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Microsatellite primers in *Oenothera harringtonii* (Onagraceae), an annual endemic to the shortgrass prairie of Colorado[1]

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- *Premise of the study:* Microsatellite markers were developed in the annual herb, *Oenothera harringtonii*, to investigate patterns of genetic diversity, gene flow, and parentage within and among populations of this Colorado endemic.
- *Methods and Results:* Ten polymorphic loci were identified in *O. harringtonii* and tested in four populations sampled across the range of the species. These loci contained trinucleotide repeats with 7–29 alleles per locus. Nine of the 10 loci also amplified in *O. caespitosa* subsp. *macroglottis*, *O. caespitosa* subsp. *marginata*, and *O. caespitosa* subsp. *navajoensis.* In addition, we optimized three markers developed for *O. biennis* and provide reports of their effectiveness in all four taxa.
- *Conclusions:* These results indicate the utility of these markers in *O. harringtonii* for future studies of genetic structure, gene flow, and parentage as well as their applicability in other members of the *O. caespitosa* species complex.

Key words: annual herb; endemic; microsatellites; *Oenothera caespitosa*; *Oenothera harringtonii*; Onagraceae.

Oenothera harringtonii W. L. Wagner, Stockh. & W. M. Klein (Onagraceae) is an annual, self-incompatible, herbaceous species endemic to the shortgrass prairies and alkaline clay soils of the middle Arkansas Valley of south-central and southeastern Colorado. This region is high in endemic plants (12 species), supporting 20 globally and 30 state-imperiled species, and has experienced rapid human development (residential, recreational, military, mining, and highway maintenance), with Fremont and Pueblo counties among the fastest growing counties in the United States (Spackman Panjabi, 2004). The combination of rare and imperiled plants and anthropogenic threats has made the middle Arkansas Valley a primary focus of conservation efforts in Colorado (Kelso et al., 2003; Spackman Panjabi, 2004; Nature Conservancy, 2006). Many extant populations of *O. harringtonii* are found throughout the range and the conservation status is currently vulnerable globally (G3; NatureServe, 2011) and in Colorado (S3; Colorado Natural Heritage Program, 2011). Populations of *O. harringtonii* occur in areas exhibiting immediate development threats and in contiguous, undeveloped natural areas. *Oenothera harringtonii* is a member of *Oenothera* sect. *Pachylophus*, along with four narrow endemic species and the *O. caespitosa* species complex (Wagner et al., 1985). Here, we characterize 10 microsatellite loci in *O. harringtonii* and their transferability in three members of the *O. caespitosa* species complex. We also optimized three primers developed for *O. biennis* L. (Larson et al., 2008) and provide reports of their utility in all four taxa.

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METHODS AND RESULTS

Microsatellite-enriched genomic libraries were developed by Genetic Identification Services (Chatsworth, California, USA) from approximately 100 ng of genomic DNA (Jones et al., 2002) using leaf tissue from 10 individuals. These plants resulted from greenhouse crosses derived from field-collected seed (populations include: Baculite Mesa = Herbarium accession number 16755, E. Hilpman, S. Todd, and K. Skogen, 38.2923N 104.55775W; Burnt Mill = see Table 1; David's Canyon = Herbarium accession number 14235, K. Skogen, E. Hilpman, S. Todd and C. Klase, 37.75643N 103.59391W; and Florence = Herbarium accession number 14225, K. Skogen, E. Hilpman, C. Klase, and S. Todd, 38.4444N 104.55775W). Libraries were enriched for four repeat motifs: $(CA)_n$, $(AAC)_n$, $(AAG)_n$, and $(ATG)_n$. From the four enriched libraries, 96 clones were sequenced, yielding microsatellites as follows: for CA- all 11 sequences contained a microsatellite, for AAC- 14 of 28 contained a microsatellite, for AAG- 24 of 28 contained a microsatellite, and for ATG 17 of 28 contained a microsatellite. Once appropriate microsatellites were located, PCR primers were designed for the flanking regions of 38 of the 66 sequences identified using DesignerPCR version 1.03 (Research Genetics, Huntsville, Alabama, USA).

