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PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF MICROSATELLITE MARKERS FOR THE CLEISTOGAMOUS SPECIES *LAMIUM AMPLEXICAULE* (LAMIACEAE)[1]

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- *Premise of the study: Lamium amplexicaule* is a cleistogamous plant that produces both closed flowers (obligately self-pollinated) and open flowers (potentially outcrossed). The conditions for the maintenance of such a mating system depend on the outcrossing rate of the open flowers, which can be estimated using neutral microsatellite markers.
- *Methods and Results:* Forty primer pairs corresponding to microsatellite motifs obtained by coupling multiplex microsatellite enrichment and next-generation sequencing were tested. Thirteen primers amplified with satisfying results. The polymorphism of these markers was studied in four French populations. Allele number varied from one to eight per locus and per population. Heterozygosity levels were significantly lower than those expected under Hardy–Weinberg equilibrium.
- *Conclusions:* Our results are consistent with a partial self-fertilization pattern. These markers will be used to estimate the outcrossing rate as well as population differentiation in *L. amplexicaule*.

Key words: cleistogamy; Lamiaceae; *Lamium amplexicaule*; microsatellite markers; outcrossing rate.

Cleistogamy is the coexistence of both closed flowers that are obligately self-pollinating and open flowers that are potentially outcrossing on the same individual. It has been generally considered as an example of a mixed mating system, and its evolutionary stability is still enigmatic (Goodwillie et al., 2005). *Lamium amplexicaule* L. is an annual cleistogamous weed of the mint family (Lamiaceae) native to Europe and Asia. The species has been introduced to all other continents where it has become invasive (USDA–ARS, 2003). It has been documented as both a winter annual (seeds dormant through autumn and winter, flowering in spring) and a summer annual (seeds dormant through spring and summer, flowering in autumn [Baskin and Baskin, 1981]). Even though nectar production and pollinators' visits of open flowers have been documented for this species (Orueta and Viejo, 1999), the species is considered as predominantly self-pollinating (Fryxell, 1957). The percentage of open flowers produced during the flowering season never exceeds 50% and can vary in response to environmental cues such as photoperiod and temperature, being as low as zero when plants are exposed to short, cold days (Lord, 1982).

Lord (1982) suggested that such a plastic production of open flowers is an adaptation to long, warm days, i.e., spring season, when pollinators are abundant. Likewise, when pollinators are scarce in the short, cold, autumn days, the production of closed flowers providing autonomous self-fertilization is favored. Such an adaptation assumes that open flowers are able to outcross substantially, thus increasing individual fitness by avoiding the deleterious effects of inbreeding (Charlesworth and Willis, 2009). To analyze the mating system of this species, we developed 13 microsatellite markers that will be used to estimate the outcrossing rate of *L. amplexicaule*.

METHODS AND RESULTS

Plant DNA was extracted from fresh plant tissue using the DNeasy Plant Mini Kit (QIAGEN, Courtaboeuf, France) following the manufacturer's protocol. An enriched DNA library was obtained by Genoscreen (Lille, France) by coupling multiplex microsatellite enrichment and next-generation sequencing on 454 GS-FLX Titanium platforms according to the method described in Malausa et al. (2011). Noncompound sequences containing microsatellite motifs longer than five repeats were retained. A total of 211 markers were returned, out of which the 40 markers with the longest repeat sequences were further tested for amplification. PCR amplification was performed in a final volume of 10 µL containing 5 µL of Multiplex PCR Master Mix (QIAGEN), 2 µM of each primer (Eurogentec, Angers, France), 2 µL of pure water, and 1 µL of DNA extraction solution (approximate concentration 50 ng/µL). PCR conditions were as follows: 15 min activation of the HotStart *Taq* DNA polymerase at 95°C, 30 cycles including 60 s initial denaturation at 94°C, 90 s at annealing temperature (Table 1), and 60 s extension at 72°C, followed by 30 min final extension at 60°C. Amplification of a DNA fragment of the expected size was

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TABLE 1. General characteristics of the amplified loci for *Lamium amplexicaule*.

