Isolation and characterization of tetranucleotide microsatellite loci in Pinus massoniana (Pinaceae)

Guan, Lanhua
Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Suharyanto
Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Shiraishi, Susumu
Faculty of Agriculture, Kyushu University

出版情報：American Journal of Botany. 98 (8), pp.e216-e217, 2011-08. Botanical Society of America Inc.
バージョン：

American Journal of Botany

American Journal of Botany: e216–e217. 2011.

AJB Primer Notes & Protocols in the Plant Sciences

Isolation and characterization of tetranucleotide microsatellite loci in *Pinus massoniana* (Pinaceae)[1]

Lanhua Guan[2], Suharyanto[2], and Susumu Shiraishi[3,4]

[2]Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka 8128581, Japan;
[3]Faculty of Agriculture, Kyushu University, Fukuoka 8128581, Japan

- *Premise of the study*: Tetranucleotide microsatellite markers were developed for the first time in *Pinus massoniana* Lamb. to facilitate studies of population and conservation biology in this species.
- *Methods and Results*: Ten tetranucleotide microsatellite primer pairs were developed using dual suppression PCR. Seven, six, and eight of the primer pairs exhibited cross-species transferability to *P. thunbergii*, *P. densiflora*, and *P. luchuensis*, respectively. The number of alleles ranged from 1 to 31 per locus across four pine species.
- *Conclusions*: Considering its advantage over dinucleotide microsatellites in generating fewer artifacts arising from stutter bands, this tetranucleotide microsatellite panel will facilitate future population and conservation biological studies in *P. massoniana*. Six to eight markers can also be used in studies of three congeneric species.

Key words: microsatellite; *Pinus massoniana*; tetranucleotide; transferability.

Pinus massoniana Lamb., one of the most ecologically and economically important coniferous species, is widely distributed in southern China. However, to date, molecular marker-based studies have been limited to genetic diversity and population structure using isozymes (Huang and Zhang, 2000) and RAPD markers (Peng et al., 2003). Synecological studies have revealed that *P. massoniana* resources are decreasing (Ding and Song, 1998). It is urgent that molecular markers be developed to assess the genetic diversity and to promote the conservation and management of *P. massoniana* resources. The high reliability and high polymorphism of tetranucleotide microsatellites are ideal molecular markers for these studies (Guan and Shiraishi, 2011). In this study, we describe the isolation of 10 tetranucleotide microsatellite loci, their characterization, and their transferability to three closely related species.

METHODS AND RESULTS

Total genomic DNA was extracted from mixed seeds (voucher deposited at Silviculture Collection of KYU, KYUS-PM-039) using the sodium dodecyl sulfate (SDS) method (0.1 M Tris-HCl, 0.01 M EDTA, 1.0% SDS, 0.5% β-Mercaptoethanol). We developed the tetranucleotide microsatellite markers using the protocol of Lian and Hogetsu (2002) with modification (Guan and Shiraishi, 2011). A genomic library was constructed by digesting total genomic DNA with *Sau*3AI (New England Biolabs, Beverly, Massachusetts, USA) and ligating it with an adaptor (consisting of a 43-mer: 5′-CTGGTGCAAGGTTCAA GCTACTGGAGGGCAGGCACGGGAGATC –3′ and a 12-mer with amino-residue-capped 3′-end and phosphorylated 5′-end: 5′-TCCCGTGCCTGC-3′). $(GATA)_n$ or $(TACA)_n$ repeat regions were screened with the 5′ anchored $(GATA)_6$ or $(TACA)_6$ primer and adaptor primer. Nine primer pairs were designed for microsatellite marker development, and 10 loci were obtained (Table 1). A primer pair (B4535) generated two loci (B4535-1 and B4535-2). One locus was monomorphic, and the remaining nine loci were polymorphic.

