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Source: Applications in Plant Sciences, 1(6)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1200564

Applications in Plant Sciences 2013 1(6): 1200564

PRIMER NOTE

CHARACTERIZATION OF MICROSATELLITE LOCI IN *CASTILLEJA SESSILIFLORA* AND TRANSFERABILITY TO 24 *CASTILLEJA* SPECIES (OROBANCHACEAE)[1]

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- *Premise of the study:* Microsatellite primers were developed in the hemiparasitic perennial forb *Castilleja sessiliflora* to investigate patterns of gene flow and genetic diversity within and among populations.
- *Methods and Results:* Twelve polymorphic loci were identified in *C. sessiliflora* and tested on three populations (32 individuals each) sampled across the range of the species. The loci amplified di- and trinucleotide repeats with 3–14 alleles per locus. To assess cross-amplification, primer pairs were also tested on 24 additional *Castilleja* species that represent the morphological and geographic diversity of the genus. We provide reports of their effectiveness in all 25 taxa.
- *Conclusions:* These results indicate the utility of these primers in *C. sessiliflora* for future studies of genetic structure and gene flow, as well as their widespread applicability in other members of the diverse and complex genus *Castilleja*.

Key words: *Castilleja*; *Castilleja sessiliflora*; cross-amplification; hemiparasite; microsatellites; Orobanchaceae.

Castilleja sessiliflora Pursh is a generalist, hemiparasitic perennial in the Orobanchaceae Vent. with a wide host range (Crosswhite and Crosswhite, 1970). It is native to the shortgrass prairies of the Great Plains from southern Canada to northern Mexico, and extends east into Wisconsin and Illinois; it is classified as endangered in Illinois. The majority of the hemiparasites in the Orobanchaceae were previously placed within the Scrophulariaceae Juss.; however, molecular systematic studies demonstrated that the traditional circumscription of Scrophulariaceae was largely artificial (e.g., Olmstead et al., 2001).

Because the seeds of *C. sessiliflora* are gravity dispersed, it is likely that pollen movement plays a more important role in gene flow than seed dispersal. Several characteristics of the flowers of *C. sessiliflora* led Pennell (1935) to speculate that it is pollinated by lepidopterans. However, Crosswhite and Crosswhite (1970) observed that flowers in Wisconsin, USA, were only visited by *Bombus fervidus* Fabr. queens, although their observations were restricted to daytime hours when bee activity is typically high and when crepuscular insects are generally inactive. More recent observations in Illinois and Colorado reveal that *C. sessiliflora* is visited by at least one hawkmoth species, *Hyles lineata* Fabr. (J. Fant and K. Skogen, unpublished data). Interestingly, *C. sessiliflora* is the only known member of the genus for which hawkmoth visitation has been documented, and the effects of moth pollination on gene flow remain largely unexplored in this important pollinator group. Despite being a relatively speciose genus, population genetic studies within *Castilleja* Mutis ex L.f. are surprisingly limited (two allozyme and one amplified fragment length polymorphism [AFLP] study) and have not used taxon-specific markers. Here, we characterize 12 microsatellite loci in *C. sessiliflora* for use in studies of gene flow, genetic structure, and diversity, and report cross-amplification in 24 additional *Castilleja* species.

METHODS AND RESULTS

Microsatellite-enriched genomic libraries were developed by Genetic Identification Services (Chatsworth, California, USA; Jones et al., 2002). Libraries were enriched for four repeat motifs—$(CA)_n$, $(AAC)_n$, $(AAG)_n$, and $(ATG)_n$—and from a total of 144 sequenced clones, microsatellites were found in 22 out of 24 sequences for CA, 31 of 40 sequences for AAC, 28 of 40 sequences for AAG, and 26 of 40 sequences for ATG. Of the 107 sequences identified as containing microsatellites, PCR primers were designed for 33 regions ($[CA]_6$, $[AAC]_9$, $[AAG]_{11}$, and $[ATG]_7$) in DesignerPCR version 1.03 (Research Genetics, Huntsville, Alabama, USA) using the default parameters. These primer pairs were tested on a subset of *C. sessiliflora* individuals.

