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PERMANENT GENETIC RESOURCES

Isolation and characterization of nine microsatellite loci in the Pale Pitcher Plant *Sarracenia alata* (Sarraceniaceae).

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Abstract

We isolated and evaluated nine microsatellite loci, for the first time in the family Sarraceniaceae, from the Pale Pitcher Plant, *Sarracenia alata*. Loci exhibit between two and nineteen alleles and mostly significant F_{IS} values (-0.18 to1.0) from populations collected across the state of Louisiana. Additionally, the primers reported here cross-amplify in four other closely related species of *Sarracenia*. These markers will be used to explore patterns of population divergence in this ecologically well-known, quickly evolving genus.

The genus *Sarracenia* comprises eleven species primarily restricted to the Gulf Coast States of North America, with one widespread species distributed through the mid-Atlantic region of the United States. This carnivorous genus has been a model system for addressing myriads of ecological questions for more than a century, however, to our knowledge no microsatellite markers have been developed in this family and no genetic studies focusing on *Sarracenia alata* Wood have been previously published. In this paper we report the development of nine polymorphic microsatellite markers in *S. alata*.

Microsatellite markers were developed using the protocol of Glenn and Schable (2005). High molecular weight DNA was extracted from dried leaf tissue of one individual of *Sarracenia alata* using a modified protocol of Sanchez-Hernandez, Gaytan-Oyarzun (2006), which utilizes both traditional CTAB methods and the DNeasy plant extraction kit (Quiagen, Valencia, CA). Genomic DNA was digested with *Rsa*I or *Bsu* (Table 1) and *Xmn*I. SuperSNX24 linkers (Glenn & Schable 2005) were ligated to fragments of genomic DNA. To ensure that this ligation was successful a PCR was performed following Glenn and Schable (2005); this PCR product was then separately hybridized to three mixes of biotinylated oligo probes (listed in Glenn & Schable 2005: Mix2 (dinucleotide)/Mix3(trinucleotide)/Mix4(tetranucleotide)). This genomic DNA-biotynylated complex was added to magnetic beads coated with streptavidin (Dynabeads® M-280 Invitrogen, Carlsbad, CA) with a 2h incubation at 33°C in a rotating oven. The bead mixture was washed twice with 2xSSC, 0.1%SDS and four times with 1xSSC, 0.1%SDS, with the latter two washes conducted at 50°C. A magnetic particle concentrator captured the beads (bound with biotin and gDNA) after each wash.

Enriched fragments were denatured at 95°C for 10min to separate them from the beads and precipitated with an ethanol wash. A recovery PCR was performed following Glenn and Schable (2005). These fragments were cloned using a Quiagen PCR cloning kit (Valencia, CA) following manufacturers' protocols. Bacterial colonies that contained vectors with gDNA were used as template for PCR. These products were cleaned using ExoAP (Glenn & Schable, 2005) and sequenced using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA). Sequencing reactions were cleaned with an ethanol precipitation and were run on an ABI PRISM® 3100. Sequences were edited in Sequencher v4.6 (Gene Codes, Ann Arbor, MI) and replicate sequences were removed. To search automatically for microsatellite repeats and develop primers, we exported double stranded products to *MSATCOMMANDER* (Faircloth, 2008), which automatically adds a M13: 5'-GGAAACAGCTATGACCAT-3' and a CAG: 5'-CAGTCGGGCGTCATCA-3' tag to the 5' end of the suggested forward and reverse primers. These universal sequence tags facilitate fluorescent labeling in later PCR reactions (Schuelke, 2000). *MSATCOMMANDER* then implements *PRIMER 3* (Rozen & Skaletsky, 2000) to assess the quality of each primer and its tag. The forward and reverse tagged primer pair with the lowest penalty score was chosen for initial primer construction. Primers flaking thirty-four candidate microsatellites were developed.

We report nine primer pairs that cleanly amplified products of expected size from silica dried leaf tissue of 29 individuals of *Sarracenia alata* collected throughout the state of Louisiana (Table 1) as well as a single individual of four additional species in the genus (*Sarracenia leucophylla*, *S. flava*, *S. minor* and *S. psittacina*). PCR conditions for

these primer pairs were initially optimized on one accession of *S. alata*. Loci were amplified under the following PCR conditions in 25µl volume reactions: 1X PCR buffer, 1.5mM MgCl, 0.2mM each dNTP, 5mM BSA, 1 unit *Taq* DNA polymerase, 0.16mM appropriately fluorescently FAM labeled primer (either M13 or CAG, see Table 1 for label and direction), 0.16mM unlabeled PCR primer, 0.04mM la(Rousset, 2008)beled PRC primer (with M13 or CAG tail) and 1-10ng genomic DNA. PCR conditions were as follows: 94°C for 4 min; 35 cycles of 94°C for 30 sec, T_a °C (Table 1) for 30 sec, 72 °C for 45 sec; followed by 8 cycles of 94°C for 30 sec, 53°C for 30 sec, 72 °C for 45 sec; with a final extension at 72 °C for 10 min. PCR products and 500ROX- labeled size standard (GeneScan™Warrington, UK) were suspended in formamide before running on an ABI PRISM® 3100. Fragment analysis was conducted with GENEMAPPER version 4.0 (ABI, Foster City, CA).

