Isolation and Characterization of Microsatellite Markers in the Endangered Species *Taxus wallichiana* Using the FIASCO Method

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**Abstract.** The Himalayan yew, *Taxus wallichiana* Zucc., is an endangered species with a scattered distribution in the Eastern Himalayas and southwestern China. In the present study, 10 microsatellite markers from the genome of *T. wallichiana* were developed using the protocol of fast isolation by amplified fragment length polymorphism of sequences containing repeats (FIASCO). Polymorphism of each locus was assessed in 28 samples from four wild populations of the Himalayan yew. The allele number of the microsatellites ranged from two to five with an average of 2.9 per allele. The observed and expected heterozygosities varied from 0.00 to 1.00 and from 0.3818 to 0.7552, respectively. Cross-species amplification in another two yew species showed eight of them holding promise for sister species. Two of the 10 loci (TG126 and TC49) significantly deviated from Hardy-Weinberg expectations. No significant linkage disequilibrium was detected between the comparisons of these loci. These polymorphic microsatellite markers would be useful tools for population genetics studies and assessing genetic variations to establish conservation strategy of this endangered species.

In previous studies, random amplified polymorphic DNA (RAPD), intersimple sequence repeat (ISSR), and amplified fragment length polymorphism (AFLP) data (Mohapatra et al., 2008, 2009; Saikia et al., 2000; Zhang et al., 2009) have been used to investigate the genetic diversity of *T. wall-chiana*. However, the dominant nature of these genetic markers can lead to an underestimation of the recessive allele frequency in a population causing bias in the estimate of genetic diversity and genetic differentiation (Nybom, 2004). This problem does not occur with microsatellite markers. Compared with other, random fingerprinting techniques such as RAPDs, AFLPs, and ISSRs, microsatellites show numerous advantages because they are locus-specific, codominant, highly reproducible and usually highly polymorphic (Powell et al., 1996). The development of microsatellite makers of congeneric species was recently reported for *Taxus sumatrana* (Miq.) de Laub. (Huang et al., 2008), *T. yunnanensis* W.C. Cheng & L.K. Fu (Miao et al., 2008), and *T. baccata* L. (Dubreuil et al., 2008) although no nuclear microsatellite primers hitherto have been reported for *T. wallichiana* Zucc. Therefore, we set out to develop microsatellite markers (simple sequence repeat) for this species using the fast isolation by AFLP of sequences containing repeats (FIASCO) protocol of Zane et al. (2002).

We describe the development of 10 microsatellite loci for *T. wallichiana*. DNA was extracted from dried leaf tissues and ground in liquid nitrogen using a modified CTAB method (Doyle and Doyle, 1987). Total genomic DNA (≈500 ng) was completely digested with *Msel* and then ligated to an *Msel* AFLP adaptor. A diluted digestion–ligation mixture (1:10) was amplified with an adaptor-specific primer (5′-GATGAGTCTC GAGTAAAN-3′). Amplified DNA fragments, with a size range of 200 to 800 bp, were enriched for repeats by magnetic bead selection with a 5′-biotinylated (AC)15 and (AG)15 primer and (AAG)10 probe. The enriched fragments were amplified again with the previously mentioned adaptor-specific primer. Polymerase chain reaction (PCR) products were purified using an EZNA Gel Extraction Kit (Omega Bio-Tek, Guangzhou, China). The purified DNA fragments were ligated into the pGEM-T vector (Promega, USA) and transformed into DH5α cells (TakaRa, Dalian, China). Positive clones were tested by PCR using (AC)10/(AG)10 and T5′Sp as primers. In total, 425 clones with positive inserts were sequenced on an ABI PRISM 3730xl DNA sequencer (Applied Biosystems Inc., USA) according to the manufacturer’s protocols. A total of 183 (42%) sequences were found to contain microsatellite repeats, and 80 of these were suitable for designing locus-specific primers using the primer 5.0 program (Clarke and Gorley, 2001).

The levels of polymorphisms of all the 64 microsatellite loci were assessed in 28 DNA samples from four natural populations of *T. wallichiana*. The PCR reactions were...
performed in a total volume of 15 μL containing, 30 to 50 ng genomic DNA, 0.6 μM of each primer pair (Table 1), 7.5 μL 2 X Taq PCR MasterMix (Tiangen (Tiangen Biotech Co., Ltd, Beijing, China); 0.1 U Taq polymerase/μL, 0.5 mM dNTP each, 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM MgCl₂]. PCR amplifications were conducted on an MJ PTC-200 Thermal Cycler (Waltham, MA) under the following conditions: 97 °C for 3 min followed by 30 to 36 cycles at 94 °C for 30 s followed by the annealing temperature for each specific primer (Table 1) for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. The PCR products were separated on 8% polyacrylamide denaturing gels using a 20-bp or 10-bp ladder molecular size standard (OrangeRuler™; Fermentas, Shenzhen, China) visualized by silver staining.

In the end, 10 of the 64 screened primer pairs displayed polymorphisms. The sequences of the 10 microsatellite markers are submitted to GenBank (Table 1). Standard genetic diversity parameters, Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between pairs of loci were estimated using GENEPOP Version 3.4 (Raymond and Rousset, 1995; http://genepop.curtin.edu.au/). The number of alleles per locus (A) was two to five with an average of 2.9. The values for observed heterozygosity (Hₒ) and expected heterozygosity (Hₑ) ranged from 0.00 to 1.00 and from 0.3818 to 0.7552 with averages of 0.4782 and 0.5685, respectively (Table 1). Two of the 10 loci (TG126 and TC49) significantly deviated from HWE proportions. This might have been the result of the small population sizes and isolated samples used in this survey. No significant LD was detected between the loci (P > 0.001) in our analysis.

To test for cross-species/varietyp application, these 10 primer pairs were used on samples of *Taxus fatea* and *T. chinensis* var. *mairei*. Eight of the 10 primer pairs successfully amplified fragments in these two taxa, also showing microsatellite polymorphisms (data not shown).

The microsatellite markers isolated here detected polymorphisms even in our small set of DNA samples. They will be very useful for further investigating the spatial genetic structure, genetic diversity, and gene flow of *T. wallachiana*, which will provide additional insight into population genetics and help to develop viable strategies for the conservation and management of this threatened medicinal plant. The promising cross-taxa applicability indicates their usefulness in allied taxa of this genus.

### Literature Cited


