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# Development of microsatellite loci for *Taxus wallichiana* var. *wallichiana* (Taxaceae) and cross-amplification in Taxaceae

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**ABSTRACT.** Nine polymorphic microsatellite loci were isolated and characterized in *Taxus wallichiana* var. *wallichiana*, an endangered species in China. The number of alleles per locus ranged from 2 to 20. Observed and expected heterozygosities varied from 0.0260 to 0.5325 and 0.3603 to 0.9231, respectively. Positive cross-amplification of the 9 loci was observed in 2 other varieties of *T. wallichiana* and 4 other Taxaceae species. These loci will be of value for studying population genetic structures and for genetic resource conservation in *T. wallichiana* and other *Taxus* species.

**Key words:** *Taxus wallichiana* var. *wallichiana*; Microsatellite loci; Cross-amplification; Genetic structure

 

## INTRODUCTION

*Taxus wallichiana* var. *wallichiana* Zucc., an evergreen shrub or tree species native to Sichuan, Tibet and Yunnan in China, frequently occurs along streams at altitudes from 2000-3500 m (Li and Fu, 1997; Fu et al., 1999; Farjon, 2001). This species is scattered in broad-leaved and mixed forests, and is often associated with bamboos (Li and Fu, 1997; Fu et al., 1999). It is widely used for Taxol (paclitaxel) extraction and production of wood for high-quality furniture and ornamental purposes. These important uses have resulted in considerable interest in the species over the last few decades and have led to over-exploitation of wild resources through destructive harvesting, which has caused substantial loss of genetic diversity. *T. wallichiana* is currently listed as a rare and endangered species on China's national Red List (Xinqiang et al., 1996). Protection of the species requires that harvesting from natural forests is prohibited immediately and measures need to be taken to conserve genetic diversity.

To understand the genetic diversity within *T. wallichiana*, it will be necessary to develop an appropriate panel of genetic markers. Simple sequence repeat (SSR) markers offer a powerful tool for characterizing genetic diversity and have been widely used for genome mapping, population genetics, and related areas (Ellegren, 2004; Kalia et al., 2011; Zalapa et al., 2012). In recent years, several SSR markers have been developed for different species of the genus *Taxus*, such as *T. sumatrana* (Huang et al., 2008), *T. yunnanensis* (Miao et al., 2014), *T. baccata* (Dubreuil et al., 2008), *T. chinensis* var. *mairei* (Zhou et al., 2009), and *T. wallichiana* (Yang et al., 2009).

However, the previously developed SSR markers for *T. wallichiana* have identified few polymorphisms. As polymorphic SSR markers are needed to characterize genetic diversity and phylogeography of the species, we initiated this study to develop and characterize new SSR markers for *T. wallichiana* var. *wallichiana*, that could be also be applied across the whole of the Taxaceae family. Here, we describe the development of new SSR markers and apply them to two *T. wallichiana* varieties (*T. wallichiana* var*. mairei and T. wallichiana* var. *chinensis*) and also show that they can be used for cross-amplification in 4 other *Taxus* species (*T. cuspidata* Sieb. et Zucc., *T. fuana* Nan Li et R. R. Mill., *Amentotaxus argotaenia* (Hance) Pilger, and *Pseudotaxus chienii* (Cheng) Cheng).

## MATERIAL AND METHODS

Leaves were collected from 77 *T. wallichiana* var. *wallichiana* individuals from 3 populations in Yunnan (100.785E, 24.474N), Hengduan Mountain Range (101.693E, 26.742N) and Himalayan Mountain Range (96.715E, 28.826N). Voucher specimens from the sampled populations were deposited in the herbarium of the Chinese Academy of Forestry (CAF). The leaves were dried using silica gel and stored at -80°C before use in experiments for analyzing SSR primer polymorphisms. Total genomic DNAs were extracted using a DNeasy Plant Kit (QIAGEN) following the manufacturer protocol.

