Development of Microsatellite Markers for Leucosceptrum japonicum and L. stellipilum (Lamiaceae)

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PRIMER NOTE

DEVELOPMENT OF MICROSATELLITE MARKERS FOR *LEUCOSCEPTRUM JAPONICUM* AND *L. STELLIPILUM* (LAMIACEAE)[1]

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- *Premise of the study:* We developed 10 new primers for amplifying microsatellite loci for both *Leucosceptrum japonicum* and *L. stellipilum* to study the genetic structure of hybrid zones between these two species.
- *Methods and Results:* We isolated DNA fragments containing microsatellites from an enriched library and designed the 10 primer pairs amplifying the fragments. The number of alleles per locus ranged from one to 11 in both *L. japonicum* and *L. stellipilum.* The observed heterozygosity ranged from 0.00 to 0.84 and 0.00 to 1.00 in *L. japonicum* and *L. stellipilum*, respectively. The expected heterozygosity ranged from 0.00 to 0.84 and 0.00 to 0.89 in *L. japonicum* and *L. stellipilum*, respectively.
- *Conclusions:* These newly developed markers will be useful in investigating genetic structure and hybridization patterns in hybrid zones between *L. japonicum* and *L. stellipilum*.

Key words: genetic structure; Lamiaceae; *Leucosceptrum japonicum*; *Leucosceptrum stellipilum*; microsatellite; natural hybridization.

Natural hybridization is a common phenomenon in plant species and plays an important role in plant evolution because it can rapidly generate novel gene combinations (Rieseberg, 1997). The genus *Leucosceptrum* Smith (Lamiaceae) is endemic to eastern Asia and consists of five species. *Leucosceptrum japonicum* (Miq.) Kitam. & Murata and *L. stellipilum* (Miq.) Kitam. & Murata, which are morphologically distinct, are endemic to Japan (Murata and Yamazaki, 1993). *Leucosceptrum japonicum* is distributed throughout the entire Japanese archipelago while *L. stellipilum* is restricted to the western part of the Japanese mainland. In localities where these two species co-occur, natural hybridization between them has been reported (Takahashi, 2001). However, this report was made merely on the basis of morphological characters, and no genetic approaches have been used to investigate the genetic structure of the hybrid zone. In this study, we developed microsatellite markers amplifiable in both *L. japonicum* and *L. stellipilum* that could be used to investigate the genetic structure of a hybrid zone between these two species.

METHODS AND RESULTS

Voucher specimens used in this study were deposited at the herbarium of Tohoku University (TUS), Sendai, Miyagi Prefecture, Japan. Genomic DNA was extracted from fresh leaves of *L. japonicum* collected from a single individual from a population at Sakunami in northeastern Japan (Miyagi Prefecture; 38°22′16″N, 140°34′48″E; voucher no. TUS 419135) using a modified cetyltrimethylammonium bromide (CTAB) method (Maki and Horie, 1999). An enriched library was constructed by a modified biotin-capture method (Fischer and Bachmann, 1998). Five 100-ng aliquots of DNA were separately digested with *Nde*II (Nippon Gene, Tokyo, Japan), *Csp*6I, *Taq*I, *Hin*fI, and *HpyF*3I restriction enzymes (Fermentas, Vilnius, Lithuania). Digestions were carried out at 37°C for 1 h, except for *Taq*I, which was at 65°C for 1 h. Fragments of 400–1000 bp were excised from 1.2% agarose gel and purified with Quantum Prep Freeze ‘N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad, Hercules, California, USA). The size-selected fragments digested by *Nde*II were ligated into the sticky-end adapter described by Bloor et al. (2001). Those fragments digested with *Csp*6I, *Taq*I, *Hin*fI, and *HpyF*3I were separately ligated into modified adapters, which have sticky ends consisting of TA, CG, ANT, and TNA, respectively. PCR amplification was performed using oligo A as a primer (Bloor et al., 2001) following the amplification steps from Bloor et al. (2001) and used the ligated fragments as templates to test for successful ligation; the PCR products were then hybridized to the 5′ biotin-labeled oligonucleotide probes $(GT)_{12}$ and $(CT)_{12}$, respectively. DNA molecules hybridized to the biotin-labeled probes were subsequently captured by streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA). After washing with buffer I (6× saline sodium citrate [SSC] and 0.1% sodium dodecyl sulfate [SDS]) and buffer II (1× SSC and 0.1% SDS), captured DNAs were eluted into 200 μL of sterile water by boiling for 5 min. The enriched single-stranded DNA fragments were amplified by PCR using oligo A as the primer following the amplification steps from Bloor et al. (2001). The PCR products were ligated into pGEM-T Easy Vector (Promega Corporation) for 16 h at 4°C and then transformed into competent cells (DH5α; Toyobo, Osaka, Japan).

