American Journal of Botany

American Journal of Botany: e349–e351. 2011.

AJB Primer Notes & Protocols in the Plant Sciences

Development and characterization of microsatellites in *Torreya jackii* (Taxaceae), an endangered species in China[1]

Jian-Hui Li[2], Jing Jiao[2], Kai Jiang[2], and Yuan-Yuan Li[2,3,4]

[2]School of Resources and Environmental Sciences, East China Normal University, Shanghai 200062, China; and [3]Tiantong National Station of Forest Ecosystems, Shanghai 200062, China

- *Premise of the study:* Polymorphic microsatellite loci were developed in *Torreya jackii*, an endangered species in China, to provide markers for further studies on the genetic diversity of this species.
- *Methods and Results:* Eight polymorphic loci and one monomorphic locus were developed and characterized in four *T. jackii* populations (Xianju, Songyang, Pujiang, and Tonglu) from Zhejiang Province, China. The number of alleles per locus ranged from one to eight across 80 *T. jackii* individuals. At the eight polymorphic loci, the observed heterozygosity ranged from 0.150 to 1.000 and the expected heterozygosity ranged from 0.185 to 0.796.
- *Conclusions:* The microsatellite loci developed and characterized in this study will facilitate future analyses of the genetic diversity of *T. jackii*. Such information will aid in designing strategies to conserve this currently endangered species.

Key words: conservation; genetic diversity; microsatellites; Taxaceae; *Torreya jackii*.

Torreya jackii Chun (Taxaceae) is a dioecious, wind-pollinated, evergreen tree that is only distributed in Zhejiang, Fujian, and Jiangxi provinces of China. Owing to the narrow distribution and limited numbers of *T. jackii*, it is listed as an endangered species in China (Yu, 1999). Populations of *T. jackii* also suffer from habitat fragmentation, which may lead to negative demographic and genetic consequences (Chen, 2000). Low genetic diversity in *T. jackii* populations has been reported using dominant random amplified polymorphic DNAs (RAPDs) and intersimple sequence repeats (ISSRs) (Li and Jin, 2007; Li et al., 2007). To develop successful conservation strategies, we need to identify distinct populations, understand gene flow (especially contemporary dispersal), and determine populations of conservation priority (Lu et al., 2007). However, due to their dominance manner and, thus, low efficiency and low resolution, RAPDs and ISSRs are of limited use in these studies. By contrast, given their high levels of polymorphism and codominant inheritance, microsatellites are powerful and commonly used molecular markers (Liu et al., 2009). In the current study, we developed and characterized microsatellite loci for *T. jackii* to facilitate the analysis of genetic diversity, gene flow, and conservation genetics in this endangered species.

[1]Manuscript received 14 April 2011; revision accepted 30 June 2011.

This research was supported by the Natural Science Foundation of China (30870361, 30700101) and the Natural Science Foundation of Zhejiang Province (Y5090258).

[4]Author for correspondence: yyli@des.ecnu.edu.cn

doi:10.3732/ajb.1100179

METHODS AND RESULTS

We sampled four wild populations of *T. jackii* from Xianju, Pujiang, Songyang, and Tonglu in Zhejiang Province, China (Appendix 1). Fresh healthy leaves were collected randomly from 18 to 22 adult trees in each population and stored in silica gel. The distances between sampled trees were from 30 m to 50 m, depending on the population size. Total genomic DNA was extracted using a Plant Genomic DNA Extraction Kit (Tiangen, Beijing, China).We used the biotin–streptavidin capture method to develop the microsatellite loci (Xu et al., 2010). Approximately 300 ng of genomic DNA from an individual tree from Songyang was digested with *Mse*I restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA) and fragments 200–1000 bp in length were fractionated. We then ligated these fragments with an *Mse*I-adaptor pair. Five-microliter volumes of the products were used as templates for PCR with *Mse*I-N primer (5′-GATGAGTCCTGAGTAAN-3′). PCR was carried out in a total reaction volume of 20 μL under the following reaction conditions: denaturation at 95°C for 3 min, followed by 17 cycles of 30 s denaturation at 94°C, 1 min annealing at 53°C, and 1 min extension at 72°C. To enrich the fragments containing simple sequence repeats, the PCR products were denatured at 95°C for 5 min, and then hybridized with a 5′-biotinylated oligonucleotide probe $(AG)_{15}$ in 250 μL hybridization solution at 48°C for 2 h. Hybridization products were selectively captured with streptavidin-coated magnetic beads (Promega, Madison, Wisconsin, USA). After stringent washing to remove the unhybridized DNA, the captured DNA fragments were eluted in 50 μL of 1× TE buffer. The enriched product was amplified with *Mse*I-N as primers for 30 cycles, as described above. After purification with a multifunctional DNA Extraction Kit (Bioteke, Beijing, China), the PCR products were ligated into pMD 19-T vector (TaKaRa Biotechnology Co., Dalian, China) and then transformed into the *Escherichia coli* strain JM109 by electroporation. A total of 748 insert-containing clones were selected and tested by PCR using $(AG)_{10}$ and $M13^+/M13^-$ as primers.