Genomic DNA was extracted from silica-preserved leaf tissue following a modified cetyltrimethylammonium bromide (CTAB) method (Khasa et al., 2000). To visualize samples, each forward primer was modified with the addition of an M13 sequence to the 5′ end (5′-CACGACGTTGTAAAACGAC-3′) (Schuelke, 2000). An initial PCR was conducted in a 10-μL reaction containing PCR MasterMix (Promega, Madison, Wisconsin, USA; final concentration of 0.025 U/μL *Taq*DNA polymerase in a proprietary reaction buffer [pH 8.5], 200 μM of each dNTP, and 1.5 mM $MgCl_2$), plus 0.5 ng/μL BSA, 5 ng template DNA, and 0.25 μM of both forward and reverse primers. This PCR was run for 3 min at 94°C; then 13 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 1 min; and a final extension of 72°C for 10 min. The reaction product was labeled through a second PCR using a 15-μL reaction containing 10 μL of the PCR product, and an additional 2.5 μL of PCR 2× MasterMix (Promega), 0.5 ng/μL BSA, 2.0 mM $MgCl_2$, and 0.25 μM of M13 primer labeled with either WellRed D2 (black), D3 (green), or D4 (blue) fluorescent dye (Sigma-Proligo, St. Louis, Missouri, USA). The PCR conditions for this reaction were 94°C for 3 min; 27 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and an extension of 72°C for 10 min. PCR products were analyzed and scored using a CEQ 8000 Genetic Analysis System version 9.0 (Beckman Coulter, Brea, California, USA). Nine primer sets previously described for *O. biennis* (Larson et al., 2008) were prelabeled with WellRed D2 (black), D3 (green), or D4 (blue) fluorescent dye

TABLE 1. Results of initial primer screening in four populations of *Oenothera harringtonii*.

	Burnt Mill[a] (38.09803N, 104.73043W)					Cone Mountain (37.566782N, 104.299439W)					Riverside (38.259159N, 104.707131W)					Stem Beach (38.18185N, 104.59006W)				
Locus	*N*	*A*	Private	H_o	H_e[b]	*N*	*A*	Private	H_o	H_e[b]	*N*	*A*	Private	H_o	H_e[b]	*N*	*A*	Private	H_o	H_e[b]
OenhaB105	29	14	1	0.86	0.90^{NS}	27	14	—	0.85	0.89^{NS}	27	11	—	0.89	0.86^{NS}	27	13	—	0.85	0.84***
OenhaC105	29	13	1	0.76	0.80^{NS}	23	13	2	0.83	0.78^{NS}	28	12	2	0.79	0.84***	28	12	2	0.75	0.72^{NS}
OenhaC106	29	21	2	0.66	0.92***	25	22	3	0.80	0.94**	26	19	—	0.81	0.87*	27	18	1	0.70	0.91^{NS}
OenhaC126	25	7	—	0.60	0.70^{NS}	29	9	—	0.55	0.85^{NS}	25	6	—	0.44	0.77***	26	7	1	0.31	0.79***
OenhaC4	27	11	2	0.52	0.78***	26	11	2	0.73	0.84^{NS}	29	9	1	0.76	0.86^{NS}	29	11	1	0.62	0.81***
OenhaD102	28	7	1	0.79	0.71^{NS}	29	7	—	0.66	0.71^{NS}	29	6	—	0.62	0.63^{NS}	28	7	—	0.64	0.71*
OenhaD111	20	17	5	0.85	0.90^{NS}	24	17	4	0.88	0.87^{NS}	24	8	—	0.67	0.77**	20	12	1	0.80	0.84*
OenhaD118	29	3	—	0.38	0.40***	29	6	3	0.24	0.28*	31	2	—	0.03	0.09***	27	4	1	0.19	0.17^{NS}
OenhaD2	27	6	1	0.78	0.77^{NS}	26	6	—	0.69	0.70^{NS}	29	7	—	0.45	0.66^{NS}	24	6	—	0.50	0.71^{NS}
OenhaD5	29	15	—	0.72	0.89^{NS}	29	21	3	0.86	0.93^{NS}	30	13	—	0.73	0.87^{NS}	30	20	1	0.90	0.93*
Oenbi2tri2	30	7	—	0.70	0.81^{NS}	29	8	—	0.76	0.83*	30	7	1	0.73	0.76^{NS}	30	7	—	0.80	0.79^{NS}
Oenbi39di2	28	6	—	0.50	0.64***	27	7	1	0.37	0.73***	30	7	1	0.33	0.62***	26	6	1	0.65	0.74^{NS}
Oenbi39tri4	27	2	—	0.04	0.04^{NS}	27	2	—	0.19	0.23^{NS}	30	2	—	0.03	0.03^{NS}	26	2	1	0.04	0.04^{NS}

Note: — = not applicable; *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; *N* = number of individuals sampled; Private = number of private alleles.