Genomic DNA was extracted from silica-dried leaf material using QIAGEN DNeasy kits (QIAGEN, Valencia, California, USA) for *C. sessiliflora* samples and the modified 2× cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987) for remaining species. DNA quantity was determined using a Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, Delaware, USA), and samples were diluted to a final concentration of 5 µg/mL. 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 10-µL PCR was conducted using 5 ng of template DNA, 25 µM of modified forward and reverse primer, and proprietary PCR MasterMix 2× (50 units/mL *Taq* DNA polymerase and buffer plus 400 µM of each dNTP; Promega Corporation, Madison, Wisconsin, USA). This PCR was run at 94°C for 3 min, followed by 15 cycles of 94°C for 40 s, 57°C for 40 s, and 72°C for 90 s, with a final extension of 72°C for 10 min. To this PCR product, an additional 5 µL of PCR mixture was added with the forward and reverse primer substituted with 25 µM of M13 primer labeled with either

[1]Manuscript received 24 October 2012; revision accepted 30 November 2012.

The authors thank J. M. Keller and J. Rossi for assistance with laboratory work and E. Hilpman and S. Todd for field collections.

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doi:10.3732/apps.1200564

TABLE 1. Characteristics of 15 microsatellite primers tested on three *Castilleja sessiliflora* populations, two located in Colorado and one in Illinois, USA.

Locus	Repeat motif	Primer sequences (5′–3′)	Size range (bp)	T_a (°C)	WellRED dye (color)	GenBank accession no.
CaSe_A01	$(AT)_6$	F: TAACGAAGTGAGGCAAGTAGTC R: ATTCCGAGACATCAAACACAT	181–193	57	D4 (blue)	JX430080
CaSe_A101	$(TC)_{11}(AC)_{13}$	F: TTGATTCCATCACAGTGAAC R: TACCATTGCTTGTGTGCATTTA	128–180	57	D4 (blue)	JX430077
CaSe_A102	$(AT)_{14}$	F: TGCTAAAAGATGTTTGGTAACC R: AATGCCCTAGAAAGTTGCC	209–253	57	D4 (blue)	JX430076
CaSe_A103	$(TG)_9$	F: CAAAATGTCGTCTGACCAAATA R: AGGAGCTGTAATTCTAACCCTG	97–113	57	D4 (blue)	JX430075
CaSe_B103	$(GTT)_8$	F: CTTGAAAACCCGTACTCAGTC R: ATGGAAATGGACATCAATGAG	282–294	57	D4 (blue)	JX430074
CaSe_B104	$(GTT)_7$	F: ATTTCCCGCAATTCAAACTAC R: AATTCACAACAATGGCATCAG	234–243	57	D3 (green)	JX430073
CaSe_B116	$(CAA)_8$	F: CAATCTGCACACCAAGTGTTC R: CTTGACGACGTGCTTGTCTAA	247–262, 283–298	57	D3 (green)	JX430072
CaSe_B04	$(GTT)_8$	F: GGAACAATATCAAGTCCTCTGA R: CTTCGACCCATTACTTCACTAA	131–163	57	D3 (green)	JX430079
CaSe_C02	$(TTC)_7$	F: CCATCACTTTGGTAGCCTGAAT R: ACGGATAAGGAGACTGACCTG	271–282	57	D3 (green)	JX430078
CaSe_C102	$(TTC)_9$	F: TGCGTAATGCTCTATTATTCAG R: GGATTAGCTGTTTCTGCACTAG	199–226	57	D3 (green)	JX430071
CaSe_C104	$(TTC)_6$	F: CTATCCCTAACGCGATACCTA R: ATTTCGACGAGTACGATTACC	239–248	57	D2 (black)	JX430070
CaSe_C105	$(TTC)_8$	F: CCTATCGAAGCATCTCATCAC R: GAGGAACTGGGATTTGATTAT	180–207	57	D2 (black)	JX430069
CaSe_D101	$(ATC)_5$	F: ATCATCATCAACCATCCATAA R: TGTACGGATCAGAGAGAAATG	100–109	57	D2 (black)	JX430068
CaSe_D103	$(ACT)_{11}(ACT)_{13}$	F: CCATCATCACAGGCTTCAG R: TGGTGGTGCTGGTAACAAC	237–252	57	D2 (black)	JX430067
CaSe_D119	$(ACT)_{11}$	F: TACCACCTCCACCAGGTTATC R: GCGGTGATCCAATTTGTATG	186–249	57	D2 (black)	JX430064

Note: T_a = annealing temperature when run individually.

