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PRIMER NOTE

DEVELOPMENT OF POLYMORPHIC MICROSATELLITE MARKERS FOR JAPANESE YEW, *TAXUS CUSPIDATA*, AND *T. CUSPIDATA* VAR. *NANA* (TAXACEAE)[1]

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- *Premise of the study: Taxus cuspidata* (Taxaceae), which is well known for the effective anticancer metabolite paclitaxel (e.g., taxol), is an evergreen needle-leaved tree widely distributed in eastern Eurasia including Japan. We developed 15 microsatellite markers from this species and confirmed their utility for the dwarf variety *nana*, which is common in alpine regions along the Sea of Japan.
- *Methods and Results:* Thirteen polymorphic loci were characterized for genetic variation in three populations of *T. cuspidata*. The number of alleles per locus ranged from 11 to 31, with an average of 18.5; the expected heterozygosity ranged from 0.78 to 0.95, with an average of 0.89. All loci were successfully amplified in *T. cuspidata* var. *nana* and showed high polymorphism.
- *Conclusions*: These markers will be useful for investigating speciation and range formation of *T. cuspidata* in Japan, and the results will provide crucial information for the conservation of *Taxus* species.

Key words: gymnosperm; microsatellite; molecular marker; Taxaceae; *Taxus cuspidata*.

Taxus cuspidata Siebold & Zucc. (Taxaceae) is an evergreen needle-leaved tree with a straight trunk (up to 20 m). It grows at low density in mixed broadleaved forests throughout the cool temperate zone of Japan, Korea, northeastern China, and the extreme southeast of Russia (Hayashi, 1954). In contrast, its dwarf variety, *T. cuspidata* var. *nana* Hort. ex Rehder, is a small to medium-sized shrub (≤2 m) that locally dominates in alpine regions with heavy snowfall along the Sea of Japan (Hayashi, 1954), thus demonstrating a clearly disjunct distribution from that of *T. cuspidata*. *Taxus* species, including *T. cuspidata* and *T. cuspidata* var. *nana*, are well known as sources of paclitaxel, an anticancer metabolite first found in the bark of the Pacific yew (*T. brevifolia* Nutt.; Wani et al., 1971). Because paclitaxel has been difficult to synthesize on an industrial scale (Glowniak et al., 1996; Sottani et al., 2000), its production has continued to rely heavily on natural resources until relatively recently. Consequently, some *Taxus* species have been overexploited.

Although the overexploitation of *T. cuspidata* and *T. cuspidata* var. *nana* has not been reported, the assessment of the distribution of genetic resources formed through speciation and of species' range formation is essential to the long-term management of economically valuable natural resources. Being derived from transcripts, expressed sequence tag (EST)–simple sequence repeat (SSR) markers are useful for assaying functional diversity in natural populations (Varshney et al., 2005). Ueno et al. (2015) developed 80 EST-SSR markers for *T. cuspidata*; however, levels of diversity are often lower in EST-SSRs than in genomic SSRs. Although genomic SSR markers were developed in some relatives of *T. cuspidata* (e.g., Dubreuil et al., 2008; Cheng et al., 2015a), their utility for *T. cuspidata* was limited and the level of polymorphism in *T. cuspidata* populations was low (Cheng et al., 2015b). Therefore, we have developed highly polymorphic, genomic microsatellite markers to investigate the spatial genetic structure of *T. cuspidata*. This paper reports 15 genomic microsatellite markers developed for *T. cuspidata* by using next-generation sequencing technology and their utility for *T. cuspidata* var. *nana*.

METHODS AND RESULTS

Three *T. cuspidata* populations were sampled throughout the species range in Japan: Mt. Rausu, Hokkaido (the north-easternmost habitat in Japan; 44°04′54″N, 145°07′33″E); Mt. Kurai, Gifu Prefecture (36°02′30″N, 137°11′80″E); and Mt. Ohnogara, Kagoshima Prefecture (the southernmost habitat; 31°29′17″N, 130°49′10″E). One *T. cuspidata* var. *nana* population was also sampled on Mt. Hyono, Hyogo Prefecture (35°21′23″N, 134°30′81″E; Appendix 1). A single leaf from each of 10 adult trees in each population was collected. Total genomic DNA was isolated from ~50 mg of leaf tissue from each tree by using the hexadecyltrimethylammonium bromide mini-prep procedure (Stewart and Via, 1993).

Approximately 200 ng of DNA extracted from one individual of *T. cuspidata* collected in Mt. Kurai was used for library preparation with a TruSeq Nano DNA Library Prep Kit (Illumina, San Diego, California, USA). Sequencing was performed on a MiSeq Benchtop Sequencer (Illumina) in 2 × 300-bp read mode. The data were assembled into contigs in fastq-join software (Aronesty, 2011). Microsatellite regions were mined among contigs of >400 bp in MSATCOMMANDER version 1.0.8 software (Faircloth, 2008). The search parameter was restricted to dinucleotide motifs with a minimum of 16 repeats. Primer pairs for microsatellite amplification were designed in Primer3 version 2.2.3 software (Rozen and Skaletsky, 1999) with default parameter settings.