A total of 240 positive clones were selected, purified, and then sequenced on an ABI 3730 DNA Sequence Analyzer (Applied Biosystems, Foster City, California, USA), and 162 were found to contain simple sequence repeats. Among these, 72 sequences were discarded because either the repeat was too short or the flanking regions of the repeat sequences were not suitable for designing primers. Finally, the remaining 90 sequences were used to design primers using Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, California, USA; http://www.premierbiosoft.com). These primers were tested for polymorphism in 21 *T. jackii* individuals randomly selected from across the

TABLE 1. Forward and reverse primers (including fluorescent dye markers), repeat motif, annealing temperature, number of alleles, fragment size range, and GenBank accession number of the nine isolated microsatellite loci in *Torreya jackii*.

Locus	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	N_a	Size range (bp)	GenBank Accession No.
TJ10	F: <FAM>GTCGTCACTTGTCATTCAACAA R: TATCTTTCCCATGCCACTTC	$(AT)_9(AG)_8$	58.5	8	215–229	JF754928
TJ21	F: <FAM>CTCAACATCTTCCAACCCAC R: AACCAGATCAACTTAGTCTTTCAGA	$(TC)_{12}$	60.1	4	180–188	JF754929
TJ28	F: <HEX>GGGAGCTAATCACGAGTCAG R: CTGTTGGAGGAGTGTTGTTG	$(CT)_8$	63.9	4	133–139	JF754930
TJ45	F: <FAM>AGAAACTTCTTTCTCCTCACA R: TAGATCCTCATAGCATGAACA	$(TC)_9$	59.4	3	125–129	JF754931
TJ55	F: <ROX>CTTGCTCCCAGAATTTGACC R: TGAACTCTTATTCCCGATGC	$(CT)_7$	60.1	4	143–149	JF754932
TJ62	F: <HEX>TTGGAGGATTGCTATTACTG R: AAATCTTGGGAGCTAATCAC	$(GA)_8$	55.2	7	141–153	JF754933
TJ75	F: <FAM>TAGGAATTTGCCATACTCTG R: CTGATTTACACTTGGTGACAC	$(GA)_7$	59.1	3	126–130	JF754934
TJ77	F: <FAM>CGTAGATGTACAGTAATTATCCCTC R: TGGTTGTCTCAAGGGTGTAA	$(TC)_7$	61.1	1	246	JF754935
TJ79	F: <FAM>GAAGGAAATACAATGAAGAAATC R: TCATAGCTTACAAGGGTGAAT	$(TC)_8$	54.2	6	132–142	JF754936

Note: N_a = number of alleles; T_a = annealing temperature.

four sampled populations. PCRs were conducted using a PTC-200 thermal cycler (MJ Research, Waltham, Massachusetts, USA) in 20 μL volume containing the following components: 50 ng of genomic DNA, 0.2 mM of each dNTPs, 0.1 μM of each primer, 1× PCR buffer (Mg^{2+} free), 1.5 mM Mg^{2+}, and 1 U of *Taq* DNA polymerase (Sangon, Shanghai, China). Microsatellite loci were amplified under the following conditions: denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, 54.2–63.9°C for 30 s (Table 1), 72°C for 30 s; and a final extension at 72°C for 10 min. Amplified PCR products were electrophoresed on 8% denaturing polyacrylamide gels and visualized by silver staining using pUC19 DNA/*Msp*I (*Hpa*II) (Fermentas, Vilnius, Lithuania) as the ladder. Of the 90 primer pairs tested, 81 loci either failed to have clear PCR products or included nonspecific amplified bands. Ultimately, nine microsatellite loci successfully amplified the target fragments (Table 1).

To further check the applicability of the nine microsatellite loci, each was tested in a total of 80 individuals from the four *T. jackii* populations. Voucher specimens were deposited in East China Normal University, China. We performed PCR using the same thermocycler program mentioned above. Forward primers were labeled with one of the following fluorescent dyes: 5′-FAM, 5′-HEX, or 5′-ROX (Sangon). To get the best resolution, we performed the PCR amplifications separately in a total volume of 15 μL with the above reactions and scanned the products on an ABI 3130 genetic analyzer using GS500(–250) LIZ as the internal lane standard. Allele discrimination was performed using the software GENEMAPPER 4.0 (Applied Biosystems). Using the software TFPGA version 1.3 (Miller, 1997), we found that the number of alleles per locus ranged from one to eight, with an average of 4.4 (Table 1). At the population level, the number of alleles per polymorphic locus ranged from two to six, the observed heterozygosity (H_o) ranged from 0.150 to 1.000, and the expected heterozygosity (H_e) ranged from 0.185 to 0.796 (Table 2). Eight of the nine loci were polymorphic, but we detected no variation at TJ77 (Table 1).