Genomic DNA (5 µg) was sequenced on a 454 Life Sciences GS-FLX (Roche) at the Meiji Biotechnology Company (Shanghai, China). The raw sequences were assembled into contigs using Newbler 2.6 software. The sample occupied 12.5% of a plate and produced 208,698 reads, with an average fragment size of 555 bp; 5.8% of the sequences contained

microsatellites. All contigs or singletons longer than 100 bp were screened for microsatellites using MSATCOMMANDER version 0.8.2 (Faircloth, 2008) with default parameters (minimum-repeats were 10, 6, 5, 5, 5, and 4 for mono-, di-, tri-, tetra-, penta-, and hexa-nucleotide, respectively). Primers were designed with the software PRIMER3 (Rozen and Skaletsky, 1999). Forward primers were synthesized with a 19 bp M13 tail (5'-CACGACGTTGTAAAACGAC-3') at the 5'-end to allow labeling with a tailed fluorescent dye. All sequences were submitted to GenBank (accession Nos. KP702222 to KP702230).

We evaluated polymorphisms at each locus from the 77 sampled individuals. Although PCR conditions were optimized for each pair of primers, overall, they were similar. Each PCR amplification was performed in a 25 µL reaction solution containing approximately 50 ng template DNA, 12.5 µL 2X mix (Genestar, Beijing, China), 5 pmol each primer, 10.5 µL ddH2O. The following amplification program was used: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s extension; with a final extension step at 72°C for 5 min. The PCR products were further monitored and genotyped using a capillary electrophoresis system (CEQ 8000 Genetic Analyzer, Beckman Coulter). The results were exported to the Genomelab Genetic Analysis System.

The microsatellite loci were assayed in 4 Taxaceae species, namely, *T. cuspidata* Sieb. et Zucc., *T. fuana* Nan Li et R. R. Mill., *A. argotaenia* (Hance) Pilger, and *P. chienii* (Cheng) Cheng), and in 2 varieties of *T. wallichiana* , namely *mairei* and *chinensis*. PCR was performed as described above. The products were visualized on a 1.5% agarose gel run at 110 V for 20 min along with DL2000 Marker (Takara Biotechnology, Beijing, China); the gel was stained with GoldenView and photographed using the Bio-Rad Gel Documentation System. The loci were considered successfully amplified when at least one band of the expected size was observed.

The number of alleles, size range, expected heterozygosity ($H_E$) and observed heterozygosity ($H_O$) were quantified using POPGENE32 software (Yeh and Boyle, 1997). Deviations from Hardy-Weinberg equilibrium (HWE) for each locus and linkage disequilibrium among all loci were assessed by GENEPOP version 4.2 software (Raymond and Rousset, 1995).

## RESULTS

Of the total of 88 primer pairs designed, 48 pairs successfully amplified the expected product but only 9 loci showed polymorphisms. The PCR conditions and the characteristics of these 9 loci are given in Table 1. The allele number ($N_A$) of these polymorphic loci ranged from 2 (T10) to 20 (T2) (mean 8.44). $H_O$ and $H_E$ varied from 0.0260 to 0.5325 (mean 0.2468) and 0.3603 to 0.9231 (mean 0.6987) (Table 1), respectively. Among the 9 new SSR makers, 8 loci in plants from the Yunnan Plateau region, 7 loci in plants from the Hengduan Mountain region, and 8 loci in plants from the Himalayan region showed significant deviation from HWE after Bonferroni correction ($P < 0.05$), most likely due to excess homozygosity, or population structure within the samples, or limited sample size and sampling strategy. No significant linkage disequilibrium was detected between any pairs of loci.

Cross-amplification tests were performed for the two other varieties of *T. wallichiana* and four Taxaceae species (Table 2). The nine loci were successfully amplified in the varieties *T. wallichiana* var. *mairei* and *T. wallichiana* var. *chinensis* and species *T. fauna* and *T. cuspidata*; two were successful in *P. chienii*, and one in *A. argotaenia*.

**Table 1.** Characteristics of nine polymorphic microsatellite markers in three populations of *T. wallichiana* var. *wallichiana*.

