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Polymorphic microsatellite markers in *Taxus chinensis* var. *mairei* (Taxaceae)

WEI FU[1], ZHI-XIN DU[2], HE REN[1], NOPPARAT BUAHOM[3], HONG-WEI LIU[4] and XIN HUANG[1]*

[1]*Institute of Plant Quarantine, Chinese Academy of Inspection and Quarantine, Beijing, 100029, People's Republic of China*
[2]*Gangxi Entry-Exit Inspection and Quarantine Bureau, Gangxi 530028, People's Republic of China*
[3]*Department of Entomology, College of Agronomy and Biotechnology, China Agricultural University, Beijing 100093, People's Republic of China*
[4]*State Key Laboratory of Tree Genetics and Breeding, The Research Institute of Forestry, Chinese Academy of Forestry, Beijing 100091, People's Republic of China*

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Introduction

Taxus chinensis var. *mairei* is an endemic evergreen conifer in China. It is the most widely distributed species in the genus *Taxus* and primarily occurs south of the Yangtze river (Zhou *et al.* 2009). It is noteworthy that this species is considered an important source for the production of taxol, which is used in the treatment of various cancers (Ru *et al.* 2006). However, this species has been severely affected by overexploitation for economic purposes and is on the verge of extinction (Wu *et al.* 2012). *T. chinensis* var. *mairei* was listed in appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in 1995 and is one of the first-grade state protection plants (The State Council of the People's Republic of China 1999). Therefore, it is urgent to develop conservation strategies for this endangered species through the investigation of its genetic structure and diversity.

Microsatellites having high levels of polymorphism and codominant features (Wu *et al.* 2009) represent a useful marker system in population genetics analysis (Walter and Epperson 2001) and have been applied to many species of the genus *Taxus* (Dubreuil *et al.* 2008; Miao *et al.* 2008; Mohapatra *et al.* 2009; Mahmoodi *et al.* 2010). To date, a total of 15 polymorphic microsatellite loci have been reported in *T. chinensis* var. *mairei* (Zhou *et al.* 2009; Xue *et al.* 2012). In this study, we developed 12 new polymorphic microsatellite markers to assess the genetic diversity of this species. These 12 ploymorphic SSRs (simple sequence repeats) would be useful for facilitating the genetic conservation of *T. chinensis* var. *mairei*.

Materials and methods

A total of 123 individuals of *T. chinensis* var. *mairei* were collected from Jiangxi and Fujian provinces in China and were identified based on morphology. Genomic DNA was extracted from dried leaves using the DNeasy Plant Mini kit (Qiagen, Germany). A small insert genomic library was constructed by digesting genome DNA with *Sau*3AI (New England Biolabs, Beijing, China) for 4 h at 37°C. The digested DNA was separated with centrifugal concentrators. Fragments of 300–1000 bp were excised and recovered using the Agarose Gel Purification Kit (TaKaRa, Dalian, China). The purified fragments were then ligated to two adaptor oligonucleotides (adaptor A: 5′-GATCGTCGACGGTACCGAATTCT-3′; adaptor B: 5′-CAGCTGCCATGGCTTAAGAACTG-3′) with T4 DNA ligase (TaKaRa) overnight at 16°C. The ligation products were amplified using the adaptor A sequences as forward and reverse primers. The recovered polymerase chain reaction (PCR) products were denatured and stringently hybridized to a biotinylated $(CA)_{15}$ probes (Invitrogen, New York, USA). These heteroduplexes were then captured using Dynabeads Z548C Streptavidin (Promega, Beijing, China), eluted and the DNA enriched by PCR, with adaptor A as primer. Microsatellite-enriched DNA fragments were ligated into pEASY-T1 Cloning Kit Easy vector (Promega, Beijing, China) and used to transform DH5α competent cells (TaKaRa). The positive clones

*For correspondence. E-mail: 7548851@163.com.

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Table 1. Characterization of 12 polymorphic microsatellite loci in *Taxus chinensis* var. *mairei.*