Data were analyzed in GENEPOP v. 4.0.7 (Rousset, 2008). The nine loci reported here were polymorphic is *S. alata*, with the number of alleles ranging from two to nineteen and the observed heterozygosity ranging from 0 to 0.8 (Table 1). Though all individuals of *S. alata* are homozygous at locus 36, each population sampled is fixed for one allele inferring east/west population structure across the state. Deviations from HWE were detected in 8 of the 9 loci, of which two loci showed significant heterozygote deficiency ($P<0.05$ using the Hardy-Weinberg exact test) across all populations. Though plants in this genus are thought to be primarily outcrossing (Schnell, 2002; Slack, 1979) these data and two previous studies of *Sarracenia* (Godt& Hamrick, 1998; Wang *et al.*, 2004) demonstrate a pattern of heterozygote deficiency. Linkage disequilibrium was

detected for two pairwise comparisons out of all possible comparisons ($P < 0.001$) across all populations. Preliminary analyses demonstrate the ability of these novel microsatellite markers to detect genetic diversity in *S. alata*. The microsatellites also amplified loci cleanly in four additional species of *Sarracenia*. Amplification of these loci in *S. alata* and other *Sarracenia* species will prove useful in population genetic studies throughout the genus and possibly in other closely related genera.

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Table 1 Characterization of nine microsatellite loci in 29 *Sarracenia alata* individuals collected throughout Louisiana. Annealing temperature (T_a), universal fluorescent-labeled primer used (CAG or M13) and the direction (F or R) of the PCR primer that had an identical tag, the enzyme used (digest) in initial genomic DNA digestion, and size range of alleles. Number of individuals genotyped (N), number of alleles (N_A) as well as observed (H_O) and expected (H_E) heterozygosity under HWE. †Significance values for deviation from HWE calculated in GenePop are indicated adjacent to F_{IS}: *P<0.05, **P<0.01, ***P<0.001.

Cover photo caption The Pale Pitcher Plant, *Sarracenia alata*, is one of eleven species in the carnivorous genus restricted to eastern North America. In the current issue Koopman et al. present microsatellite loci for *S. alata*, these markers also amplify in four additional congeners. Photo: M. Koopman.

Locus		Primer Sequence (5'-3')	Ta	Repeat Unit	Label, direction	Digest	Size (bp)	N	N_A	H_O	H_E	F_{IS}†	Genbank Accession #
7	F	GCATCATTCGTCGATCCG	60	(CT)^12	CAG, F	Rsa	233-319	29	7	0.2413	0.589	0.614***	
	R	TGCATAGGAACAAAGCACGC											
18	F	CACGCTCTTTTGGGCAATTC	60	(GTTTT)^6	CAG, R	Bsu	202-223	29	7	0.6551	0.842	0.225**	
	R	GTGCCTTCAATCTGGGTTCG											
19	F	CTGTGAATATCGCCGACGC	54	(CT)^17	M13, R	Rsa	176-222	26	16	0.759	0.9	0.159**	
	R	GAATAGTCGCCGTTCGGTC											
21	F	TTTTGGATTGGACCCAGCG	60	(GT)^22	CAG, F	Rsa	146-230	25	13	0.793	0.817	0.030**	
	R	TCAAAGGGTAGGGCACCTG											
27	F	GTGAGTTTTGAGGAATTTCGTTTTG	60	(GT)^6	CAG, F	Rsa	163-267	20	3	0.034	0.099	0.657	
	R	GTCTGGTCTCAACCCGTTATG											
36	F	CTAGCACCTCCGGAACTCTC	60	(GTTT)^5	M13, F	Bsu	214-218	24	2	0	0.35	1.00***	
	R	GATGTCCATGACGTGTGCG											
44	F	GGGCCTAGCTATGTTGGG	54	(GT)^8	CAG, F	Rsa	181-185	26	4	0.655	0.641	-0.022*	
	R	CCGAAGGCCAAATGGAGAC											
47	F	ATCACCCACCAGAAACGGG	60	(GGAAA)^2	CAG, R	Bsu	238-252	23	7	0.621	0.728	0.149*	
	R	GCGTGGTAGGCAGGTAAATG											
5	F	GAACAAGAGCACTACATTTGC	60	(AG)^10	CAG, F	Rsa	247-255	23	4	0.727	0.682	-0.068*	
	R	TCGAGCTTCCTCCTTGTGG											

Table 1