| Locus | Primer sequences (5'-3') | Repeat motif | Total | | | Yunnan Plateau region (N = 22) | | | Hengduan Mountain region (N = 25) | | | Himalayan region (N = 30) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $N_A$ [Size range (bp)] | $H_O$ | $H_E$ | $N_A$ | $H_O$ | $H_E$ | $N_A$ | $H_O$ | $H_E$ | $N_A$ | $H_O$ | $H_E$ |
| T2 | F: AACGTTGTAAATCATTTGGACTCA<br>R: CGGCATGAAATAGGATCAAAC | $(AT)_{14}$ | 20 (138-184) | 0.5325 | 0.9231 | 5 | 0.6364 | 0.8087* | 12 | 0.4000 | 0.8767 | 12 | 0.5667 | 0.8729 |
| T10 | F: ACCATGGGATGAGGAAGATG<br>R: TCACCCCTCCATCTCAAAAG | $(AGG)_{10}$ | 2 (160-187) | 0.2468 | 0.3675 | 2 | 0.3182 | 0.4852 | 2 | 0.2400 | 0.2155 | 2 | 0.2000 | 0.3638* |
| T20 | F: TCTTAGCCCTTTGGTTCTACACA<br>R: ATTCTAGAGGGTTGATGCGAGA | $(TC)_7$ | 4 (174-180) | 0.3636 | 0.7353 | 4 | 0.7273 | 0.6797* | 4 | 0.2400 | 0.5935* | 4 | 0.2000 | 0.7237* |
| T38 | F: CAGATTTCAAACCTTTCGTGAG<br>R: ATCCATTTATGGCTTGGTGA | $(CATA)_{10}$ | 12 (113-186) | 0.4156 | 0.8550 | 6 | 0.6364 | 0.8034* | 8 | 0.2000 | 0.449 | 10 | 0.4333 | 0.8576* |
| T40 | F: CGATATCGTATCGGTTGAGACA<br>R: ATCGACGAGTCGCAAGATTTAT | $(CATA)_5$ | 7 (186-234) | 0.1688 | 0.7360 | 3 | 0.1364 | 0.6459* | 6 | 0.1200 | 0.7927* | 4 | 0.2333 | 0.4944* |
| T41 | F: CTTCTTGTTTTGTTGCAGGTTG<br>R: GCCGCCTAATTAAAACGTAGTG | $(CA)_9CG(CA)_7$ | 9 (180-221) | 0.2987 | 0.8021 | 9 | 0.3636 | 0.7896* | 6 | 0.4400 | 0.8155* | 4 | 0.1333 | 0.7051* |
| T42 | F: ACCCCATAGTTCAGGGTCGA<br>R: AGAAGGAGGCTCGGCTCTAA | $(AGATA)_{10}$ | 14 (180-230) | 0.0909 | 0.9079 | 4 | 0 | 0.6638* | 11 | 0.1200 | 0.8792* | 11 | 0.1333 | 0.8469* |
| T78 | F: CCGCTTCTCCACTCCTTCTT<br>R: GTGTTTGTGTTTGCGTTCGG | $(ATAG)_6$ | 3 (45-158) | 0.0779 | 0.6009 | 3 | 0.0909 | 0.4175* | 2 | 0.0800 | 0.2743* | 3 | 0.0667 | 0.5808* |
| T85 | F: TATAGGATAGGGGCTCGGGT<br>R: AACTTCACTATGGCACGTCG | $(TATC)_9$ | 5 (152-166) | 0.0260 | 0.3603 | 3 | 0.0455 | 0.3943* | 4 | 0.0400 | 0.5265* | 3 | 0.0000 | 0.1288* |

*Departs significantly from HWE at P < 0.05 (Bonferroni-corrected value).

**Table 2.** Cross-species amplification of other species and varieties in Taxaceae.

| | Locus | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Species/Variety | T2 | T10 | T20 | T38 | T40 | T41 | T42 | T78 | T85 |
| *Taxus wallichiana* var. *mairei* | + | + | + | + | + | + | + | + | + |
| *Taxus wallichiana* var. *chinensis* | + | + | + | + | + | + | + | + | + |
| *Taxus fuana* | + | + | + | + | + | + | + | + | + |
| *Taxus cuspidata* | + | + | + | + | + | + | + | + | + |
| *Pseudotaxus chienii* | - | - | ± | ± | ± | - | - | + | + |
| *Amentotaxus argotaenia* | - | - | - | ± | ± | - | - | ± | + |

Two individuals of each species were screened. (+) = expected size band amplification; (±) = unexpected size band amplification; (-) = no amplification.

## DISCUSSION

All novel markers in the present study showed high levels of polymorphism, indicating that these markers will of value to ongoing research into the population genetic structures, population structures, patterns of gene flow, and mating systems of all the species of the genus *Taxus* in China.

### Conflicts of interest

The authors declare no conflict of interest.

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