Locus name (accession no.)	Primer sequence (5′–3′)	Repeat motif	Allele no.	T_m (°C)	Allele size (bp)	H_O	H_E	PIC
HDS1 (KC545815)	F:TGCGGAGTCATGTACAACTTAA R:CCCTTGCTTTGTACCATATGTGA	$(CA)_5$	14	58	151–200	0.545	0.759	0.733
HDS24 (KC545816)	F: TCATAACCCTTGGTGAGCCT R: GAGTGGGGTTCACAAAATGAT	$(CT)_8$	7	68	267–282	0.465	0.758	0.718
HDS27 (KC545817)	F:TGAGAAGGAGATGAATATGCAAG R:GTGCTTTGGTTCACACTTGAC	$(TG)_3T\ldots(CA)_5$	12	60	149–224	0.837	0.789	0.756
HDS63 (KC545818)	F: TCTCATCCATGATTAGTTTAGTAGC R: GGAGACCAACAATGAAGGAAT	$(CA)_5$	9	58	279–300	0.281*	0.304	0.284
HDS94 (KC545819)	F: GCCCGCAGATTGTGTTGT R: CATTCTAGAAGACCCCACAAGA	$(TC)_{17}$	10	68	144–165	0.650	0.759	0.724
HDS100 (KC545820)	F: GGATGGGTTTGATAAGGTGGT R: CCTTGTTCCTTGTTGCATTCT	$(CA)_5$	9	68	138–188	0.190*	0.541	0.500
HDS113 (KC545821)	F:GATTATCATAACTCAAGAAGTGTGC R:CCTGTTGTATTCACTACCCCTTAT	$(GT)_{5\ldots}(TG)_8$	20	58	355–412	0.784	0.896	0.884
HDS118 (KC545822)	F: TGAACCTCACACACCACATACT R: CGACATCATAAAAGTGCAAAG	$(TCAC)_5$	4	54	287–306	0.375	0.498	0.399
HDS131 (KC545823)	F: GTAAGTGTCTATTCCTCCTCGTT R: ACACACGCAAACGCAAAT	$(GT)_{5..}(TG)_{6..}(GT)_{5..}(GT)_5$ $(GT)_{5..}(CGTGTG)_{5..}(GT)_5$	11	58	293–360	0.317	0.369	0.355
HDS134 (KC545824)	F: CATGTGTGTGTTTGTGTGCAT R: GCAGACGTAGATGCATACATTG	$(GT)_{6..}(TG)_{6..}(TG)_{6..}(TG)_{10}$	7	58	246–263	0.647	0.696	0.641
HDS143 (KC545825)	F: ACCTGCAATAAAGCATTCACT R: CGACGATCATGTAATCTCTCTC	$(TG)_{7..}(TG)_{5..}(GA)_7$	6	56	264–282	0.508	0.555	0.486
HDS145 (KC545826)	F: AATGTACTCTTCTCATATGGGCA R: GACGATCTGTTTGGAGTTGTTCT	$(TG)_5$	19	68	151–251	0.287*	0.737	0.696

T_m (°C), annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphic information content; *deviation from HWE at $P < 0.05$ after Bonferroni correction.

were screened by PCR. Approximately 5.49% of the positive clones contained microsatellite sequences and 182 clones were sequenced on an ABI 3730XL DNA Analyser (Microread Company, Beijing, China). Eighty-seven clones yielded sequences of appropriate length and sufficient quality for primer design. Primers pairs were designed for these 66 putative loci using the Primer Premier 5.0 (Rozen and Skaletsky 2000), and 10 produced well-defined robust products of the expected size.

Amplification was performed in a 15 μL volume with 75 ng genomic DNA as template, 2.5× multiplex buffer, 1.0 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Microread, Beijing, China) and 5 μM of each primer (Sangon, Shanghai, China), one of which was labelled with a fluorescent dye. Amplifications were carried out in a veriti 96-well thermal cycler (Applied Biosystems, Foster City, USA) with the following conditions: 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 56°C for 45 s, and 72°C for 45 s; 10 cycles at 94°C for 30 s, 53°C for 45 s, 72°C for 45 s and a final extension at 72°C for 12 min. Fluorescently labelled fragments were visualised on an ABI Prism 377 DNA Sequencer with GeneScan-500 ROX size standard (Applied Biosystems). Allele size was called with GeneScan software, ver. 3.7 (Applied Biosystems).

Cervus ver. 2.0 (Marshall *et al.* 1998) was used to calculate the observed and expected heterozygosity (H_O and H_E) and to evaluate the deviation from Hardy–Weinberg equilibrium (HWE) and the polymorphic information content (PIC). The percentage of null alleles was tested with Micro-Checker ver. 2.2.3 (Van Oosterhout *et al.* 2004). Tests of linkage disequilibrium between pairs of loci were conducted with Arlequin 3.1 (Excoffier *et al.* 2005).

Results and discussion

Microsatellite variability was genotyped across 123 individuals of *T. chinensis* var. *mairei*, and 12 polymorphic microsatellites were developed. The details of the polymorphic microsatellite loci are summarized in table 1. The observed heterozygosity ranged from 0.287 to 0.837, and the expected heterozygosity ranged from 0.304 to 0.896, with means of 0.491 and 0.638, respectively. The values of PIC generally ranged from 0.284 to 0.884 (mean = 0.598). Eight loci were highly polymorphic (PIC $>$ 0.5). The number of alleles ranged from 4 to 20 at each locus, with an average of 10. These findings indicated that these markers will be useful for the analysis of the population structure and genetic diversity of *T. chinensis* var. *mairei*. Significant deviations from HWE because of a deficit of heterozygotes were detected at three loci HDS63, HDS100 and HDS145 ($P < 0.05$) after the application of a Bonferroni correction. An analysis conducted using Micro-Checker ver. 2.2.3 showed that this heterozygote deficiency may be due to the presence of null alleles at the three loci ($P < 0.001$). No significant linkage disequilibrium ($P < 0.01$) was observed for any pair of loci after Bonferroni correction. These loci showed high levels of polymorphism because the polymorphism per locus was positively related to the motif repeat size (Xue *et al.* 2012). In this study, the motif repeat size was large in these novel developed loci (table 1), and may have led to relatively more polymorphisms.

The microsatellite markers reported here represent an efficient tool for the evaluation of the genetic diversity and structure of *T. chinensis* var. *mairei* populations. We are using these loci to investigate gene flow and to assess the population genetic structure of this species. All 12 microsatellite markers will provide useful information for monitoring the introduction and spread of *T. chinensis* var. *mairei*.

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