[a]Vouchers of Burnt Mill population = Herbarium accession number 14223, K. Skogen and J. Fant; deposited at the Nancy Poole Rich Herbarium at the Chicago Botanic Garden, Glencoe, Illinois, USA. Vouchers for Cone Mountain, Riverside, and Stem Beach were not collected due to small population size and conservation status.

[b]Significant departures from HWE are indicated at the following levels: **P* = 0.05, ***P* = 0.01, and ****P* = 0.001; NS = not significant.

labels. A single PCR was conducted using the same reagent concentrations as above and the following conditions: 95°C for 2 min; 35 cycles of 95°C for 50 s, 55°C for 1 min, and 72°C for 2 min; and an extension of 72°C for 10 min.

We tested a total of 47 primer pairs on a subset of *O. harringtonii* individuals to identify primers that were polymorphic and amplified reliably. Of the 38 primers developed in *O. harringtonii*, 12 did not amplify, 12 amplified but were unreliable (GenBank accession numbers: JQ679459, JQ679477, JQ679479, JQ679466, JQ679468, JQ679469, JQ679470, JQ679471, JQ679474, JQ679484, JQ679475 and JX430063), six were monomorphic (GenBank accession numbers: JQ679458, JQ679460, JQ679462, JQ679475, JQ679481, and JQ679482), and 14 were polymorphic. Of the nine primers developed in *O. biennis*, four were polymorphic and five did not amplify. The remaining 18 reliable, polymorphic primers were characterized using four populations of *O. harringtonii*, one population of *O. caespitosa* Nutt. subsp. *macroglottis* (Rydb.) W. L. Wagner, Stockh. & W. M. Klein, two populations of *O. caespitosa* subsp. *marginata* (Nutt ex. Hook. & Arn.) Munz, and two populations of *O. caespitosa* subsp. *navajoensis* (W. L. Wagner, Stockh. & W. M. Klein) Cronq. (Tables 1 and 2).

We report the following descriptive parameters: sample size (*N*), number of alleles per locus (*A*), number of private alleles (Private), observed heterozygosity (H_o), expected heterozygosity (H_e), and departure from expected proportions under Hardy–Weinberg equilibrium (HWE) (Tables 1 and 2, calculated in GenAlEx; Peakall and Smouse, 2006). Each primer was tested for the potential of null alleles using exact tests in Micro-Checker (van Oosterhout et al., 2004) for each locus, population, and globally. Potential null alleles were identified in five of the 18 primers tested, four from those derived from *O. harringtonii* (GenBank accession numbers: JQ679461, JQ679476, JQ679463, and JQ679478) and one from those derived from *O. biennis* (Oenbi2tri6). Linkage disequilibrium was tested for each pair of loci across all populations using Fisher's method in GENEPOP (Raymond and Rousset, 1995). Of the 91 possible primer pairs, only comparisons between primer sets Oenbi39di2 and OenhaD111 showed significant linkage disequilibrium ($P < 0.05$). When tested within each population using the log likelihood ratio statistic in GENEPOP, significant linkage disequilibrium was observed in these two primer sets in only two of the four populations (Burnt Mill and Stem Beach).

Excluding those with null alleles, 13 loci showed reliable amplification and allelic polymorphisms that could be consistently scored, 10 loci developed in *O. harringtonii* and three developed in *O. biennis* (Larson et al., 2008) (Table 3). The 10 markers developed for *O. harringtonii* varied from seven to 29 alleles per locus, while the three developed for *O. biennis* revealed lower variation, with three to 12 alleles per locus. All 13 loci were polymorphic within *O. harringtonii* populations, with two to 21 alleles per locus and one to three private alleles (Table 1). Significant departure from expected proportions under HWE was observed in all loci in at least one population, except Oenbi39tri4 and OenhaD5, although no loci showed significant deviation in all populations (Table 1); observed deviations might be due to strong bottlenecks (resulting from seasonal variability) and inbreeding in small populations.

All 13 markers were polymorphic in the three *O. caespitosa* subspecies except OenhaD118, which was monomorphic in *O. caespitosa* subsp. *macroglottis*, and OenhaC4, which did not amplify consistently in any of the three subspecies. Allelic diversity by population for all polymorphic markers ranged from two to 11 alleles per locus in *O. caespitosa* subsp. *macroglottis*, two to 15 alleles per locus in *O. caespitosa* subsp. *marginata*, and two to 15 alleles per locus in *O. caespitosa* subsp. *navajoensis* (Table 2).