The PCR amplification for the assessment of these microsatellite loci and the cross-amplification for three congeneric species, *P. thunbergii* Parl., *P. densiflora* Siebold & Zucc., and *P. luchuensis* Mayr, were performed according to the protocol of Guan and Shiraishi (2011). In *P. massoniana*, 24 individuals were sampled from a natural population in Hubei Province, China (31°01′48″N, 113°06′26″E). Sixteen individuals from each of the other three pine species were collected throughout Kyushu Island and the western part of Honshu Island (*P. thunbergii*: 31°15′35″N–34°10′57″N, 129°40′50″E–135°20′46″E; *P. densiflora*: 32°29′53″N–34°42′04″N, 129°49′32″E–134°38′05″E), and Okinawa Island, Japan (*P. luchuensis*: 26°35′39″N–26°35′55″N, 127°59′11″E–127°59′19″E).

The characteristics and transferability of 10 loci are shown in Table 2. Seven, six, and eight loci were successfully amplified in *P. thunbergii*, *P. densiflora*, and *P. luchuensis*, respectively. Locus B4640 was monomorphic in all species including *P. massoniana*. Locus B4546 was monomorphic in the three closely related species. The number of alleles ranged from 1 to 31 per locus across the four species. The observed and expected heterozygosities were estimated using CERVUS (Kalinowski et al., 2007) and are shown in Table 2. Except for monomorphic loci, the average observed heterozygosities (H_O) in *P. massoniana*, *P. thunbergii*, *P. densiflora*, and *P. luchuensis* were 0.67, 0.77, 0.62, and 0.67, respectively. Loci B4535-2 and B4631 showed significant deviation (GENEPOP; Rousset, 2008) from Hardy–Weinberg equilibrium (HWE) in three and two species, respectively.

Since megagametophyte (haploid tissue) samples of *P. massoniana* were not available, the Mendelian inheritance of these loci was confirmed by megagametophyte analyses in closely related species. Polymorphic loci B4521, B4535-2, B4630, B4631, and B4637 showed Mendelian segregations. In monomorphic loci B4535-1, B4546, and B4640, all megagametophytes of each haploid family had the same banding pattern. It is probable that the loci (B4638 and B4645), which were detected only in *P. massoniana*, also exhibit Mendelian inheritance (Table 2). The banding patterns generated by the B4535 primer pair seemed to be composed of a monomorphic locus (B4535-1) and a polymorphic locus (B4535-2), which indicates that these two loci may have arisen from one region by a duplication event (Kinlaw and Neale, 1997).

CONCLUSIONS

Ten tetranucleotide microsatellite markers were developed in *P. massoniana*, and six to eight of them demonstrated cross-species transferability. The tetranucleotide microsatellite shows fewer genotyping errors than the dinucleotide microsatellite.

[1]Manuscript received 13 February 2011; revision accepted 2 April 2011.
[4]Author for correspondence: sushi@agr.kyushu-u.ac.jp

doi:10.3732/ajb.1100076

TABLE 1. Characteristics of 10 tetranucleotide microsatellite markers developed in *Pinus massoniana*. For each primer pair, the forward and reverse sequences, repeat motif, size range (bp), annealing temperature (T_a) and GenBank accession number are shown.

Locus	Primer sequence (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	GenBank Accession No.
B4521	F: TGAGAATACCAATGACTAAGGAGG	$(GATA)_{12}$	193–233	58	AB607036
	R: CCTTCAAGGTGTCCAAATTGT				
B4535-1[a]	F: TGAATACCCTCAATAATTATAGCACAG	$(TA)_{10}(GATA)_{11}$	161–193	50	AB607037
B4535-2[a]	R: GATTTCGTGCTTCTAGTTTACCAA		235–297		
B4546	F: TCATTTCAAACTAAGATAAATTTATGCATT	$(GATA)_{11}$	202–218	50	AB607038
	R: ACCAGATTCATAAAAATATCTTAAGGTGGA				
B4630	F: AGCATGGGGAAATATTGGCAAGT	$(TACA)_8$	157–217	53	AB607040
	R: GATCATTTAATCTTGTAAGCGTTTTTG				
B4631	F: AAATATATGAAGAATGTGCAA	$(TACA)_{10}$	291–331	50	AB607041
	R: ATGATACATAATGCACGTTA				
B4637	F: GGAAATAATCCTTCTCTCGCCTCC	$(TACA)_{15}$	255–271	53	AB607042
	R: CTAGCCCCAAATCTGTCTAAGTGA				
B4638	F: AATTTTTCATATTCTTAGATAGGAGT	$(TACA)_6$	139–151	50	AB607043
	R: TTTACAAAGTAGGAGAAACTGAGCA				
B4640	F: CTGTACACATGGCCCACTCG	$(TACA)_4$	299	50	AB607044
	R: TTCATCATCTAACAAGGTCGTCAGC				
B4645	F: CCGTTGGGTAGATTGATTCCACT	$(TC)_9(TACA)_{14}$	244–280	56	AB607045
	R: AACACTTGATAAATATGGGTTACGATG				

