Microsatellite Markers in the Western Prairie Fringed Orchid, Platanthera praeclara (Orchidaceae)

Authors: Ross, Andrew A., Aldrich-Wolfe, Laura, Lance, Stacey, Glenn, Travis, and Travers, Steven E.

Source: Applications in Plant Sciences, 1(4)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1200413

Applications in Plant Sciences 2013 1(4): 1200413

PRIMER NOTE

MICROSATELLITE MARKERS IN THE WESTERN PRAIRIE FRINGED ORCHID, *PLATANTHERA PRAECLARA* (ORCHIDACEAE)[1]

ANDREW A. ROSS[2], LAURA ALDRICH-WOLFE[3], STACEY LANCE[4], TRAVIS GLENN[4], AND STEVEN E. TRAVERS[2,5]

[2]Department of Biological Sciences, NDSU Dept. 2715, North Dakota State University, Fargo, North Dakota 58108 USA; [3]Biology Department, Concordia College, Moorhead, Minnesota 56562 USA; and [4]Environmental Health Science, University of Georgia, Athens, Georgia 30602 USA

- *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 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 *Rsa*I (New England Biolabs, Ipswich, Massachusetts, USA) and then ligated to double-stranded SuperSNX linkers (SuperSNX24 Forward 5′-GTTTAAGGCCTAGCTAGCAGCAGAATC-3′ and SuperSNX24 Reverse 5′-GATTCTGCTAGCTAGGCCTTAAACAAAA-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′-CAGTCGGGCGTCATCA-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 H_2O, 4 μL of 5× GoTaq Flexi Buffer (Promega Corporation, Madison, Wisconsin, USA), 1.2 μL of 25 mM $MgCl_2$, 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,

[1]Manuscript received 6 August 2012; revision accepted 21 September 2012.

The authors thank Marion Harris at North Dakota State University (NDSU), Kenneth L. Jones at University of Colorado Denver, the U.S. Fish and Wildlife Service, and The Nature Conservancy for assistance with this project. Funding was provided by the San Diego County Orchid Society and National Science Foundation Experimental Program to Stimulate Competitive Research (EPSCoR) grant EPS0814442 to NDSU.