In total, 493 recombinant clones were sequenced with the universal M13 primers (forward: 5′-GTTTTCCCAGTCACGAC-3′, reverse: 5′-CAGGAAACAGCTATGAC-3′) using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) on an ABI 3100 Genetic Analyzer (Applied Biosystems). Microsatellite sequences were found in 73 clones, of which 46 with sufficient flanking regions were chosen for primer pair designs using the software Primer Premier 6.0 (Premier Biosoft International, Palo Alto, California, USA). A modified version of the method by Schuelke (2000) was used, employing three primers in the PCR reaction: the fluorescent-labeled universal primer, a locus-specific forward primer with a universal tail attached at its 5′ end, and a normal locus-specific reverse primer. Four primer sequences (tail A: 5′-FAM-GCCTCCCTCGCGCCATCAG-3′, tail B: 5′-VIC-GCCTTGCCAGCCCGC-3′, tail C: 5′-NED-CAGGACCAGGCTACCGTG-3′, and tail D: 5′-PET-CGGAGAGCCGAGAGGTG-3′) used by Blacket et al. (2012) were employed as universal primers.

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TABLE 1. Characteristics of the 10 microsatellites developed from *Leucosceptrum japonicum*.

Locus	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	Tail sequence[a]	GenBank accession no.
Leu1[b]	F: GCGGATTGCCAGAAAGTTTAGA R: AGAAAGTGCTCGACAACCTCAT	$(CA)_5CG(CA)_7CG(CA)_{12}C(GA)_7$	60	Tag-C	AB819716
Leu2[c]	F: AGCTAACACGGTCAGGAGAG R: CCTTGTGAACATGCAGTCCA	$(AC)_6N_n(AG)_8$	65	Tag-C	AB819717
Leu3[b]	F: AAGTTGACGACAGTCCATCTGC R: CAAGATATTCACGATGCGGACG	$(CA)_{10}$	60	Tag-D	AB819718
Leu4[c]	F: ACTCCTACCACACTACCATACT R: GCCTTCGCTCAATGTCCAT	$(GT)_{13}$	60	Tag-D	AB819719
Leu5[b]	F: TTGCTGTTCTGTTGTGCTCTT R: ACACAATTTCTCCAAACACACC	$(TG)_9$	65	Tag-A	AB819720
Leu6[c]	F: GTGAGCTTAGGAAATTGAGTCG R: GGGTTTAGGAGGAGGTTTGTG	$(TC)_{11}$	60	Tag-A	AB819723
Leu7[d]	F: ACGTGGGTTGGTTGTTGG R: CATCTTTGACTACTGCGACT	$(GA)_{12}$	60	Tag-B	AB819721
Leu8[d]	F: GACTGAGGAGGGTTATCT R: TCCGAAAGTTCTTAGTATTG	$(GT)_8(GA)_5AA(GA)_5$	60	Tag-C	AB819722
Leu9[c]	F: AGCGAGCCTTCTAACTCTTCAA R: TTGATGTAAGTGTGCGGCAGAA	$(TC)_{16}(CA)_4TG(CA)_3$	60	Tag-B	AB819714
Leu10[d]	F: ACTCTTTGCTCACACACTTCAG R: CGGCTATGGGTGATGTGAAG	$(GA)_3GT(GA)_4TCA(GA)_{11}$	60	Tag-A	AB819715

Note: T_a = annealing temperature.

[a]The tail sequences Tag-A (GCCTCCCTCGCGCCA), Tag-B (GCCTTGCCAGCCCGC), Tag-C (CAGGACCAGGCTACCGTG), and Tag-D (CGGAGAGCCGAGAGGTG) are attached at the 5′ end of each forward primer.

[b, c, d]Denote the three post-PCR multiplex sets designed by considering the fragment size and dye.

