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PRIMER NOTE

DEVELOPMENT OF POLYMORPHIC MICROSATELLITE MARKERS FOR INDIAN TOBACCO, *LOBELIA INFLATA* (CAMPANULACEAE)[1]

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- *Premise of the study:* Nuclear microsatellite markers were developed for *Lobelia inflata* (Campanulaceae), an obligately self-fertilizing plant species, for use in the study of temporal fluctuation in allele frequency and of the genetic structure within and among populations.
- *Methods and Results:* We developed 28 primer pairs for *L. inflata*, all of which amplify CT dinucleotide repeats. We evaluated amplification of these loci in 53 *L. inflata* individuals at three sites in eastern North America and found that 24 loci showed microsatellite polymorphism. We also found that 16 loci amplified successfully in *L. cardinalis*, and 11 amplified successfully in *L. siphilitica*.
- *Conclusions:* These primers will be useful for assessing allelic diversity within and among populations of *L. inflata*, and show potential for use in congeneric species.

Key words: Campanulaceae; *Lobelia*; *Lobelia cardinalis*; *Lobelia inflata*; *Lobelia siphilitica*; microsatellite.

Lobelia inflata L. (Campanulaceae) is a herbaceous plant native to North America in the cosmopolitan genus *Lobelia* L., which contains more than 400 species (Lammers, 2011). *Lobelia inflata* is monocarpic and is capable of expressing either an annual or biennial life history; in the latter case, the plant overwinters as a frost-hardy rosette. Systematists have placed *L. inflata* in *Lobelia* sect. *Lobelia*, along with 20 other (primarily) North American species (Murata, 1992). Of these species, *L. cardinalis* L., *L. siphilitica* L., *L. kalmii* L., *L. nuttallii* Roem. & Schult., *L. spicata* Lam., and *L. dortmanna* L. all coexist sympatrically with *L. inflata* in the northeastern United States and Canada. The phylogenetic relationships between these species are not well understood, but morphological analysis has suggested that *L. inflata* is most closely related to *L. cardinalis*, *L. siphilitica*, and *L. dortmanna* (Lammers, 2011).

Populations of *L. inflata* consist of myriad distinct genetic lineages, which are expected to be reproductively isolated from one another. This is because *L. inflata* is assumed to be obligately autogamous—i.e., it is incapable of outcrossing—because it possesses a closed anther tube, which permits pollen release only onto the stigma of the same flower. As such, plants will produce offspring that are genetically identical to the parent, and therefore heterozygosity is expected to be zero in natural populations (Simons and Johnston, 2006). Although quantitative genetic variation is present for some traits, the extent of genetic variation among *L. inflata* ecotypes is unknown (Simons and Johnston, 2000). Highly variable molecular labels (i.e., polymorphic microsatellite loci) would permit the genotyping of reproductive lineages and the tracking of gene flow among populations. In this study, we characterized 28 microsatellite loci for *L. inflata*, tested these markers in 58 individuals from three eastern North American populations, and assessed cross-amplification success in two congeneric species within *Lobelia* sect. *Lobelia*: *L. cardinalis* and *L. siphilitica.*

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METHODS AND RESULTS

Genomic DNA was extracted from a single *L. inflata* specimen collected from the Petawawa Research Forest (individual DAO-887897; see Appendix 1). To obtain high-quality DNA for enrichment, total genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) extraction method of Murray and Thompson (1980). We prepared a CT microsatellite-enriched DNA library using the method of Hamilton et al. (1999) and linker sequences of Glenn and Schable (2005). Ninety-six clones from this library were sequenced using the BigDye 3.1 kit on an ABI 3730 DNA analyzer (Applied Biosystems, Carlsbad, California, USA). Twenty-eight clones contained dinucleotide motifs with at least seven repeat units and were used to design primer pairs using the Primer3Plus software package (Untergasser et al., 2007). Selection of the final 28 primer sets was based on: (1) reliable and repeatable amplification by PCR (using the PCR parameters listed below); and (2) distinct banding pattern visualization on 3% agarose gel. All 28 primer pairs met these inclusion criteria and were used to assess genetic diversity.