CONCLUSIONS

All 10 microsatellite primers developed in *O. harringtonii* and three developed in *O. biennis* were polymorphic in the samples analyzed. In addition, nine of the 10 loci developed in *O. harringtonii* and three developed in *O. biennis* were polymorphic in three other members of the *O. caespitosa* species complex: *O. caespitosa* subsp. *macroglottis*, *O. caespitosa* subsp. *marginata*, and *O. caespitosa* subsp. *navajoensis.* These markers will be useful for assessing patterns of genetic diversity and structure within and among populations, conducting parentage studies, and in comparisons between narrow endemics and widespread members of the *O. caespitosa* species complex.

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Table 2. Results of initial primer screening in *Oenothera caespitosa* subsp. *macroglottis* (one population), *O. caespitosa* subsp. *marginata* (two populations), and *O. caespitosa* subsp. *navajoensis* (two populations).[a]

	O. caespitosa subsp. *marginata*										*O. caespitosa* subsp. *navajoensis*										*O. caespitosa* subsp. *macroglottis*				
	Paragonah[b] (37.89184N, 112.7297W)					Ruth (39.285314N, 114.964339W)					MM 11 (38.670047N, 109.498039W)					MM 16 (38.682048N, 109.479004W)					Black Canyon[b] (38.526883N, 107.660995W)				
Locus	*N*	*A*	Private	H_o	H_e[c]	*N*	*A*	Private	H_o	H_e[c]	*N*	*A*	Private	H_o	H_e[c]	*N*	*A*	Private	H_o	H_e[c]	*N*	*A*	Private	H_o	H_e[c]
OenhaB105	15	11	—	1.00	0.83^{NS}	11	9	—	0.91	0.81^{NS}	10	13	3	0.60	0.91^{NS}	9	7	—	0.44	0.85*	21	5	—	0.62	0.66^{NS}
OenhaC105	14	5	1	1.00	0.68^{NS}	11	8	1	0.73	0.83^{NS}	13	13	2	0.69	0.89*	10	12	—	0.80	0.90^{NS}	21	11	2	0.62	0.82***
OenhaC106	13	13	3	0.92	0.88^{NS}	10	15	6	1.00	0.92^{NS}	12	5	—	0.75	0.63***	18	5	1	0.61	0.58***	9	11	5	0.89	0.86**
OenhaC126	12	2	—	0.08	0.08^{NS}	5	5	—	1.00	0.76^{NS}	22	15	3	0.68	0.91^{NS}	19	10	—	0.79	0.85^{NS}	16	8	2	0.50	0.84**
OenhaD102	11	3	—	0.45	0.37^{NS}	11	5	—	0.64	0.63^{NS}	11	5	1	0.09	0.77***	13	6	1	0.31	0.74*	24	3	—	0.25	0.29***
OenhaD111	15	5	—	1.00	0.67*	11	11	1	0.91	0.87^{NS}	15	10	2	0.73	0.78***	11	4	2	0.91	0.62**	23	8	2	0.35	0.75***
OenhaD118	12	2	—	0.08	0.08^{NS}	5	2	—	0.40	0.32^{NS}	12	7	3	0.25	0.62***	7	4	—	0.71	0.64^{NS}	24	1	—	0.00	0.00^{NS}
OenhaD2	14	5	—	0.86	0.66^{NS}	10	6	1	0.80	0.75^{NS}	16	8	3	0.69	0.71**	15	8	3	0.47	0.68^{NS}	17	2	1	0.06	0.06^{NS}
OenhaD5	10	9	3	1.00	0.86^{NS}	10	5	—	0.70	0.71^{NS}	5	6	—	0.40	0.80^{NS}	5	2	2	0.40	0.48^{NS}	16	5	2	0.56	0.53^{NS}
Oenbi2tri2	2	2	—	0.00	0.50^{NS}	7	6	—	0.71	0.81^{NS}	12	8	1	0.67	0.82^{NS}	23	9	2	0.61	0.76***	14	6	—	1.00	0.75**
Oenbi39di2	12	3	—	0.25	0.54*	11	3	—	0.36	0.31^{NS}	4	3	—	0.50	0.53^{NS}	13	6	1	0.85	0.78^{NS}	3	3	—	0.33	0.61^{NS}
Oenbi39tri4	13	2	—	0.23	0.20^{NS}	11	2	1	0.09	0.09^{NS}	23	2	—	0.04	0.04^{NS}	24	2	—	0.08	0.08^{NS}	19	2	—	0.08	0.08^{NS}

Note: — = not applicable; *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; *N* = number of individuals sampled; Private = number of private alleles.