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	Dye	Multiplex	GenBank accession no.
LA-Di02	F: CATATAACCTACATACCAAACCCTC R: GCCGGAGAGGTATTTTTGGT	$(CA)_{13}$	200–204	58	PET	1	JX050158
LA-Di05	F: ATTCAATTTTAGGGGGTCGG R: GTGATTCATTCCTTACAACTTTACC	$(GT)_{13}$	105–118	58	PET	1	JX050160
LA-He02	F: AGTTTCTCCACCGCAAACC R: ATCCCATCCACATCCATCAC	$(CCATCT)_7$	94–124	58	NED	1	JX050161
LA-He03	F: CGAAAGATGGACTGTTGTTCTG R: GGAGGCTAACAATTGCCATT	$(AAGAGG)_8$	89–131	58	FAM	1	JX050162
LA-Tri02	F: AGACAGAAGGCAAAGCTGGA R: ATTCCCTCGTATTCCAACCC	$(CTT)_{11}$	154–172	58	VIC	1	JX050166
LA-Tri07	F: CTGGGGGTGAAGGAATGAAT R: TCAATCTCATCCACAAGGCA	$(CTT)_{17}$	139–203	58	FAM	1	JX050168
LA-Te04	F: TGAGAACAATGTAATGCCAGAAA R: GGCACTTCTCCGACAAACTC	$(ATGT)_8$	155–204	58	FAM	2	JX050163
LA-Te05	F: GGGTTTTTCCCGATCTGAAT R: CTCTGTCCCATAAAAATATGTTCAGA	$(TACA)_9$	96–134	58	NED	2	JX050164
LA-Tri05	F: GAGTGGCGGCTCTAACTCAG R: TCTGCGAATTCACCCTTTCT	$(CTT)_{12}$	137–163	58	VIC	2	JX050167
LA-Te07	F: CTAATTGGGGATGTGAGATAAA R: CTCAACATTCGTTTCACCCA	$(ATAC)_{11}$	175–199	55	VIC	3	JX050165
LA-Tri08	F: AAGCAAGAAGTGGCCAAGTTA R: TGGTCTTAAATAGATTTCTTGT	$(TGT)_{19}$	243–278	55	VIC	3	JX050169
LA-Tri11	F: CAAAATCTACATAAACCCGAGA R: AGGAAGGATGCATACCATGC	$(TGA)_{23}$	85–123	55	FAM	3	JX050170
LA-Di03	F: TTAGTCGTCTGACCTTGGG R: AGTTGAGAGTTAAAACACTTAGTAAG	$(GA)_{13}$	162–189	52	PET	3 post PCR	JX050159

Note: T_a = annealing temperature.

obtained for 14 primer pairs. The forward primers of the 14 loci were labeled with a FAM, VIC, PET, or NED fluorescent dye (Applied Biosystems, Life Technologies SAS, Courtaboeuf, France). Using the same PCR protocol as above, each primer pair was tested on five individuals originating from five different locations in France. Out of the five individuals tested, three came from the populations M, C, and E used for the detailed polymorphism study below (GPS coordinates in Table 2), and two came from populations that were not used for further analysis (individual A1, coordinates: 43°38′19.86″N, 3°51′52.84″E; and individual B1, coordinates: 49°19′03.28″N, 05°04′24.34″E). PCR products were then diluted in water. Dilution varied from 1/50 to 1/200 according to the concentration of the PCR product. Three microliters of diluted PCR product were pooled in 15 µL of deionized formamide (Applied Biosystems) and 0.2 µL of 500 LIZ GeneScan size standard (Applied Biosystems). PCR products prepared this way were sized using an ABI PRISM 3100 sequencer (Applied Biosystems) and the software GeneMapper version 4.1. Thirteen primer pairs out of the 14 tested were polymorphic on the five individuals and had easily readable chromatograms (Table 1). These primer pairs were combined into three multiplexes for further PCR: mix 1 contained LA-Di02, LA-Di05, LA-He02, LA-He03, LA-Tri02, and LA-Tri07; mix 2 contained LA-Te04, LA-Te05, and LA-Tri05; and mix 3 contained LA-Te07, La-Tri08, LA-Tri11, and LA-Di03. Locus LA-Di03 was added in mix 3 after PCR amplification (Table 1).

A more detailed study of polymorphism was performed on individuals from four different populations of *L. amplexicaule* in France—two large populations with several hundred individuals each (population E around Montpellier, and population M around Dijon) and two small populations with fewer than 40 individuals

TABLE 2. Results of initial primer screening in four French populations[a] of *Lamium amplexicaule*.

