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

Authors: Li, Yue, and Maki, Masayuki

Source: Applications in Plant Sciences, 1(10)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1300038
Development of microsatellite markers for *Leucosceptrum japonicum* and *L. stellipilum* (Lamiaceae) \(^1\)

YUE LI \(^2,3\) AND MASAYUKI MAKI \(^2\)

\(^2\)Botanical Gardens, Tohoku University, Aoba, Sendai 980-0862, Japan

\(^3\)Institute of Life Sciences, Tohoku University, Aoba, Sendai 980-0862, Japan

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 *Leucosceptrum japonicum* (Miq.) Kitam. & Murata, which are morphologically distinct, 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; 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, *Hinf*I, and *Hpy*FIII 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, *Hinf*I, and *Hpy*FIII 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 1 (6x saline sodium citrate [SSC] and 0.1% sodium dodecyl sulfate [SDS]) and buffer 2 (1x 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’-GTTTTCATCGACTGAC-3’, reverse: 5’-CAGGAAACAGCTTAGAC-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 Biosoftware 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’-AMG-GCCTCCTCGCCGAGTCG-3’, tail B: 5’-VIC-GCCCTTGCCAGCAGGCTAG-3’, tail C: 5’-NED-CAGGACTCCATCCGTCGTGG-3’, and tail D: 5’-PET-CGGAGAGCCCAG-3’) were used by Blacket et al. (2012) were employed as universal primers.
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 reverse 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; 60°C for 30 s on the iCycler (Bio-Rad). Three post-PCR multiplex combinations (Table 1) were designed by considering the fragment size and dye.

Table 1. Characteristics of the 10 microsatellites developed from Leucosceptrum japonicum.

| Locus   | Primer sequence (5′–3′) | Repeat motif | T_a (°C) | Tail sequence* |
|---------|-------------------------|--------------|----------|----------------|
| Leu1b   | F: GCGGATTGCAGAAAAGTATTAGA | (CA)_4(CG)(CA)_5(GA)_5 | 60       | Tag-C          |
|         | R: AGAAAGTGGTTCGCAAACTCTAT |              |          |                |
| Leu2    | F: AGCCAGGGCTGACAGGAGAG | (AC)_2N(AG)_4 | 65       | Tag-C          |
|         | R: CCTTGGAAGTGGCGTCTCAA |              |          |                |
| Leu3b   | F: AAGTTGACAGATCGCCTACGTCG | (CA)_10 |          | Tag-D          |
|         | R: CAAGATATTCCGATGCGCAGGCG |              |          |                |
| Leu4    | F: ACTCTTACACACTACATCT | (GT)_5 | 60       | Tag-D          |
|         | R: GCCCTGTCCTAAGTCCTCA |              |          |                |
| Leu5    | F: TTGCCTCTCTGTTGCTCTTCTT | (TG)_9 | 65       | Tag-A          |
|         | R: AGACATTCTCCTGCAAAAGCACC |              |          |                |
| Leu6    | F: GTGAGCTTAGAAAAATGAGCTG | (TC)_11 | 60       | Tag-A          |
|         | R: GGGTTAGAGGAGGTTTGTG |              |          |                |
| Leu7d   | F: ACGCTGAGGTTGGTCTGG | (GA)_12 | 60       | Tag-B          |
|         | R: CATCTTGTACTGAGCGACT |              |          |                |
| Leu8d   | F: GCCTGAGGAGGTTATCT | (GA)_13 | 60       | Tag-C          |
|         | R: TCCGAAATCTTCGTATGGTTTG | (TG)_9 |          |                |
| Leu9    | F: AGCCAGGGCTTACTCTTCAAA | (TC)_8(CA)_5(TG)_6 | 60       | Tag-B          |
|         | R: TTGATGTAAGTGTGCTCTT | (TG)_9 |          |                |
| Leu10   | F: ACTCTTCTGTCGACACACACAG | (GA)_14(TG)_5(TA)_5(CA)_5 | 60       | Tag-A          |
|         | R: CCGCTATGGGTAGGTGAAAG |              |          |                |

Note: T_a = annealing temperature.
*The tail sequences Tag-A (GCCTCCCTCGCGCCA), Tag-B (GCCTTGCCAGCCCGC), Tag-C (CAGGACCAGGCTACCGTG), and Tag-D (CGGAGACCGCAGAGGT) are attached at the 5′ end of each forward primer.

Denote the three post-PCR multiplex sets designed by considering the fragment size and dye.

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 0.84 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. 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_e** | **H_s** | **P** | **Allele length range (bp)** | **A** | **H_e** | **H_s** | **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    | 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    | 0.00  | 258                           |
| Leu8                  | 2                      | 0.29    | 0.50    | 0.07  | 346–354                      | 1     | 0.00    | 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_s = 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*.

LITERATURE CITED

BLACKET, M. J., C. ROBIN, R. T. GOOD, S. F. LEE, AND A. D. MILLER. 2012. Universal primers for fluorescent labelling of PCR fragments: An efficient and cost-effective approach to genotyping by fluorescence. *Molecular Ecology Resources* 12: 456–463.

BLOOR, P. A., F. S. BARKER, P. C. WATTS, H. A. NOYES, AND S. J. KEMP. 2001. Microsatellite libraries by enrichment. Website http://www.genomics.liv.ac.uk/animal/MICROSAT.PDF [accessed 8 May 2012].

FISCHER, D., AND K. BACHMANN. 1998. Microsatellite enrichment in organisms with large genomes (*Allium cepa* L.). *BioTechniques* 24: 796–802.

MAKI, M., AND S. HORIE. 1999. Random amplified polymorphic DNA (RAPD) markers reveal less genetic variation in the endangered plant *Cerastium fischerianum* var. molle than in the widespread conspecific *C. fischerianum* var. *fischerianum* (Caryophyllaceae). *Molecular Ecology* 8: 145–150.

MURATA, G., AND T. YAMAZAKI. 1993. *Leucosceptrum* Smith. In K. Iwatsuki, T. Yamazaki, D. E. Boufford, and H. Ohba [eds.], Flora of Japan, vol. Illa, 84–85. Kodansha, Tokyo, Japan.

RICE, W. 1989. Analyzing tables of statistical tests. *Evolution; International Journal of Organic Evolution* 43: 223–225.

RIESEBERG, L. H. 1997. Hybrid origins of plant species. *Annual Review of Ecology and Systematics* 28: 359–389.

ROUSSET, F. 2008. GENEPOL’007: A complete re-implementation of the GENEPOL software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

SCHUELKE, M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* 18: 233–234.

TAKAHASHI, H. 2001. A preliminary study on hybrids between *Leucosceptrum stellatum* and *L. japonicum* (Labiatae). *Bulletin of the Floral Society of Gifu Prefecture* 17: 95–101.