[a] Indicates the two loci generated by the B4535 primer pair.

TABLE 2. Results of initial primer screening in a population of *P. massoniana* and the transferability of the primer pairs to *P. thunbergii*, *P. densiflora*, and *P. luchuensis*. For each primer pair the number of alleles (A), the mean values of observed (H_O) and expected (H_E) heterozygosity, and the total number of alleles (A_T) across the four species are shown. The sample size for each population is shown in parentheses.

	P. massoniana (24)			*P. thunbergii* (16)			*P. densiflora* (16)			*P. luchuensis* (16)			
Locus	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E	A_T
B4521	12	0.875	0.883		No product			No product		5	0.786	0.675	13
B4535-1	5	0.667	0.573	1	0	0		No product		1	0	0	5
B4535-2	20	0.542**	0.918	14	0.800*	0.943	9	0.467**	0.811	11	0.500	0.720	31
B4546	5	0.500	0.636	1	0	0	1	0	0	1	0	0	6
B4630	10	0.792	0.813	8	0.733	0.818	7	0.867	0.775	10	0.933	0.897	18
B4631	10	0.875	0.871	9	0.625	0.776	4	0.250**	0.498	7	0.500*	0.675	14
B4637	5	0.583	0.570	15	0.933	0.906	8	0.875	0.847	5	0.636	0.645	19
B4638	3	0.375	0.488		No product			No product			No product		3
B4640	1	0	0	1	0	0	1	0	0	1	0	0	1
B4645	7	0.833	0.764		No product			No product			No product		7

Note: * and ** Significant deviation from Hardy–Weinberg equilibrium at $P < 0.05$ and $P < 0.01$, respectively.

Therefore, these markers will serve as a useful resource in population and conservation biological studies of *P. massoniana* and its closely related pine species.

LITERATURE CITED

DING, S. Y., AND Y. C. SONG. 1998. Declining causes of *Pinus massoniana* in the processes of succession of evergreen broad-leaved forest. *Acta Botanica Sinica* 40: 755–760 (in Chinese with English abstract).

GUAN, L., AND S. SHIRAISHI. 2011. Tetranucleotide microsatellite markers in *Cryptomeria japonica* D. Don. *Conservation Genetics Resources* 3: 283–285.

HUANG, Q. Q., AND Y. X. ZHANG. 2000. Study on the genetic structure in *Pinus massoniana* (Masson pine) populations. *Silvae Genetica* 49: 190–195.

KALINOWSKI, S. T., M. L. TAPER, AND T. C. MARSHALL. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* 16: 1099–1106.

KINLAW, C. S., AND D. B. NEALE. 1997. Complex gene families in pine genomes. *Trends in Plant Science* 2: 356–359.

LIAN, C., AND T. HOGETSU. 2002. Development of microsatellite markers in black locust (*Robinia pseudoacacia*) using a dual-supression-PCR technique. *Molecular Ecology Notes* 2: 211–213.

PENG, S. L., Q. F. LI, D. LI, Z. F. WANG, AND D. P. WANG. 2003. Genetic diversity of *Pinus massoniana* revealed by RAPD markers. *Silvae Genetica* 52: 60–63.

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