGCCTGTGAGTTTGAGA | AG | 378 | |
| PP26 | F: CCAACACAAGAGGGAGGTTTC R: TAACACTGGCGTTAGCCG | AG | 388 | |
| PP28 | F: GCATGGCAAGTATGTTGTGC R: TGCCAGCACTTTAACACAC | GT | 442 | |

^aProbe PUID = probe identification number. Probe Database accessions for the first 16 loci (successful amplification) are available at <http://www.ncbi.nlm.nih.gov/sites/entrez?db=probe>.

Coralville, Iowa, USA), 0.2 µL of GoTaq polymerase (Promega Corporation), and 2 µL of template DNA. The touchdown (Don et al., 1991) PCR profile consisted of an initial denaturation at 94°C for 2 min, followed by 16 cycles of

denaturation at 94°C for 30 s, annealing starting at 65°C for 30 s and decreasing 0.5°C each cycle, and elongation at 72°C for 30 s. This was followed by 20 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, elongation

TABLE 2. Microsatellite marker characteristics in four populations of *Platanthera praeclara*.

| Locus | Bluestem (N = 25) | | | Ulen (N = 30) | | | A-annex (N = 30) | | | Viking (N = 30) | | |
|---------|-------------------|----------------|----------------|---------------|----------------|----------------|------------------|----------------|----------------|-----------------|----------------|----------------|
| | A | H _o | H _e | A | H _o | H _e | A | H _o | H _e | A | H _o | H _e |
| PP02 | 8 | 0.680 | 0.759 | 8 | 0.567 | 0.771 | 9 | 0.700 | 0.812 | 9 | 0.786 | 0.824 |
| PP05 | 5 | 0.696 | 0.718 | 5 | 0.586 | 0.674 | 6 | 0.759 | 0.735 | 4 | 0.690 | 0.652 |
| PP07 | 3 | 0.280 | 0.463 | 3 | 0.300 | 0.645 | 3 | 0.433 | 0.562 | 4 | 0.433 | 0.717 |
| PP12 | 10 | 0.480 | 0.824 | 19 | 0.900 | 0.923 | 23 | 0.900 | 0.932 | 16 | 0.750 | 0.871 |
| PP17 | 2 | 0.292 | 0.353 | 1 | 0.000 | 0.000 | 2 | 0.100 | 0.095 | 1 | 0.000 | 0.000 |
| PP27 | 6 | 0.320 | 0.407 | 6 | 0.630 | 0.779 | 6 | 0.500 | 0.663 | 8 | 0.600 | 0.764 |
| PP13 | 10 | 0.560 | 0.714 | 12 | 0.500 | 0.858 | 10 | 0.700 | 0.583 | 11 | 0.286 | 0.825 |
| PP23 | 6 | 0.800 | 0.778 | 11 | 0.862 | 0.871 | 9 | 0.759 | 0.857 | 11 | 0.933 | 0.842 |
| PP30 | 10 | 0.840 | 0.800 | 12 | 0.690 | 0.855 | 14 | 0.967 | 0.888 | 11 | 1.000 | 0.837 |
| PP31 | 4 | 0.714 | 0.715 | 5 | 0.778 | 0.741 | 7 | 0.821 | 0.795 | 7 | 0.704 | 0.763 |
| Average | 6.4 | 0.566 | 0.653 | 8.2 | 0.583 | 0.712 | 8.9 | 0.664 | 0.692 | 8.2 | 0.618 | 0.710 |

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

at 72°C for 30 s, and a final elongation for 5 min. PCR products were visualized on 2% agarose gels stained in ethidium bromide to verify a successful amplification. PCR products were analyzed for fragment length using an Applied Biosystems 3730 analyzer at the Plant Microbe Genomics Facility at Ohio State University with the GS600LIZ size standard.

The data were exported in electropherogram form using Peak Scanner version 1.0 software (Applied Biosystems) and visually scored to determine the alleles in each amplified sample. Thirty-one loci were tested as primer pairs in PCR to determine if they produced amplified product (Table 1). Sixteen loci were amplified successfully, 10 of which were polymorphic for *P. praeclara* (PP02, PP05, PP07, PP12, PP13, PP17, PP23, PP27, PP30, PP31). Sequences for all 16 loci have been archived (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=probe>). Significant linkage disequilibrium was found using GENEPOP version 4.0.10 (Raymond and Rousset, 1995; Rousset, 2008) for four of the polymorphic loci, resulting in six loci suitable for a population genetic study of *P. praeclara* (PP02, PP05, PP07, PP12, PP17, PP27).

The individuals sampled were from two populations in western Minnesota (Ulen and Bluestem) and two populations in eastern North Dakota (A-annex and Viking). These samples were characterized with the six polymorphic loci that were not in linkage disequilibrium. Observed and expected heterozygosity (H_o and H_e, respectively) were calculated using GenAlEx version 6 (Peakall and Smouse, 2006). H_o at each locus per population ranged from 0 to 1 (Table 2). An AMOVA was calculated with GenAlEx version 6, which classified 90% of the genetic variation within populations. The program FSTAT 2.9.3.2 (Goudet, 1995) was used to calculate the overall genetic diversity among populations (F_{ST}), which was low (F_{ST} = 0.069), suggesting the presence of gene flow among populations.

CONCLUSIONS

Of the 31 loci screened, six microsatellites were polymorphic and not in linkage disequilibrium. Overall, there was considerable

polymorphism at these loci, averaging between six and nine alleles per locus. These populations represent a small portion of the range of *P. praeclara* and a single year of sampling but still demonstrate considerable variation. The number and variability of these microsatellite loci suggest that they are useful molecular markers for conservation studies of this relatively rare orchid.

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