Development of polymorphic microsatellite markers for Japanese yew, Taxus cuspidata, and T. cuspidata var. nana (Taxaceae)

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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′30″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 hexadeeytrimethylammonium 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.

doi:10.3732/apps.1600020

Applications in Plant Sciences 2016 4(7): 1600020; http://www.bioone.org/loi/apps © 2016 Kondo. Published by the Botanical Society of America. This work is licensed under a Creative Commons Attribution License (CC-BY-NC-SA).
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: TCCACAAACTCAATAGACGCTTGT (AT)24 | VIC | 154–204 |
|         | R: TCCTCTGTTAAACCTGTTTATTCCTGT | (AT)23 | 297–383 | LC111519 |
| TC18856 | F: TTCCCTTGTGGCACCCTT | VIC | 190–254 | LC111520 |
| TC20343 | F: TGACCATGATGATTTTGACT | VIC | 166 | LC127206 |
|         | R: AGACGATATACCTGCTCTGT | (AT)21 | 74–116 | LC111521 |
| TC23535 | F: CTTTCACCTTGTGGGACGCTT | (AT)24 | 108 | LC127207 |
| TC35366 | F: CCAAAGTTGCTGGATTAAGC | (AT)24 | 190–254 | LC111520 |
| TC39117 | F: GGGAGAGAGAAAGTGGGGGA | (AT)20 | 74–116 | LC111521 |
| TC43389 | F: GCCACAGTCAATGGTACCCT | AGGCA | 247–283 | LC127210 |
| TC47222 | F: AGTGGGACTCAACACATGC | AGGCG | 250–298 | LC111522 |
| TC48340 | F: TGAGGCGGACGCATGCTG | (AT)26 | 124–276 | LC111523 |
| TC63749 | F: GCAACCAGATGAGATCTGCT | (AT)22 | 214–302 | LC111524 |
| TC71760 | F: AGTGGGACTCAACATGC | AGGCA | 227–263 | LC111525 |
| TC74830 | F: TGCCCTGAAAGGTTTATGGCT | (AT)22 | 274–336 | LC111526 |
| TC82541 | F: TGGAGTCCAGCAATGGTTGT | AGGCA | 305–379 | LC111527 |
| TC84266 | F: TGGAGTCCAGCAATGGTTGT | (AT)26 | 351–397 | LC111528 |
| TC99217 | F: CCAAAGGTGTGGGCTTAAGC | (AT)20 | 351–397 | LC111528 |

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 of the 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). The PCR products were analyzed by electrophoresis on 8% polyacrylamide gels with a denaturation at 72°C for 10 min and a final extension at 60°C for 1 min. PCR products were visualized using an ABI 3130XL Genetic Analyzer (Life Technologies) by GeneMapper software (Life Technologies, Carlsbad, California, USA). Amplification 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 of the 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). The PCR products were analyzed by electrophoresis on 8% polyacrylamide gels with a denaturation at 72°C for 10 min and a final extension at 60°C for 1 min. PCR products were visualized using an ABI 3130XL Genetic Analyzer (Life Technologies) by GeneMapper software (Life Technologies, Carlsbad, California, USA). Amplification 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 of the 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). The PCR products were analyzed by electrophoresis on 8% polyacrylamide gels with a denaturation at 72°C for 10 min and a final extension at 60°C for 1 min. PCR products were visualized using an ABI 3130XL Genetic Analyzer (Life Technologies) by GeneMapper software (Life Technologies, Carlsbad, California, USA).

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

| Locus   | Aa | A  | H  | H  | H   | H   |
|---------|----|----|----|----|-----|-----|
| TC00388 | 16 (17) | 9 | 0.400* | 0.860 | 9 | 0.400* | 0.855 |
| TC18856 | 17 (21) | 10 | 0.600* | 0.860 | 5 | 0.600 | 0.770 |
| TC35366 | 13 (18) | 7 | 0.500 | 0.750 | 6 | 0.700 | 0.770 |
| TC39117 | 17 (19) | 13 | 0.900 | 0.900 | 9 | 0.600 | 0.825 |
| TC47222 | 19 (19) | 11 | 0.800 | 0.885 | 10 | 0.600 | 0.870 |
| TC43389 | 11 (13) | 8 | 0.500* | 0.835 | 4 | 0.000* | 0.640 |
| TC48340 | 31 (34) | 16 | 1.000 | 0.930 | 13 | 0.900 | 0.905 |
| TC63749 | 25 (31) | 15 | 0.800 | 0.920 | 13 | 1.000 | 0.905 |
| TC71760 | 17 (17) | 10 | 1.000 | 0.865 | 10 | 0.900 | 0.820 |
| TC74830 | 25 (28) | 15 | 0.700 | 0.895 | 13 | 1.000 | 0.905 |
| TC82541 | 19 (23) | 7 | 0.900 | 0.740 | 11 | 0.900 | 0.830 |
| TC84266 | 18 (20) | 8 | 0.400* | 0.665 | 9 | 0.600* | 0.825 |
| TC99217 | 12 (17) | 7 | 0.800 | 0.760 | 8 | 0.800 | 0.805 |

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

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

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

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At the population level, number of alleles ranged from four to 16 (average: 8.8), (average: 18.5), and hypervariable (Table 2), with 11 (TC43389) to 31 (TC48340) alleles per locus and two were monomorphic. PCR error was not observed. The evaluation of strong, single band for each allele (Table 1). Of the 15 loci, 13 were polymorphicdomly selected loci with a minimum of 16 repeats identified 15 loci with a clear, reads. A total of 1,797,717 contigs were assembled, and dinucleotide motifs with

Appendix

The natural distribution of important trees, indigenous to Japan. Conifers report III (In Japanese with English abstract). Bulletin of Governmental Forestry Experimental Station 75: 1–173.