WellRED Black (D2), Green (D3), or Blue (D4) fluorescent dye (Sigma-Aldrich, St. Louis, Missouri, USA). With the additional label, the PCR was rerun at 94°C for 3 min, 27 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 10 min. Products were analyzed and scored using a CEQ 8000 Genetic Analysis System version 9.0 (Beckman Coulter, Brea, California, USA).

We tested a total of 33 primer pairs on a subset of *C. sessiliflora* individuals to identify primers that were polymorphic and amplified reliably. Of these, seven did not amplify (GenBank accession no.: JX983112, JX983116–JX983119, JX983121, JX983129), seven were weak or did not amplify consistently (GenBank accession no.: JX983114, JX983122–JX983127), one was monomorphic (GenBank accession no.: JX983120), three produced multiple peaks (GenBank accession

TABLE 2. Results of initial primer screening in three populations of *Castilleja sessiliflora*.[a]

	Population CC						Population DC						Population IBSP					
Locus	*N*	*A*	Prv	H_o	H_e	HWE[b]	*N*	*A*	Prv	H_o	H_e	HWE[b]	*N*	*A*	Prv	H_o	H_e	HWE[b]
CaSe_A01	30	3	—	0.60	0.52	ns	30	4	—	0.63	0.51	ns	27	4	—	0.85	0.56	**
CaSe_A101	22	14	7	0.68	0.86	**	29	15	7	0.55	0.89	***	16	7	1	0.44	0.75	**
CaSe_A102	29	10	2	0.41	0.76	***	30	11	2	0.43	0.80	***	26	10	1	0.42	0.75	***
CaSe_A103	29	10	1	0.83	0.75	ns	29	19	10	0.66	0.90	***	30	5	—	0.60	0.71	*
CaSe_B04	31	6	—	0.77	0.70	ns	29	7	2	0.62	0.63	ns	30	4	—	0.57	0.67	ns
CaSe_B103	29	5	—	0.66	0.62	ns	27	5	1	0.67	0.64	ns	21	4	—	0.57	0.66	ns
CaSe_B104	27	4	—	0.81	0.64	ns	27	6	3	0.70	0.70	ns	27	4	—	0.44	0.64	ns
CaSe_B116	29	5	—	0.69	0.70	ns	28	6	3	0.61	0.62	ns	22	6	1	0.36	0.36	ns
CaSe_C02	27	7	2	0.56	0.70	ns	26	5	—	0.62	0.64	ns	19	5	—	0.79	0.71	*
CaSe_C102	29	5	—	0.45	0.61	*	28	6	—	0.80	0.67	ns	22	5	—	0.64	0.68	ns
CaSe_C104	29	3	—	0.21	0.35	ns	32	3	1	0.13	0.12	ns	29	1	—	0.00	0.00	ns
CaSe_C105	32	9	3	0.78	0.78	ns	27	7	—	0.67	0.67	ns	28	6	—	0.64	0.79	ns
CaSe_D101	28	3	—	0.44	0.54	ns	28	4	1	0.64	0.61	ns	29	3	—	0.60	0.60	ns
CaSe_D103	32	5	—	0.44	0.54	**	31	6	—	0.45	0.59	ns	27	6	—	0.52	0.56	ns
CaSe_D119	28	9	2	0.79	0.84	ns	29	11	1	0.83	0.83	ns	24	11	3	0.58	0.80	**

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = departure from Hardy–Weinberg equilibrium; *N* = number of individuals sampled; ns = not significant; Prv = number of private alleles.

[a]Voucher and location information for the study populations: CC = Colorado City, Colorado, USA, 37.75643°N, 103.59391°W, *Hilpman & Todd s.n.* (CHIC 15799); DC = David's Canyon, Colorado, USA, 37.96726°N, 104.83026°W, *Hilpman & Todd s.n.* (CHIC 16794); IBSP = Illinois Beach State Park, Illinois, USA (no herbarium specimens collected; coordinates withheld).

[b]Significant departures from HWE are indicated at the following levels: * = 0.05, ** = 0.01, and *** = 0.001.