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TABLE 1. Characteristics of 15 microsatellite loci for *Taxus cuspidata* and *T. cuspidata* var. *nana*.

Locus	Primer sequences (5′–3′)	Repeat motif	Fluorescent label	Allele size range (bp)	GenBank accession no.
TC00388	F: TCCAACAAATCTAACATAGACCCTGT	$(AT)_{24}$	VIC	154–204	LC111519
	R: TCTCTGTGTAAAACTGGTTTATTGCT				
TC18856	F: TTCCCCTTTGTGCACCCTTT	$(AT)_{23}$	VIC	297–383	LC127209
	R: TGGGATTTGTATGGGAGCAAGT				
TC20343	F: TGCAACCATGAATGTATTTGTACT	$(AC)_{21}$	VIC	166	LC127206
	R: AGAGCATAAAGTCGGTTCGTT				
TC23535	F: CCTTACCCTTGTGGACGTGT	$(AT)_{24}$	FAM	108	LC127207
	R: CCAAGCAGTGAAAAATTCAAGCA				
TC35366	F: CCAAAGGTGTGGGCTTAAGC	$(AT)_{24}$	VIC	190–254	LC111520
	R: AACCATATCCCTCAGGTGCA				
TC39117	F: GGGAGAGAGAAAGTGGGGGA	$(AG)_{20}$	FAM	74–116	LC111521
	R: TCCAGGATTCAGTAGGGGCA				
TC47222	F: GTTGTGAGCCTTCTCTGCCT	$(AT)_{22}$	FAM	250–298	LC111522
	R: AGGCTTGATTCCTTTTTAGCCT				
TC43389	F: GCCACAGTCAATGGTACCCT	$(AT)_{22}$	FAM	247–283	LC127210
	R: AGGAAACAAAATTTAGCTACCCCA				
TC48340	F: TGGAGTCCAGCAATGGTTGT	$(AT)_{26}$	VIC	124–276	LC111523
	R: ACAAGAATGGTTCGGACTTGT				
TC63749	F: GCAACATGGACATCTCTTGCT	$(AT)_{22}$	VIC	214–302	LC111524
	R: GCCACAAAAACGAGACACTCA				
TC71760	F: AGTGTGAGAGGATGCATATGC	$(AT)_{23}$	FAM	227–263	LC111525
	R: AGAACCGGGTCAAACCAATGT				
TC74830	F: TGCTCCAATGGGTCTATGGTC	$(AT)_{22}$	FAM	274–336	LC111526
	R: CCATTCGACCCAAGACCACA				
TC82541	F: TGGAAAGGCATGAAGAGGGG	$(AG)_{18}$	VIC	305–379	LC111527
	R: TCCTCTTGAGGTGCACCCTA				
TC84266	F: AGTGGGACTCAACACCATGC	$(AT)_{23}$	FAM	137–177	LC127208
	R: TGCTCACATGGTTTTGCATGG				
TC99217	F: TGTTGTAGTGACCCAATATGGT	$(AT)_{20}$	VIC	351–397	LC111528
	R: ACAATGGAGTTTGGAGCCCA				

PCR amplifications followed the standard protocol of the QIAGEN Multiplex PCR Kit (QIAGEN, Valencia, California, USA) in a final volume of 10 µL, which contained 5 ng of extracted DNA, 5 µL of 2× Multiplex PCR Master Mix, and 0.2 µM of each multiplexed primer. Forward primers were labeled with fluorochromes 6-FAM or VIC (Life Technologies, Carlsbad, California, USA). Amplification was performed in a Veriti Thermal Cycler (Life Technologies) under the following conditions: initial denaturation at 95°C for 15 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min 30 s, and extension at 72°C for 1 min; and a final extension at 60°C for 30 min. The size of the PCR products was measured in an ABI PRISM 3130XL Genetic Analyzer (Life Technologies) by GeneMapper software (Life Technologies). Because microsatellite markers with high numbers of repeat units may cause unstable results due to PCR error (Shinde et al., 2003), we conducted PCR amplifications twice in four randomly selected individuals.

The genetic polymorphism at each locus was assessed by calculating the observed number of alleles, observed heterozygosity (H_o), and expected heterozygosity (H_e). Genotypic linkage disequilibrium was tested for all combinations of locus pairs within a population by using the Markov chain method provided by Web version 4.2 of GENEPOP software (Raymond and Rousset, 1995). Significance values were computed for each population by using Fisher's method for combining independent test results. The significance of deviations from Hardy–Weinberg equilibrium (HWE) in each population, represented by the deviation of the fixation index from 0, was tested by 1000 random permutations of alleles in each population at each locus in FSTAT version 2.9.3 (Goudet, 1995).

TABLE 2. Genetic variation of the 13 polymorphic microsatellite loci for three populations of *Taxus cuspidata* and one population of *T. cuspidata* var. *nana*.

