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Isolation and characterization of microsatellite loci in the common milkweed, *Asclepias syriaca* (Apocynaceae)[1]

Susan M. Kabat[2,6], Christopher W. Dick[3,4,5], and Mark D. Hunter[3]

[2]School of Natural Resources and Environment, University of Michigan, Ann Arbor, Michigan 48109 USA; [3]Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, Michigan 48109 USA; [4]University of Michigan Herbarium, 1600 Varsity Drive, Ann Arbor, Michigan 48108-2287 USA; [5]Smithsonian Tropical Research Institute, PO Box 0843-03092, Balboa, Ancón, Republic of Panama

- *Premise of the study:* Microsatellite primers were developed for the common milkweed, *Asclepias syriaca* L., to assist in genet identification and the analysis of spatial genetic structure.
- *Methods and Results:* Using an enrichment cloning protocol, eight microsatellite loci were isolated and characterized in a Michigan population of *A. syriaca*. The primers amplified di- and trinucleotide repeats with 4–13 alleles per locus.
- *Conclusions:* The primers will be useful for studies of clonality and gene flow in natural populations.

Key words: Apocynaceae, *Asclepias syriaca*, microsatellite, milkweed.

Common milkweed, *Asclepias syriaca* L. (Apocynaceae), is one of the most common and widely distributed of approximately 100 North American milkweed species. *Asclepias syriaca* can be found throughout the Great Plains from southern Canada south to northeastern Oklahoma, northwestern Georgia, and Texas, and east from North Carolina to Maine. Its range continues to move south; in the last two decades specimens have been collected from Georgia and Louisiana (Wyatt et al., 1993; Wyatt, 1996), and it has become naturalized in the Western U.S. and invasive in parts of Europe. Although *A. syriaca* produces milky latex with toxic steroid glycosides, it hosts several specialist insect herbivores, including the monarch butterfly (*Danaus plexippus*), the milkweed beetle (*Tetraopes tetraophtalmus*), large milkweed bug (*Oncopeltus fasciatus*), small milkweed bug (*Lygaeus kalmii*), and milkweed leaf beetle (*Labidomera clivicollis*). Its broad geographic range and specialized ecological interactions makes *A. syriaca* an ideal species with which to examine geographic patterns of coevolution.

Asclepias syriaca reproduces both asexually and sexually. It is self-incompatible (Morse and Fritz, 1983), and, during sexual reproduction, pollen grains are packaged in discrete units called pollinia, which contain enough pollen to ensure full seed-set of a single flower (Ivey et al., 2003). The wind-dispersed seeds are attached to long, white flossy hairs and encased in large follicles. Asexual reproduction occurs by the elaboration of underground rhizomes. Accordingly, sexual reproduction gives rise to new genets, whereas asexual reproduction can produce multiple ramets per genet. Assigning ramets to genets is challenging under field conditions, and microsatellite markers have been used to differentiate among genets in a variety of systems [e.g., quaking aspen (*Populus tremuloides*) (Namroud et al., 2005)]. Using molecular markers to distinguish among genets of *A. syriaca* would facilitate studies of its ecology and evolutionary biology (Helms et al., 2004; Van Zandt and Agrawal, 2004). Previous microsatellite markers have been isolated from *A. syriaca* (O'Quinn and Fishbein, 2009), and here we describe additional markers that will increase resolution of clonality and genetic structure in natural populations.

METHODS AND RESULTS

DNA was extracted (DNeasy Plant Kit, Qiagen, Valencia, CA) from one *A. syriaca* ramet collected at the University of Michigan Biological Station (UMBS), Pellston, Michigan (45°33′30 N, 84°40′39 W). DNA was enriched twice for simple sequence repeats using the Oligomix 2 mixture of repeat units [$(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$] and protocol of Glenn and Schable (2005). Polymerase chain reaction (PCR) products were ligated to a plasmid vector using the TOPO TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA). Plasmid inserts were amplified and sequenced (BI Model 3730 Sequencer). Thirty-four of the 105 sequenced clones (32%) contained microsatellites. Primers were designed using the software OligoCalc (Kibbe, 2007). Polymorphism was screened in 30 *A. syriaca* genets from the University of Michigan Biological Station for 12 loci.

PCR was carried out in a volume of 10 μL containing ~30 ng of template DNA, 2 μL 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U *Taq* polymerase and 0.2 μm of each primer. Hot Start *Taq* Polymerase (Qiagen, Valencia, CA) was used for loci AS94 and ASF2, whereas GoTaq (Promega Corporation, Madison, WI) was used for all other loci. PCR for ASC5 and ASG5 included 25 μg/mL of BSA. The thermal cycle began with a 4-min denaturation step at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s at 52°C, and 60 s at 72°C, and a final extension at 72°C for 10 min. A 15-min denaturation step was used for loci AS94 and ASF2.