TABLE 3. Results of cross-amplification of primers on 24 *Castilleja* species (two individuals screened per species).[a,b]

Species	Range	Habit	CaSe_ A01	CaSe_ A101	CaSe_ A102	CaSe_ A103	CaSe_ B04	CaSe_ B103	CaSe_ B104	CaSe_ B116a[c]	CaSe_ B116b[c]	CaSe_ C02	CaSe_ C102	CaSe_ C104	CaSe_ C105	CaSe_ D101	CaSe_ D103	CaSe_ D119
C. affinis	Western North America	Perennial	207–209	147–177	244–259*	102–129	157–189	304–311	252–256*	269	305	289–292	221–232	261	221–243*	118–121	210–213	262–265
C. angustifolia	Western North America	Perennial	205–209*	135–147	236–242*	115–125	151–188	310–313	252–255*	266–269*	305–311	289–292	220	258–261	196–221	124–127	197–208	253–262*
C. applegatei	Western North America	Perennial	200–207	153–163	239–245	118–150	151–179	310–316	252–255*	266–275	302–311	289	220–223*	246–264	208–221	118–121	208–212	253–271
C. chromosa	Western North America	Perennial	199–207	—	235–237	133–142	150–170	—	255	269	305	289	220	261	217–238	121–124	209	267–273
C. cusickii	Western North America	Perennial	—	150–156*	235–244*	105–113*	160–176	—	255	266–269	302–305	289	226–226	261	217–220	124–127	202–224	253
C. fissifolia	Western North America	Perennial	207–209	153–193	236–241	109–122	176–179	310	252–255	269–272	305–311	289–295	226–229	261–267	210–216*	121–124	185–220	264–267
C. flava var. *rustica*	Western North America	Perennial	207	135–176	237–246	115–118	150–171*	307–310	252–255*	269	305	289–298*	214–223	258–264*	205–239	124–127	196–209*	261–266
C. foliolosa	South America	Perennial	193	153–189	239–269	115–118	151	313–316	252–255*	266	302	291–298	220–229	261	210	118–124	215–230	262–269
C. haydenii	Western North America	Perennial	209	135–160	185–266*	113–124*	151–176	304–310	255	269	305–315	291–298	220–229*	264–267	217–223*	121–124*	205–208	253–264
C. hispida var. *hispida*	Western North America	Perennial	205	153–155	249–271*	120–126	171–182*	313–322	252–255	263–269	305	289–295	220–226	257–263	200–207	118–127*	209–224*	255–265*
C. integra	Western North America	Perennial	207	163–175	240–244	112–122	151–169*	307–310*	245–260	266–275	302–308	291	223–244	258	204–214*	124–127*	203–218*	264–269
C. integrifolia	Latin America	Perennial	207	147	—	105–135	—	307–316	—	266–272	305	289–298	226	258–261*	216	115–124	185–216*	259
C. irasuensis	Central America	Perennial	207	163	252–254*	118–124	179–197	310–313	255–256	269	305	289–295	226–229	264–267	211–235	121–127*	185–220	250–259
C. lemmonii	Western North America	Perennial	209	163–166	240–283	118–124*	166–201	310	253–258	269	305	289–298	226–229	267–270	225–229*	121–130	186–238	253–264
C. linarifolia	Western North America	Perennial	208	155	240–242	116–129	148–151	310–313	255	266–278	302–314	289–292	220–226	258–264	211–216	127	208–224	264–267
C. lineariloba	Western North America	Annual	208	155	189	118–125	160	—	248–253	266–270	302–307	283–298*	220–229	257–260	213–223*	115	209	260–271
C. miniata	Western North America	Perennial	199–207	—	—	116–122	176	303–313	253–256	266–269	303–305	289	220–230	267–274	208–217	115–124*	196–208*	254–259
C. minor	Western North America	Perennial	207–209	162	234	124–129	148–157	—	249–253*	269	305	292	223–223	261	220–232	118	211–239	267
C. occidentalis	Western North America	Perennial	205–209	149–156	234–240	109–155*	167–185*	—	253–256	269–275	305–311*	289–292	214–223	258–267*	210–217*	115–130	209	253
C. pallescens var. *inverta*	Western North America	Perennial	205–209*	155–168	234–242	109–124	—	—	255–258	266–269	302–305	289–295	220–226	258–261	211–217	121–124	209–214	261–265
C. peruviana	South America	Annual	198–207	139–155	232–239	121	160–164	307	260–267	260–266*	—	289–295*	226–229	249–258	214–229	115–121	198	235–259*

TABLE 3. Continued.