		T. cuspidata									*T. cuspidata* var. *nana*		
		Mt. Rausu (n = 10)			Mt. Kurai (n = 10)			Mt. Ohnogara (n = 10)			Mt. Hyono (n = 10)		
Locus	A[a]	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
TC00388	16 (17)	9	0.400*	0.860	9	0.400*	0.855	7	0.600	0.775	6	0.400	0.775
TC18856	17 (21)	10	0.600*	0.860	5	0.600	0.770	6	0.600	0.650	11	0.600	0.890*
TC35366	13 (18)	7	0.500	0.750	6	0.700	0.770	7	0.600	0.695	10	1.000	0.870
TC39117	17 (19)	13	0.900	0.900	9	0.600	0.825	5	0.700	0.675	7	0.900	0.800
TC47222	19 (19)	11	0.800	0.885	10	0.600	0.870	8	0.700	0.830	5	0.500	0.590
TC43389	11 (13)	8	0.500*	0.835	4	0.000*	0.640	6	0.400*	0.770	6	0.200	0.765*
TC48340	31 (34)	16	1.000	0.930	13	0.900	0.905	7	0.700	0.745	9	0.600	0.835
TC63749	25 (31)	15	0.800	0.920	13	1.000	0.900	5	0.400	0.740	14	0.900	0.885
TC71760	17 (17)	10	1.000	0.865	10	0.900	0.820	8	0.900	0.820	5	0.900	0.745
TC74830	25 (28)	15	0.700	0.895	13	1.000	0.905	9	0.800	0.855	8	0.700	0.735
TC82541	19 (23)	7	0.900	0.740	11	0.900	0.830	10	1.000	0.860	9	1.000	0.865
TC84266	18 (20)	8	0.400*	0.665	9	0.600*	0.825	8	0.800	0.745	8	0.800	0.720
TC99217	12 (12)	7	0.800	0.760	8	0.800	0.805	4	0.600	0.635	6	0.700	0.725

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.

* Significant deviation from Hardy–Weinberg equilibrium expectations ($P < 0.01$).

[a] Numbers in parentheses are the total numbers of alleles observed among all four populations.

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Bonferroni's correction was applied to all pairwise test results to adjust for multiple comparisons. The potential presence of null alleles was assessed in MICRO-CHECKER version 2.2.3 software (van Oosterhout et al., 2004), using the second method of Brookfield (1996) to calculate the expected frequency of null alleles.

One individual of *T. cuspidata* provided a total of 2046 Mbp and 6,796,562 reads. A total of 1,797,717 contigs were assembled, and dinucleotide motifs with a minimum of 16 repeats were identified in 3981 contigs. Screening of 24 randomly selected loci with a minimum of 16 repeats identified 15 loci with a clear, strong, single band for each allele (Table 1). Of the 15 loci, 13 were polymorphic and two were monomorphic. PCR error was not observed. The evaluation of polymorphism in the 30 adult trees showed that the 13 polymorphic loci were hypervariable (Table 2), with 11 (TC43389) to 31 (TC48340) alleles per locus (average: 18.5), and H_e from 0.78 (TC99217) to 0.95 (TC48340) (average: 0.89). At the population level, number of alleles ranged from four to 16 (average: 8.8), H_o from 0.00 to 1.00 (average: 0.70), and H_e from 0.64 to 0.93 (average: 0.80). Null alleles were significant at two loci (TC43389 and TC63749), at a frequency of 0.21 and 0.20, respectively. Among all loci in the three full-size populations, eight of 39 combinations deviated significantly from HWE ($P < 0.01$, Table 2). This lack of equilibrium could be explained by the small number of samples in each population. There was no evidence of significant linkage disequilibrium ($P < 0.05$) in any pair of loci. All 15 loci were successfully amplified in the *T. cuspidata* var. *nana* population and showed high polymorphism (Table 2).

CONCLUSIONS

In this study, 13 novel polymorphic microsatellite markers for *T. cuspidata* and *T. cuspidata* var. *nana* were developed. These microsatellite markers will be useful for investigating speciation and range formation of *T. cuspidata* and *T. cuspidata* var. *nana* in Japan, and the results will provide crucial information for conservation of *Taxus* species as sources of antitumor agents.

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Appendix 1. Voucher and location information for *Taxus cuspidata* and *T. cuspidata* var. *nana* populations used in this study. One voucher was collected from each population sampled.

Species	Collection locality	Geographic coordinates	Voucher collection no.[a]
T. cuspidata Siebold & Zucc.	Mt. Rausu, Hokkaido, Japan	44°04′54″N, 145°07′33″E	*T. Kondo 0053*
T. cuspidata	Mt. Kurai, Gifu Prefecture, Japan	36°02′30″N, 137°11′80″E	*T. Kondo 0067*
T. cuspidata	Mt. Ohnogara, Kagoshima Prefecture, Japan	31°29′17″N, 130°49′10″E	*T. Kondo 0073*
T. cuspidata var. *nana* Hort. ex Rehder	Mt. Hyono, Hyogo Prefecture, Japan	35°21′23″N, 134°30′81″E	*T. Kondo 0068*

[a]All vouchers were deposited in the Herbarium of the Graduate School for International Development and Cooperation, Hiroshima University, Hiroshima, Japan.

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