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[6] Author for correspondence: kabats@umich.edu

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TABLE 1. Characteristics of 8 microsatellite primers developed for *Asclepias syriaca*. Shown for each primer pair are the forward and reverse sequences, repeat type in the cloned fragment, size range (bp) in a sample of 30 individuals, annealing temperature (T_a) and the GenBank accession number.

Locus	Sequence	Repeat Motif	Size Range (bp)	T_a	GenBank Acc No.
ASC5	F: TTGGAAGCTCAATTCTATACT	$(GAT)_{20}$	102-121	52	HM004507
	R: CAAAGATGTAGAGGGTAAGTC				
ASF2	F: TGAACAAGATCCTGCGAATG	$(AGA)_{10}$	87-118	52	HM004509
	R: TCATTAGCAACAAAGGTATCC				
ASF9	F: CACAGAAACAAGGTGAAATG	$(AAG)_9$	107-125	52	HM004508
	R: TACTTTGCTTAATCAGCTCC				
ASH8	F: AAATCGCATACAGTGGAAAG	$(AAG)_{11}$	157-171	52	HM004502
	R: GACTACTTTCGCTAAATCAG				
ASG6	F: CTATGCAAACTCCTCATGAT	$(TGG)_9$	171-205	52	HM004506
	R: GAAGGCTGTTTCAGATCTTG				
AS94	F: TTCTTCGAGTAGGTAGGAATG	$(AAG)_{19}$	139-171	52	HM004505
	R: CACCCCTACAAACAATCCT				
ASB5	F: CCATGAAATTAGCTCAAGATC	$(GAA)_{11}$	187-193	52	HM004504
	R: CAAAGTCCGATTCGGGTAA				
ASG5	F: CTGACAGAATCACTGCTC	$(TTC)_{11}$	157-176	52	HM004503
	R: CTTTATATCGCTGACATTACT				

Forward or reverse primers were end-labeled with FAM, HEX or TAMRA. Eight of the 12 loci were found to be polymorphic and generated consistent and easily scored amplification products of the expected size range (Table 1). Amplified products were genotyped on an ABI 3730 Sequencer and analyzed using GeneMarker v 1.8 (SoftGenetics LLC, State College, PA).

GenAlEx v 6.2 (Peakall and Smouse, 2006) was used to calculate the mean number of alleles observed (A), observed and expected heterozygosity (H_o and H_E) (Table 2) and Probability of Identity (PI) for each locus. Linkage disequilibrium (LD) and deviations from Hardy-Weinberg (HW) were assessed in GenePop v 4 (Raymond and Rousset, 1995) using the Markov chain method with 1000 dememorizations, 100 batches, and 1000 iterations per batch.

The loci contained 3 to 13 alleles in a sample of 30 individuals from the UMBS, with observed heterozygosity ranging from 0.33 to 0.83 (Table 2). Three loci (ASG6, ASB5, ASG5) showed significant excess of heterozygotes. Two loci (ASH8 and AS94) showed a significant excess of homozygotes. Significant LD was detected between loci ASF9 and ASH8. Probability of identity (PI) using all eight markers was 1.5E-7. No identical multilocus genotypes were found in the dataset, which suggests that the sampled individuals represent distinct genets.

CONCLUSIONS

The excesses in heterozygosity in three loci may reflect biological properties particular to the UMBS population, such as the level of clonality, while the excess homozygosity in two loci may be caused by null alleles. Even though *A. syriaca* can reproduce asexually, there is no indication that the samples used in this study came from repeated genets. However due to the relative isolation from other populations of *A. syriaca*, it is possible that inbreeding could cause some of the homozygote excess. Inbreeding would be expected to affect all loci equally, however, yielding homozygote excess at all loci, not just two of five examined. The high probability of identity indicates that these markers will provide clear resolution of genet and ramet structure in the UMBS and other natural populations of *A. syriaca*. These markers will also be useful in assessing population genetic structure and gene flow at local and regional spatial scales.

TABLE 2. Results of initial primer screening in a single population of *Asclepias syriaca*. Shown for each locus are the number of alleles (A), observed heterozygosity (H_o) and expected heterozygosity (He) in a population of 30 putative genets. Deviations from Hardy-Weinberg * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$

Locus	A	H_o	H_e
ASC5	4	0.70	0.55
ASF2	9	0.77	0.74
ASF9	8	0.77	0.62
ASH8	7	0.33*	0.49
ASG6	12	0.83***	0.82
AS94	13	0.53***	0.75
ASB5	3	0.80***	0.50
ASG5	11	0.83*	0.78

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