Species	Range	Habit	CaSe_ A01	CaSe_ A101	CaSe_ A102	CaSe_ A103	CaSe_ B04	CaSe_ B103	CaSe_ B104	CaSe_ B116a[c]	CaSe_ B116b[c]	CaSe_ C02	CaSe_ C102	CaSe_ C104	CaSe_ C105	CaSe_ D101	CaSe_ D103	CaSe_ D119
C. pilosa	Western North America	Perennial	208–210	155–176	240–252	118–126	—	310–316*	252–258*	266–269	302–305	289	214–220	258–261	211–229	118–130*	208–211	257–264
C. pulchella	Western North America	Perennial	207–209	146–146	242–242	107–114*	165–174*	—	255	269–292	305–328	289–298	220–226	255–261	213–223	127–130	209–224	259–269
C. tenuis	Western North America	Annual	207	155–176	188–229	116–122*	151–168	—	246–253	263–269	299–305	295–298	229	255	207–211	115–121	204–207	259
	Monomorphic		11	5	2	1	3	3	5	8	9	7	4	7	2	2	3	4
	No Product		1	2	2	2	2	7	0	0	0	0	0	0	0	0	0	0

[a] Numbers shown are the size range of alleles (bp) recorded in each taxon.

[b] Voucher information: ***Castilleja affinis*** Hook. & Arn. (*Tank 2002-10* [University of Washington Herbarium (WTU)]; *Colwell 05-06* [National Park Service Herbarium (YM)]), ***C. angustifolia*** (Nutt.) G. Don (*Smith 8332* [Boise State University (SRP)]; *Tank 1037* [University of Idaho Herbarium (ID)]), ***C. applegatei*** Fernald (*Tank 2009-10* [ID]; *Mansfield 10-624* [College of Idaho Herbarium (CIC)]), ***C. chromosa*** A. Nelson (*Tank 2009-2* [ID]; *Egger 1419* [WTU]), ***C. cusickii*** Greenm. (*Tank 1059* [ID]; *Tank 1047* [ID]), ***C. fissifolia*** L.f. (*Garcia-Robledo 035* [Universidad de los Andes (ANDES)]; *Olmstead 2009-22* [WTU]), ***C. flava*** S. Watson **var. *rustica*** (Piper) N. H. Holmgren (*Egger 1373* [WTU]; *Smith 8399* [SRP]), ***C. foliolosa*** Hook. & Arn. (*Colwell 04-02* [YM]; *Tank 2002-05* [WTU]), ***C. haydenii*** (A. Gray) Cockerell (*Legler 10089* [University of Wyoming (RM)]; *Egger 1176* [WTU]), ***C. hispida*** Benth. **var. *hispida*** (*Tank 2001-21* [WTU]; *Duke s.n.* [no voucher]), ***C. integra*** A. Gray (*Legler 10819* [RM]; *Tank 2001-58* [WTU]), ***C. integrifolia*** L.f. (*Uribe-Convers 14b* [ANDES]; *Uribe-Convers 20* [ANDES]), ***C. irasuensis*** Oerst. (*Egger 1304* [WTU]; *Tank 03-77* [WTU]), ***C. lemmonii*** A. Gray (*Tank 2001-51* [WTU]; *Colwell 03-29* [YM]), ***C. linarifolia*** Benth. (*Egger 1390* [WTU]; *Tank 2001-54* [WTU]), ***C. linariloba*** (Benth.) T. I. Chuang & Heckard (*Tank 2002-04* [WTU]; *Colwell 04-143* [YM]), ***C. miniata*** Douglas ex Hook. (*Tank 2001-23* [WTU]; *Egger 1376* [WTU]), ***C. minor*** (A. Gray) A. Gray (*Egger 763* [var. *exilis*] [WTU]; *Egger 1453* [var. *stenantha*] [WTU]), *C. occidentalis* (*Egger 1398* [WTU]; *Tank 1052* [ID]), ***C. pallescens*** (A. Gray) Greenm. **var. *inverta*** (A. Nelson & F. R. Macbr.) Edwin (*Smith 8366* [SRP]; *Tank 1032* [ID]), ***C. peruviana*** T. I. Chuang & Heckard (*Tank 2005-17* [WTU]; *Tank 2005-26* [WTU]), ***C. pilosa*** (S. Wats.) Rydb. (*Hinchliff 469* [ID]; *Hinchliff 456* [ID]), ***C. pulchella*** Rydb. (*Egger 1385* [WTU]; *Egger 1372* [WTU]), ***C. tenuis*** (A. Heller) T. I. Chuang & Heckard (*Egger 1235* [WTU]; *Tank 2001-13* [WTU]).