[a] Primer OenhaC4 did not amplify in any of the *O. caespitosa* subspecies tested.

[b] Vouchers of populations: Paragonah = Herbarium accession number 13306, K. Skogen and J. Fant; Black Canyon = Herbarium accession number15699, J. Fant, E. Hilpman, and S. Todd. Vouchers are deposited at the Nancy Poole Rich Herbarium at the Chicago Botanic Garden, Glencoe, Illinois, USA. Vouchers were not collected at Ruth (small population size), MM11 and MM16 (ongoing studies; Artz et al., 2010).

[c] Significant departures from HWE are indicated at the following levels: *P = 0.05, **P = 0.01, and ***P = 0.001; NS = not significant.

TABLE 3. Characteristics of 13 microsatellite primers tested on *Oenothera harringtonii* populations; 10 of the primers were developed in *O. harringtonii* and three were developed in *O. biennis*.[a]

Locus	Repeat motif	Primer sequences (5′–3′)	Size (bp)	T_a (°C)	Reaction mix[b]	Label[c]	GenBank accession no.
OenhaB105	$(TTG)_{17}$	F: TAGCCTCCTCAAGAGACACA R: CGATGCTGGAACTTCAAAG	155–199	55	C	D2	JQ266359
OenhaC4	$(TCT)_{10}$	F: TCCACCAGTCTCTGTTGTCT R: GGATTTTACCTTGAACTCAGC	169–226	55	D	D4	JQ266366
OenhaC105	$(TTC)_{10}$	F: CCTGCTCTTTTGTCAATCTG R: CCGTATGATGTCCTTTATCG	205–293	55	C	D4	JQ266360
OenhaC106	$(TTC)_2(TTC)_{31}$	F: ACTTCGCAGTCCCTTTCAAC R: CCAACTTTCCCTTCAACGATA	240–349	55	B	D4	JQ266361
OenhaC126	$(CTG)_3(TCT)_{13}$	F: TCCAATCCAACCCATACTTAG R: GTTGCTGAAGAAGATGTCAATC	265–292	55	D	D3	JQ266362
OenhaD2	$(ATC)_{10}(AGA)_3(GAA)_3$	F: AGCCATCTTGAACTAACCAAC R: CAACAACTAAGCCCAACACTA	184–202	55	C	D3	JQ266367
OenhaD5	$(GA)_3(GA)_3(CAT)_{19}$	F: ATGATGAGAGTCATGGCTAATC R: ATTTCTGGTCAATCGTAGGAG	268–359	55	A	D3	JQ266368
OenhaD102	$(ATG)_8$	F: GCACAAATCCGAAACTCA R: TGCCGCATCTAACCATAC	243–264	55	B	D3	JQ266363
OenhaD111	$(TGC)_3(GAT)_5$	F: CGTAGGTGTGGTGTGTATTTG R: CATCGTCATTTTCGTTTAGTG	137–235	55	B	D2	JQ266364
OenhaD118	$(GA)_4(GCA)_4(ATG)_9$	F: GATTGCTGAAGGGTATTTCGTC R: CACTGGCACTGCTACTCACC	254–278	55	D	D2	JQ266365
Oenbi2tri2*	$(GTT)_8$	F: TTCAGCTCCGATTGCCAAATGATGG R: ATTCGCTGATGGAGCTGGGTTGTTCTT	400–427	55	A	D4	EF988083
Oenbi39di2*	$(GT)_{10}$	F: TAACTTCGTGGAACCAGACAGACACTCTC R: GAAGCGACAAGGCATACACTTTCTCCT	185–203	55	A	D4	EF988088
Oenbi39tri4*	$(CTT)_{16}$	F: TTTCCTCTCCTTATCTCCTCAATTCTTCTCA R: CTTGCGTTATCTTATGCGTAGCCGTTAGTTTAT	188–194	55	A	D2	EF988090

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals sampled; T_a = annealing temperature when run individually.

[a] All values are based on samples from four populations located in Pueblo and Huerfano counties in Colorado, USA (Table 1).

[b] Reaction mix in which the locus was amplified (multiplex reaction A, B, C, and D).

[c] Fluorescent label attached to reverse end of primer: D2 = black; D3 = green; D4 = blue.

* Developed by Larson et al. (2008).

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