To assess amplification, we performed PCR in 20-μL reaction volumes, using the Phire II Direct PCR Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Unlike two-step protocols that require separate DNA isolation and PCR steps, direct PCR allows amplification directly from plant material (Bellstedt et al., 2010). Here, the genomic DNA template for the direct PCR reaction was taken from a circular punch of dried leaf or fruit capsule material using a 0.35-mm Harris Uni-Core Micro-Punch (Thermo Fisher Scientific). Each 20-μL reaction contained 0.5 μM primers and 1.5 mM $MgCl_2$. A standard three-step PCR protocol was used, including an initial denaturation at 98°C for 5 min, followed by 30 cycles of denaturation at 98°C for 5 s, annealing at 50–58°C for 5 s, extension at 72°C for 20 s, and a final extension step of 72°C for 10 min. The annealing

temperatures for each primer set used in this study are given in Table 1. The PCR reactions were performed using a T-3000 ThermoCycler (Biometra, Goettingen, Germany). We initially screened primer sets for polymorphism using 58 individuals from three *L. inflata* populations: Petawawa, Ontario ($n = 43$); Martock, Nova Scotia ($n = 8$); and Petersham, Massachusetts ($n = 7$). We then tested cross-amplification of these primer sets in *L. cardinalis* ($n = 12$) and *L. siphilitica* ($n = 3$) (see Appendix 1 for collection location and voucher deposition information). Amplicon size was determined using a GeneRuler 100-bp DNA Ladder (Thermo Fisher Scientific). Gel images for all samples were compared via Sequentix GelQuest (Sequentix Digital DNA Processing, Klein Raden, Germany) from 3% agarose gel electrophoresis (run at 60 V for 90 min).

Genetic diversity parameters for the three populations are presented in Table 2. Among *L. inflata* individuals, 24 loci were polymorphic, with two to four alleles per locus. Four of the loci (Linflata1, Linflata6, Linflata11, and Linflata24) were monomorphic in all *L. inflata* individuals tested. Notably, some loci showed substantial differences in allele size; for example, two alleles were found for Linflata5 in the Petawawa population—one was 215 bp and the other 331 bp. Although the variability of simple sequence repeats (SSRs) undermines

TABLE 1. Characteristics of microsatellite markers developed for *Lobelia inflata.*