	Population C (N = 19)				Population E (N = 40)				Population M (N = 36)				Population P (N = 19)			
Locus	A	H_o	H_e	A_p	A	H_o	H_e	A_p	A	H_o	H_e	A_p	A	H_o	H_e	A_p
Di02	1	—	—		2	0.051	0.097*	1	1	—	—		1	—	—	1
Di05	1	—	—		3	0.026	0.370*	1	2	0.000	0.475*		2	0.333	0.500	
He02	1	—	—		2	0.000	0.142*	2	2	0.000	0.056*	1	1	—	—	
He03	1	—	—		3	0.075	0.388*	2	3	0.028	0.344*	1	1	—	—	1
Tri02	1	—	—		2	0.026	0.204*	1	2	0.000	0.375*	1	1	—	—	1
Tri07	3	0.056	0.156*	2	7	0.231	0.681*	4	9	0.029	0.741*	4	1	—	—	
Te04	1	—	—		2	0.053	0.051	1	1	—	—		2	0.053	0.051	2
Te05	1	—	—		3	0.200	0.301*	3	1	—	—		1	—	—	
Tri05	1	—	—		3	0.075	0.119*	1	1	—	—		1	—	—	
Te07	1	—	—		2	0.077	0.163*		2	0.000	0.490*		2	0.000	0.198*	
Tri08	1	—	—		3	0.075	0.423*	2	1	—	—		0	NA	NA	
Tri11	2	0.059	0.057*	1	4	0.077	0.334*		2	0.000	0.424*	1	0	NA	NA	
Di03	1	—	—		2	0.086	0.082		2	0.000	0.312*	1	0	NA	NA	

Note: A = number of alleles; A_p = number of private alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of plants sampled; NA = no amplification.

[a]Populations: C = Dijon, small population (47°16′37″N, 5°03′43″E); E = Montpellier, large population (43°44′56″N, 3°51′06″E); M = Dijon, large population (47°15′58″N, 4°59′11″E); P = Montpellier, small population (43°46′16″N, 3°47′28″E).

* Significant deviation from Hardy–Weinberg equilibrium after Bonferonni correction.

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(population P around Montpellier, and population C around Dijon). GPS coordinates of the four populations are in Table 2. For each of the four populations a voucher has been deposited in the Botanical Institute of Montpellier herbarium collection (voucher accession numbers: population C, MPU000543; population E, MPU000544; population M, MPU000545; and population P, MPU000546). Nineteen to 40 individuals were sampled per population (Table 2). Total DNA was extracted from dried plant tissue using the DNeasy96 Plant Kit (QIAGEN) following the manufacturer's protocol for dry plant tissue, with an additional lysis step of incubation at 65°C for 90 min. Multiplex PCR amplification was performed with the same protocol as cited above.

The proportion of amplified individuals was high for most of the loci. In population P, loci LA-Di05, LA-Di03, LA-Tri08, and LA-Tri11 amplified poorly or not at all probably because of a substitution in the primer sequence. In the other three populations, the missing results were found mainly in one or two individuals that did not amplify throughout most of the loci. When the poorly amplified loci in population P were excluded, the mean proportion of individuals successfully amplified was 0.9 or higher for all populations. Expected heterozygosity and Hardy–Weinberg equilibrium tests were performed using GenAlEx (Peakall and Smouse, 2006). Number of alleles for each locus in the four populations, heterozygosity levels, and private alleles are shown in Table 2.

CONCLUSIONS

These newly developed microsatellite markers showed high amplification success even though their polymorphism levels within populations are not very high, especially in the small populations. Expected heterozygosity for polymorphic loci was generally low, and most of the polymorphic loci showed a significant deficit in observed heterozygotes. On the other hand, there are important differences in allelic patterns between populations. These observations are consistent with partially self-pollinating populations. Markers developed in this study can thus be used to (1) study structure and differentiation within populations and (2) calculate estimates of the outcrossing rate of open flowers in the large populations of *L. amplexicaule*. Estimating the outcrossing rate of open flowers will allow the relationship between cleistogamy rate and individual outcrossing rate to be determined and will thus relate the plasticity of cleistogamy to mating system plasticity.

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