[5]Author for correspondence: Steven.Travers@ndsu.edu

doi:10.3732/apps.1200413

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	CT	177–199	12324941
	R: CCACGGGATCTCCTTCCAAT			
PP03	F: TGGAGATCAACCACGCGATA	AAAC	189	12324946
	R: ACTTCAGGTAAGCAGGCTTTG			
PP05	F: TACCCGAGTTCCTTGCTGAC	CT	202–214	12324949
	R: CCTCTCGACAACAACCAGT			
PP07	F: ACCCTCGTAGATCGTTTCGG	AG	239–245	12324950
	R: GTGGATTTCGTGTGCCTT			
PP09	F: CCATCTCTCCGTGGATACG	GTTT	253–263	12324951
	R: GGACATGCACTAATCGGCAC			
PP12	F: GGTGCGGTCACTAACTTTGA	AC	256–328	12324936
	R: GGCGCAACCCACATTGATT			
PP13	F: TTGTGGCGCTCGATCATCTT	GT	248–318	12324937
	R: TTTCCCTCACCGCCTCTTT			
PP15	F: TCCGGGTTTCCTTTGACGTA	GTTT	293–298	12324938
	R: AGGTGCTTCAACGATCCAAAC			
PP17	F: GCATGTCTCAAGCTCTCACG	GTTT	311–323	12324939
	R: TCGCTCTCATTTCCACCG			
PP19	F: GCTTCACTGACATTTCTTGGGT	GTTT	335–339	12324940
	R: TTCAGCAATCATTCCGCACA			
PP20	F: CGATCCGCGAGAGTGTAGAA	AG	386	12324942
	R: GTCGCCTGTGAGTTTGGAGA			
PP23	F: GAAGTGTCCGCAGCTCTTTC	AG	368–392	12324943
	R: CATCACGGTTGCGAGGTATC			
PP27	F: CAATGGTTGTGCTCTGAATGAC	GT	435–451	12324944
	R: CCGGTTCCAACAAAGTGC			
PP29	F: TTTCATCAGCGCCAAGAACT	AGAT	465	12324945
	R: TCCTACATTGGCCGCTACTC			
PP30	F: CTGAGCAAAGAGGCGTAGA	GT	475–507	12324947
	R: TGCCACATTCCTGAGCTACC			
PP31	F: AGAAGGCACGGTTACTCAAA	AC	503–517	12324948
	R: TCTCCATTCCTGAATCCTTGTG			
PP01	F: TTTATTTCTTCTCTGGGCCG	GT	167	
	R: TATACCTTCAGCACACACTC			
PP04	F: GGGCACAATGGAGACAAAGG	GTT	190	
	R: GAGAGGGCAGACCCAAGAAT			
PP06	F: GGTCTGTTTCCAACCTCCC	GTTT	202	
	R: GACCACCAACCTACATGCTG			
PP08	F: GGTGGGAATCAGGTGGCTAA	CT	237	
	R: TCTTCGCGGATCTCTCCTTG			
PP10	F: TGCCTGGAGTCAAGTCTCAT	AC	248	
	R: GACCAAGGAGGAGGGTTCAG			
PP11	F: CCATCTCTCCGTGGATACG	GTTT	249	
	R: TGGGCATGCACTAATCAACAC			
PP14	F: GAGTGCCAAAGTCCATCGTG	ACAG	275	
	R: AGCCTCGTATGGTTCCATCT			
PP16	F: TCGAGGTGCTTCAACGATCC	AAAC	283	
	R: TCCGGGTTTCCTTTGACGTA			
PP18	F: GATCGGGACGTGAAACCTCT	GT	311	
	R: CGAAAGTCATATGTGGCATCCT			
PP21	F: ATCAATGCCTCATCAGACCA	AG	352	
	R: GATTTCTCCAAACCCGCACT			
PP22	F: CTGTGGGAGATTCGCGAGAG	CTT	358	
	R: CAGAAGGGCTCAGATGACCG			
PP24	F: TGGGTTCTTCCGTGGGTTT	AG	366	
	R: AGGCACCTGTTAGACGTTGT			
PP25	F: GATCTTGCAACCCTTCAGGC	AG	378	
	R: GTCGCCTGTGAGTTTGGAGA			
PP26	F: CCAACACAAGAGGGAGGTTC	AG	388	
	R: TAACACTGGCGTTAGCCG			
PP28	F: GCATGGCAAGTATGTTGTGC	GT	442	
	R: TGCCCAGCACTTTAACACAC			

[a]Probe 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*.

	Bluestem (N = 25)			Ulen (N = 30)			A-annex (N = 30)			Viking (N = 30)		
Locus	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.

LITERATURE CITED

DON, R., P. COX, B. WAINWRIGHT, K. BAKER, AND J. MATTICK. 1991. 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research* 19: 4008.

FAIRCLOTH, B. 2008. MSATCOMMANDER: Detection of microsatellite repeat arrays and automated, locus-specific primer design. *Molecular Ecology* 8: 92–94.

GLENN, T., AND N. SCHABLE. 2005. Isolating microsatellite DNA loci. *Methods in Enzymology* 395: 202–222.

GOUDET, J. 1995. FSTAT version 1.2: A computer program to calculate F-statistics. *Journal of Heredity* 86: 485–486.

PEAKALL, R., AND P. SMOUSE. 2006. GenAlEx 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288–295.

RAYMOND, M., AND F. ROUSSET. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248–249.

ROUSSET, F. 2008. GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

SHEVIAK, C., AND M. BOWLES. 1986. The prairie fringed orchids: A pollinator-isolated species pair. *Rhodora* 88: 267–290.