[c] As CaSe_B116 produced two distinct peak regions, these were recorded separately as CaSe_B116a and CaSe_B116b.

* Denotes taxa that showed multiple peaks.

no.: JX983113, JX983115, JX983128), and 15 were polymorphic (Table 1). The 15 polymorphic primer pairs were further tested on 32 individuals from each of three populations of *C. sessiliflora* (Colorado City [CC], Colorado, USA, *Hilpman & Todd s.n.*, Chicago Botanic Garden Herbarium [CHIC] 15799; David's Canyon [DC], Colorado, USA, *Hilpman & Todd s.n.*, CHIC 16794; and Illinois Beach State Park [IBSP], Illinois, USA, for which no herbarium specimens were collected and specific GPS coordinates are withheld, due to its conservation status in Illinois; Table 2). To evaluate the utility of these primers beyond the target species, the primer pairs were also tested on two individuals from a diverse sampling of *Castilleja* species representing the morphological and geographic diversity of the genus, with special attention given to the North American species from the rapidly radiating perennial clade of *Castilleja* (Tank and Olmstead, 2008). This sampling included 20 species from western North America (two annual and 18 perennial) and four species from Central and South America (one annual and three perennial; Table 3).

Primers were tested for the potential of null alleles, by population and globally, using exact tests in MICRO-CHECKER (van Oosterhout et al., 2004). Potential null alleles were identified in three of the 15 loci tested (CaSe_A01, CaSe_A101, and CaSe_A102; Table 1). Linkage disequilibrium was tested for each pair of loci across all populations using Fisher's method in GENEPOP (Raymond and Rousset, 1995). Of the 107 possible loci pairs, significant linkage disequilibrium ($P < 0.05$) was identified between CaSe_B103 and CaSe_D119 and between CaSe_B116 and CaSe_C104, although this was nonsignificant when sequential Bonferroni corrections were applied. For all loci, we report the following descriptive parameters: sample size, mean number of alleles, number of private alleles, observed heterozygosity, expected heterozygosity, and departure from Hardy–Weinberg equilibrium (HWE) (Tables 1 and 2; calculated in GenAlEx; Peakall and Smouse, 2006). The 12 loci that showed reliable amplification and allelic polymorphisms varied from three to 20 alleles per locus (Table 1). One locus, CaSe_B116, produced two separate peak regions separated by 36 bp; this may represent a duplicate annealing site within close vicinity. Significant departure from expected proportions under HWE was observed in five of the 12 loci for at least one population, although no loci showed significant deviation in all populations (Table 2). Finally, 15 loci (including CaSe_A01, CaSe_A101, and CaSe_A102) were tested on two individuals from different populations of 24 additional *Castilleja* species (Table 3). Nine loci produced bands in all species tested, while of the remaining loci, one worked in 23 species, four worked in 22 species, and one worked in 16 species. Monomorphism by loci varied from one to 10 (Table 3). Some loci produced more than two bands; this may suggest evidence of differences in ploidy, which is common among perennial *Castilleja* species, or, alternatively, these extra bands may be a result of stutter or spurious peaks that might disappear with more stringent and optimized PCR conditions.

CONCLUSIONS

Twelve microsatellite loci developed in *C. sessiliflora* were polymorphic and amplified reliably in the samples analyzed. In addition, all loci cross-amplified in 24 additional *Castilleja* species, with most loci revealing polymorphisms in more than half of the species tested. These loci will be useful for assessing patterns of gene flow, genetic diversity, and structure within and among populations of *C. sessiliflora* and other *Castilleja* species, and will contribute to investigations of species delimitation in this diverse and complex genus.

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