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. Journal of Heredity 86: 248–249.

Rozen, S., and H. Skaletsky. 1999. Primer3 on the WWW for general users and for biologist programmers. In S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

Shinde, D., Y. Lai, F. Sun, and N. Arnhem. 2003. Taq DNA polymerase slippage mutation rates measured by PCR and quasi-likelihood analysis: (CA/GT), and (A/T), microsatellites. Nucleic Acids Research 31: 974–980.

Sottani, C., R. Turel, G. Micolli, M. L. Fiolentino, and C. Minola. 2000. Rapid and sensitive determination of paclitaxel in environmental samples by high performance liquid chromatography tandem mass spectrometry. Rapid Communications in Mass Spectrometry 14: 930–935.

Stewart, C. N. Jr., and L. E. Via. 1993. A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other applications. BioTechniques 14: 748–750.

Ueno, S., Y. Wen, and Y. Tsumura. 2015. Development of EST-SSR markers for Taxus cuspidata from publicly available transcriptome sequences. Biochemical Systematics and Ecology 63: 20–26.

van Oosterhout, C., W. F. Hutchinson, D. P. M. Wills, and P. Shipley. 2004. MICRO-CHECKER. Software for identifying and correcting genotype errors in microsatellite data. Molecular Ecology Notes 4: 535–538.

Varshney, R. K., A. Graner, and M. E. Sorrells. 2005. Genic microsatellite markers in plants: features and applications. Trends in Biotechnology 23: 48–55.

Wang, M. C., H. L. Taylor, M. E. Wall, P. Coggon, and A. T. Mepham. 1971. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. Journal of the American Chemical Society 93: 2325–2327.

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.

LITERATURE CITED

Aronesty, E. 2011. ea-utils: Command-line tools for processing biological sequencing data. Website: http://code.google.com/p/ea-utils [accessed 17 June 2016].

Brookfield, J. F. Y. 1996. A simple new method for estimating null allele frequency from heterozygote deficiency. Molecular Ecology 5: 453–455.

Cheng, B. B., Q. W. Sun, and Y. Q. Zheng. 2015a. Development of microsatellite loci for Taxus wallichiana var. wallichiana (Taxaceae) and cross-amplification in Taxaceae. Genetics and Molecular Research 14: 16018–16023.

Cheng, B. B., Y. Q. Zheng, and Q. W. Sun. 2015b. Genetic diversity and population structure of Taxus cuspidata in the Changbai Mountains assessed by chloroplast DNA sequences and microsatellite markers. Biochemical Systematics and Ecology 63: 157–164.

Dubreuil, M., F. Sebastiani, M. Mayol, S. C. González-Martínez, M. Riba, and G. G. Vendramin. 2008. Isolation and characterization of polymorphic nuclear microsatellite loci in Taxus baccata L. Conservation Genetics 9: 1665–1668.

Farbrot, B. C. 2008. MSATCOMMANDER: Detection of microsatellite repeat arrays and automated, locus-specific primer design. Molecular Ecology Resources 8: 92–94.

Gloeckner, K., G. Zoroka, A. Jozeffczyk, and M. Furmanowa. 1996. Sample preparation for taxol and cephalomannine determination in various organs of Taxus sp. Journal of Pharmaceutical and Biomedical Analysis 14: 1215–1220.

Goudet, J. 1995. FSTAT (version 1.2): A computer program to calculate F-statistics. Journal of Heredity 86: 485–486.

Hayashi, Y. 1954. The natural distribution of important trees, indigenous to Japan. Conifers report III (In Japanese with English abstract). Bulletin of Governmental Forestry Experimental Station 75: 1–173.

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. Journal of Heredity 86: 248–249.

Rozen, S., and H. Skaletsky. 1999. Primer3 on the WWW for general users and for biologist programmers. In S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

Shinde, D., Y. Lai, F. Sun, and N. Arnhem. 2003. Taq DNA polymerase slippage mutation rates measured by PCR and quasi-likelihood analysis: (CA/GT), and (A/T), microsatellites. Nucleic Acids Research 31: 974–980.

Sottani, C., R. Turel, G. Micolli, M. L. Fiolentino, and C. Minola. 2000. Rapid and sensitive determination of paclitaxel in environmental samples by high performance liquid chromatography tandem mass spectrometry. Rapid Communications in Mass Spectrometry 14: 930–935.

Stewart, C. N. Jr., and L. E. Via. 1993. A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other applications. BioTechniques 14: 748–750.

Ueno, S., Y. Wen, and Y. Tsumura. 2015. Development of EST-SSR markers for Taxus cuspidata from publicly available transcriptome sequences. Biochemical Systematics and Ecology 63: 20–26.

van Oosterhout, C., W. F. Hutchinson, D. P. M. Wills, and P. Shipley. 2004. MICRO-CHECKER. Software for identifying and correcting genotype errors in microsatellite data. Molecular Ecology Notes 4: 535–538.

Varshney, R. K., A. Graner, and M. E. Sorrells. 2005. Genic microsatellite markers in plants: features and applications. Trends in Biotechnology 23: 48–55.

Wang, M. C., H. L. Taylor, M. E. Wall, P. Coggon, and A. T. Mepham. 1971. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. Journal of the American Chemical Society 93: 2325–2327.

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. |
|---------|---------------------|------------------------|-----------------------|
| 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 |

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