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
Linflata1	F: TTCCAGAGACATGCTTCACG R: TTTCTCAAAACTGGCACATGA	$(CT)_{38}$	181	59.3	KF855960
Linflata2	F: CTCTCCCCCTTCGTTTTCTC R: GCAACAACACGAACACGATT	$(CT)_{18}$	165–201	59.6	KF855961
Linflata3	F: GGCCATTGGGTTGCTTTTAT R: GTTTCGGCAACCAACAGAAT	$(CT)_{24}$	145–163	59.9	KF855962
Linflata4	F: GCCTATGCTGGGGTTACTTG R: ACACGGTAGCGGAAAAATGT	$(GA)_7$	158–166	59.5	KF855963
Linflata5	F: TTTCAACCTTCATGAGGAAATATG R: TTGCTTAGTGCTTATGGTTGAAG	$(GA)_{37}$	215–331	58.6	KF855964
Linflata6	F: TGGGAATTGTATTTCCCTCAA R: AGAGGCTATTCGCGATCTTG	$(GA)_{23}$	189	59.2	KF855965
Linflata7	F: TCGATCTAATTGTGTCAAACTGC R: ATTGGGCTCATGCATTCTCT	$(CT)_{28}$	144–154	59.2	KF855966
Linflata8	F: TGTCCTTTAATCAGGTCAACCA R: GGTCAACTTTGCAAGCCATT	$(CT)_{32}$	207–225	59.4	KF855967
Linflata9	F: AAATTGACCAACATACCCCTAGTC R: CGCTGCTCCTCCCTTACTC	$(CT)_7$	207–311	59.5	KF855968
Linflata10	F: AAGAAAGTTTTGAGGCCATCC R: AACCAAGGGGACTGAAGCAT	$(GA)_9TA(GA)_{11}$	113–231	59.5	KF855969
Linflata11	F: ATGAGTGCTGGTCGGCTTT R: CTAATCCCATCCCCCATTTT	$(GA)_{15}$	162	59.8	KF855970
Linflata12	F: AACAAGGAGACGGGGATTTT R: TGCCTTTCTACGGCTACTTTG	$(CT)_{18}$	126–184	59.5	KF855971
Linflata13	F: ACAAACATCATCAGTCACCCATA R: GGGGGTTTCCTCTCTCCATA	$(GA)_{22}$	102–118	59.2	KF855972
Linflata14	F: GGCAACTGCATTTAGGGATG R: TCAAAGGCCCTTCTCTCTGA	$(CT)_{35}$	185–207	60.0	KF855973
Linflata15	F: TTTCAGGCTGTCCTTTGACC R: TTCAAATACTTGTTTATTCGACGTTC	$(GT)_9(GA)_8$	168–180	59.8	KF855974
Linflata16	F: TGTTGCAGAAACAAACAACCTT R: AGCCCACCTGGTATTCAAGA	$(GA)_{12}$	178–256	59.5	KF855975
Linflata17	F: CTGGTGAACAATTGCCAAAA R: GGTTGTTTGTTTCTGCAACATT	$(GA)_{15}$	175–191	59.0	KF855975
Linflata18	F: CCGTATCCTATCCATCTCCTACA R: AGGGGAGTGAGGAAGACGTT	$(CT)_{25}$	176–184	59.3	KF855976
Linflata19	F: GACCCTCCAAATAACCAAGTGA R: CACACTGCCCATAATCCTCA	$(CT)_{17}(CA)_8$	164–168	59.5	KF855977
Linflata20	F: AAGGGTGAGGAAGTCCGATT R: ACCTTCAAAACCACCAGCAC	$(GA)_{16}$	129–189	59.9	KF855978
Linflata21	F: ACTGCTATCGCTCTCGATCTC R: TTTCCCAACAAACCTCCATC	$(GA)_{19}$	180–214	58.8	KF855979
Linflata22	F: CTTCATTTGAGCCTCGTAACC R: ACCTATATAAAAGACAATGTCAGCAA	$(GT)_{10}(GA)_{18}$	93–119	57.6	KF855980
Linflata23	F: CTCTCCCCCTTCGTTTTCTC R: GCAACAACACGAACACGATT	$(CT)_{18}$	165–244	59.6	KF855961
Linflata24	F: TCGTCAATTTTGTGGTTGTCA R: GCGGCAGTGAAGGAGAAATA	$(CT)_{21}(CA)_{20}$	208	60.0	KF855981
Linflata25	F: TTCAGGACATAAGCCTTGAACTC R: AAAAGAAATTTGCGCCACAC	$(CT)_{13}(CA)_{17}$	155–177	59.7	KF855982
Linflata26	F: GGATCATGCCTTGAATCTGC R: AACGGAGAAGCAACCCTTTT	$(CT)_{14}$	104–143	60.1	KF855983
Linflata27	F: CCACCTCTCCAGCAAAGAAC R: TCCCTCCCCATATTGTGTGT	$(GA)_{20}$	175–181	59.8	KF855984
Linflata28	F: CAAACACATGCAAACACACAA R: AGCAGTGCCCGTTTAGATTG	$(GA)_9$	183–197	59.0	KF855984

Note: T_a = annealing temperature.

TABLE 2. Allele numbers and cross-amplification for *Lobelia inflata* microsatellite loci. Data for *L. inflata* includes 58 individuals from three populations.