CONCLUSIONS

The nine developed microsatellite loci reported here for *T. jackii* have been shown to amplify successfully across all populations and individuals tested. These markers will be useful tools for analyzing genetic variation, gene flow, and parentage. For example, using these markers we can identify the parents of seedlings and saplings in natural populations, and thus infer pollen and seed dispersals. Furthermore, tracking the survival and reproduction of these individuals can provide essential data for fitness estimation and its link to parentage relatedness. Such information will help to improve conservation practices for endangered species in fragmented habitats (Lu et al., 2006).

TABLE 2. Number of samples genotyped, number of alleles, and observed and expected heterozygosities of the eight polymorphic microsatellite loci in four *Torreya jackii* populations.

	Xianju (28°33′N, 120°24′E)				Pujiang (29°25′N, 119°52′E)				Songyang (28°27′N, 119°29′E)				Tonglu (29°45′N, 119°50′E)			
Locus	N	N_a	H_o	H_e	N	N_a	H_o	H_e	N	N_a	H_o	H_e	N	N_a	H_o	H_e
TJ10	18	4	0.778	0.640	20	6	0.750	0.787	20	5	0.650	0.796	22	6	0.636	0.592
TJ21	18	2	0.667	0.457	20	3	0.800	0.563	20	4	0.900	0.664	22	3	1.000	0.554
TJ28	18	3	0.833	0.560	20	3	0.850	0.586	20	2	0.200	0.185	22	3	0.227	0.213
TJ45	18	3	0.167	0.481	20	3	0.150	0.578	20	2	0.250	0.481	22	3	0.273	0.595
TJ55	18	2	0.389	0.322	20	4	0.200	0.191	20	3	0.600	0.641	22	3	0.227	0.210
TJ62	18	5	0.611	0.651	20	6	0.500	0.728	20	6	0.400	0.767	22	6	0.455	0.702
TJ75	18	2	0.889	0.514	20	2	0.750	0.501	20	2	0.950	0.512	22	3	0.818	0.574
TJ79	18	5	0.889	0.791	20	5	0.950	0.722	20	5	0.750	0.572	22	5	0.955	0.645

Note: H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of samples genotyped; N_a = number of alleles.

LITERATURE CITED

CHEN, X. Y. 2000. Effects of habitat fragmentation on genetic structure of plant populations and implications for the biodiversity conservation. *Acta Ecologica Sinica* 20: 884–892.

LI, J. H., Z. X. JIN, AND J. M. LI. 2007. Genetic diversity of endangered plant *Torreya jackii*: A study with RAPD markers. *Chinese Journal of Applied Ecology* 18: 2661–2667.

LI, J. M., AND Z. X. JIN. 2007. Genetic variation and differentiation in *Torreya jackii* Chun, an endangered plant endemic to China. *Plant Science* 172: 1048–1053.

LIU, M., M. M. SHI, M. H. LIU, AND X. Y. CHEN. 2009. Isolation and characterization of microsatellite loci in *Fagus longipetiolata* Seem. (Fagaceae). *Conservation Genetics* 10: 1981–1983.

LU, H. P., Y. W. CAI, X. Y. CHEN, X. ZHANG, Y. J. GU, AND G. F. ZHANG. 2006. High RAPD but no cpDNA sequence variation in the endemic and endangered plant, *Heptacodium miconioides* Rehd. (Caprifoliaceae). *Genetica* 128: 409–417.

LU, H. P., H. H. WAGNER, AND X. Y. CHEN. 2007. A contribution diversity approach to evaluate species diversity. *Basic and Applied Ecology* 8: 1–12.

MILLER, M. P. 1997. Tools for Population Genetic Analyses (TFPGA), version 1.3. A Windows program for the analysis of allozyme and molecular population genetic data and documentation distributed by the author. Website http://www.marksgeneticsoftware.net/tfpga.htm [accessed 9 November 2011].

XU, N. N., S. YU, J. G. ZHANG, P. K. E. TSANG, AND X. Y. CHEN. 2010. Microsatellite primers for *Halophila ovalis* and cross-amplification in *H. minor* (Hydrocharitaceae). *American Journal of Botany* 97: e56–e57.

YU, Y. F. 1999. A milestone of wild plant conservation in China. *Plants* 5: 3–11.

APPENDIX 1. Information on voucher specimens deposited in East China Normal University. Information presented: taxon—voucher specimens, locality in eastern China.

Torreya jackii—*XJ1*, Xianju, Zhejiang, China; *PJ2*, Pujiang, Zhejiang, China; *SY3*, Songyang, Zhejiang, China; *TL4*, Tonglu, Zhejiang, China.