Initial testing of amplification of the primer pairs and loci polymorphisms was performed on three to four individuals of each species. PCR reactions were performed separately for each locus in 3-μL volumes containing 15–30 ng of genomic DNA, 1× Type-it Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), 0.075 μM tailed forward primer, 0.1 μM fluorescent-labeled universal primer, and 0.25 μM reverse primer. The reactions were started with an initial denaturation at 95°C for 5 min; followed by 32 cycles at 95°C for 30 s, locus-specific annealing temperature for 90 s (Table 1), and 72°C for 30 s; and 60°C for 30 min on the iCycler (Bio-Rad). Three post-PCR multiplex combinations (Table 1) were designed by considering the fragment size and dye. A mixture of 0.5 μL of each of the PCR products was diluted 1 : 20 with distilled water and run with GeneScan 600 LIZ Size Standard (Applied Biosystems) on an ABI 3100 Genetic Analyzer (Applied Biosystems). Allele sizes were assigned using GeneMapper version 3.7 software (Applied Biosystems).

Successful amplification of *L. japonicum* was detected at 15 loci, of which 10 were reliably amplified in *L. stellipilum.* These 10 loci were subsequently used for genotyping 25 individuals of *L. japonicum* collected from the Sakunami population and 11 individuals of *L. stellipilum* collected from the Kumano population (33°53′38″N, 135°52′12″E; voucher no. TUS 419136) (Table 2).

The number of alleles per locus ranged from one to 11 in both *L. japonicum* and *L. stellipilum.* A total of 50 and 51 alleles were detected at the 10 microsatellite loci among 25 individuals of *L. japonicum* and 11 individuals of *L. stellipilum*, respectively. All 10 loci were polymorphic among the 25 *L. japonicum* individuals, with the exception of one monomorphic locus (Leu4). Excluding Leu7 and Leu8, eight loci were polymorphic among the 11 *L. stellipilum* individuals. The observed heterozygosity ranged from 0.00 to 0.84 and 0.00 to 1.00 in *L. japonicum* and *L. stellipilum*, respectively. The expected heterozygosity ranged from 0.00 to 0.84 and 0.00 to 0.89 in *L. japonicum* and *L. stellipilum*, respectively. Tests for deviation from Hardy–Weinberg equilibrium (HWE) at each locus and the linkage disequilibrium (LD) of all combinations of the loci were conducted with GENEPOP 4.2 (Rousset, 2008). All 10 loci were not significantly deviated from HWE in either species, and no combination of the loci was in LD ($P > 0.05$) after sequential Bonferroni correction (Rice, 1989). Because the flowers of both *L. japonicum* and *L. stellipilum* were very frequently visited by bumblebees (Li and Maki, personal observation), these species appear to be predominantly outcrossing, which is consistent with their high genetic diversities and the lack of deviation from HWE.

TABLE 2. Results of primer screening of the 10 newly developed microsatellite loci in one population of *Leucosceptrum japonicum* and one population of *L. stellipilum*.

	L. japonicum (N = 25)					*L. stellipilum* (N = 11)				
Locus	A	H_o	H_e	P	Allele length range (bp)	A	H_o	H_e	P	Allele length range (bp)
Leu1	7	0.80	0.78	0.48	211–231	11	1.00	0.89	1.00	203–235
Leu2	6	0.43	0.43	0.12	142–162	6	0.64	0.65	0.29	148–174
Leu3	3	0.44	0.46	0.20	238–241	6	0.64	0.75	0.07	211–241
Leu4	1	0.00	0.00	—	258	5	0.64	0.63	0.11	256–268
Leu5	3	0.40	0.48	0.59	155–161	3	0.64	0.54	1.00	155–161
Leu6	6	0.52	0.56	0.42	156–176	3	0.18	0.43	0.06	162–168
Leu7	6	0.73	0.66	0.50	267–283	1	0.00	0.00	—	258
Leu8	2	0.29	0.50	0.07	346–354	1	0.00	0.00	—	336
Leu9	6	0.64	0.75	0.07	229–249	6	0.55	0.62	0.74	216–243
Leu10	10	0.84	0.84	0.39	127–201	9	0.73	0.86	0.33	138–157

Note: A = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals genotyped; P = probability of departure from Hardy–Weinberg equilibrium.

CONCLUSIONS

Reliable amplification in both *L. japonicum* and *L. stellipilum* and the relatively high level of diversity demonstrate the potential application of the microsatellite markers described here to investigate the genetic structure and hybridization patterns in hybrid zones between *L. japonicum* and *L. stellipilum*.

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