	L. inflata (N = 58)									No. of alleles detected for cross-amplification[a]	
	Petawawa, ON (n = 43)			Martock, NS (n = 8)			Petersham, MA (n = 7)				
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	*L. cardinalis* (n = 12)	*L. siphilitica* (n = 3)
Linflata1	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0
Linflata2	3	0.000	0.651	2	0.000	0.469	1	0.000	0.000	2	1
Linflata3	2	0.000	0.214	1	0.000	0.000	2	0.000	0.490	0	0
Linflata4	2	0.000	0.467	1	0.000	0.000	1	0.000	0.000	1	0
Linflata5	2	0.000	0.089	2	0.000	0.219	2	0.000	0.245	0	0
Linflata6	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	1
Linflata7	3	0.000	0.662	3	0.000	0.594	3	0.000	0.653	1	1
Linflata8	3	0.000	0.467	1	0.000	0.000	2	0.000	0.408	0	0
Linflata9	2	0.000	0.500	2	0.000	0.469	1	0.000	0.000	0	0
Linflata10	2	0.000	0.130	1	0.000	0.000	2	0.000	0.245	0	0
Linflata11	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0
Linflata12	4	0.000	0.586	2	0.000	0.375	2	0.000	0.408	2	0
Linflata13	2	0.000	0.478	1	0.000	0.000	2	0.000	0.490	0	0
Linflata14	2	0.000	0.439	1	0.000	0.000	1	0.000	0.000	1	1
Linflata15	1	0.000	0.000	1	0.000	0.000	2	0.000	0.490	0	0
Linflata16	2	0.000	0.273	2	0.000	0.219	1	0.000	0.000	1	1
Linflata17	2	0.000	0.130	2	0.000	0.469	2	0.000	0.490	1	0
Linflata18	2	0.000	0.576	1	0.000	0.000	1	0.000	0.000	0	0
Linflata19	2	0.000	0.169	1	0.000	0.000	1	0.000	0.000	0	1
Linflata20	2	0.000	0.283	2	0.000	0.469	1	0.000	0.000	0	0
Linflata21	3	0.000	0.580	2	0.000	0.469	3	0.000	0.388	1	1
Linflata22	2	0.000	0.423	1	0.000	0.000	1	0.000	0.000	0	1
Linflata23	2	0.000	0.357	1	0.000	0.000	1	0.000	0.000	0	0
Linflata24	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0
Linflata25	1	0.000	0.000	2	0.000	0.375	1	0.000	0.000	1	1
Linflata26	3	0.000	0.597	2	0.000	0.500	2	0.000	0.408	2	1
Linflata27	2	0.000	0.498	1	0.000	0.000	2	0.000	0.490	1	1
Linflata28	2	0.000	0.498	1	0.000	0.000	2	0.000	0.245	1	0

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; MA = Massachusetts; N = entire population size; n = individual population size; NS = Nova Scotia; ON = Ontario.

[a]Zero alleles detected for cross-amplification indicates failed amplification.

their usefulness as precise molecular clocks, in the absence of outcrossing, differences in SSR allele size may be proportional to lineage divergence time (Neff, 2004). We used GENEPOP version 4.2 to test linkage disequilibrium and Hardy–Weinberg equilibrium (Rousset, 2008). Observed heterozygosity was zero across all loci in *L. inflata*, a finding that supports the hypothesis that outcrossing is rare or nonexistent in field populations of *L. inflata*.

Cross-amplification success of these 28 loci in *L. cardinalis* and *L. siphilitica* is also presented in Table 2. There was successful amplification at 16 loci for *L. cardinalis*, with two to three alleles per locus, and at 11 loci for *L. siphilitica*, with one to two alleles per locus. For *L. cardinalis*, we found three loci (Linflata2, Linflata12, and Linflata26) to be polymorphic. We found no polymorphism for any loci in *L. siphilitica*.

CONCLUSIONS

These microsatellite markers are the first to be developed for *L. inflata*, and offer a new opportunity to investigate allelic diversity and gene flow within and among populations of *L. inflata*. Observed homozygote excess is likely due to lack of gene flow between *L. inflata* reproductive lineages. Successful cross-amplification of these loci in both *L. cardinalis* and *L. siphilitica* suggests that these loci may also be used to assess genetic diversity in congeneric species.

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Appendix 1. Voucher specimens used to characterize microsatellite markers in *Lobelia inflata*. All specimens were collected in Canada and the United States and are deposited at the Canadian National Collection of Vascular Plants (DAO), in Ottawa, Canada. Information listed below: taxon, voucher specimen, collection site, and geographic coordinates.

Lobelia inflata sect. *Lobelia* (Campanulaceae); voucher accession number DAO-887897; cultivated at Carleton University (45.3854°N, 75.6922°W)

Lobelia inflata sect. *Lobelia* (Campanulaceae); voucher accession number DAO-887899; collected from Petawawa Research Forest, Ontario (45.9902°N, 77.4413°W)

Lobelia inflata sect. *Lobelia* (Campanulaceae); voucher accession number DAO-887901; collected from Martock, Nova Scotia (44.9560°N, 64.1060°W)

Lobelia inflata sect. *Lobelia* (Campanulaceae); voucher accession number DAO-887902; collected from Harvard Forest, Petersham, Massachusetts (42.5314°N, 72.1899°W)

Lobelia siphilitica sect. *Lobelia* (Campanulaceae); voucher accession number DAO-887903; cultivated at Carleton University (45.3854°N, 75.6922°W)

Lobelia cardinalis sect. *Lobelia* (Campanulaceae); voucher accession number DAO-887904; cultivated at Carleton University (45.3854°N, 75.6922°W)