

## DEVELOPMENT AND CHARACTERIZATION OF 11 POLYMORPHIC MICROSATELLITE MARKERS IN *TAPISCIA SINENSIS* (STAPHYLEACEAE)<sup>1</sup>

PENGFEI ZHANG<sup>2,6</sup>, ZHIGUANG WANG<sup>2,6</sup>, YALING LIU<sup>3</sup>, HUA TIAN<sup>4</sup>, XIAOHONG YAO<sup>4</sup>,  
AND JINJU ZHANG<sup>5,7</sup>

<sup>2</sup>College of Horticulture, Shanxi Agriculture University, Taigu 030801, Shanxi, People's Republic of China; <sup>3</sup>College of Life Science, Shanxi Agriculture University, Taigu 030801, Shanxi, People's Republic of China; <sup>4</sup>Key Laboratory of Plant Germplasm Enhancement and Specialty Agriculture, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan 430074, Hubei, People's Republic of China; and <sup>5</sup>College of Life Sciences, Jiangxi Normal University, Nanchang 330022, Jiangxi, People's Republic of China

- *Premise of the study:* We developed and characterized five polymorphic microsatellite loci from an AC-enriched genomic library of a rare tree, *Tapiscia sinensis*, and six compound microsatellite primers using a dual-suppression-PCR-based approach, to investigate geographical genetic structure.
- *Methods and Results:* The polymorphism of these loci was assessed in 102 samples collected from three populations. The number of alleles per locus ranged from one to nine. Expected heterozygosity per locus ranged from 0.000 to 0.709 and observed heterozygosity ranged from 0.000 to 0.750. Five loci deviated from Hardy–Weinberg equilibrium after Bonferroni correction.
- *Conclusions:* This set of microsatellites may be useful for investigating broad-scale genetic variation in the extant populations of the endangered tree species *T. sinensis*.

**Key words:** conservation genetics; microsatellite; Staphyleaceae; *Tapiscia sinensis*.

*Tapiscia sinensis* Oliv. (Staphyleaceae) is a geographically widespread, woody angiosperm found in the southwest and central subtropical mountains of China at elevations of 250–2200 m. This species is a Tertiary relict endemic to subtropical China. Therefore, it is a good candidate for inferring refugial location and colonization histories of plant species in subtropical China. Moreover, *T. sinensis* is also an economically valuable timber and landscaping tree. Currently, the natural populations of *T. sinensis* have been declining dramatically, and it has become scarce due to habitat degradation and deforestation over the past several decades (Fu and Jin, 1992). *Tapiscia sinensis* was therefore listed in the red list of endangered plant species of China (Fu and Jin, 1992). Understanding genetic variation and genetic structure of the natural populations is crucial to provide conservation strategies for this endangered species. However, to our knowledge, little is known about genetic diversity in *T. sinensis*. Because microsatellite markers have proven to be highly efficient molecular tools in conservation genetic studies, we constructed a useful set of microsatellites

for studying the genetic diversity of this endangered species; 11 polymorphic microsatellite loci from the genome of *T. sinensis* are described here.

### METHODS AND RESULTS

Whole genomic DNA was extracted from leaf tissue of a single individual of *T. sinensis* using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). An enriched partial genomic library was constructed using a Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol described by Zane et al. (2002), with some modifications. A total of 250 ng of genomic DNA was digested with the restriction endonuclease *MseI* (New England Biolabs, Beverly, Massachusetts, USA) and ligated with *MseI* AFLP adapter (5'-TACTCAGGACTCAT-3'/5'-GACGATGAGTCCTGAG 3') using the T4 DNA ligase (TaKaRa Biotechnology Co., Dalian, China). The digestion-ligation mixture was diluted (1 : 10) and PCR amplified using AFLP adapter-specific primers (5'-GATGAGTCCTGAGTAAN-3', i.e., *MseI*-N) for 18 cycles in a total volume of 20  $\mu$ L. The thermocycler conditions were as follows: 30 s denaturation at 94°C, annealing at 53°C for 60 s, and a final extension at 72°C for 60 s. After denaturation at 95°C for 5 min, 500 ng of amplified DNA fragments with a size range of 500–750 bp were hybridized with 150 pmol of 5'-biotinylated (AC)<sub>15</sub> probes in 250  $\mu$ L of hybridization buffer containing saline sodium citrate (SSC) 4.7 $\times$  and sodium dodecyl sulfate (SDS) 0.07%. The DNA was denatured for 5 min at 95°C and incubated at 48°C for 2 h. The hybridization products were selectively captured with 300  $\mu$ L streptavidin-coated beads (Promega Corporation, Madison, Wisconsin, USA). The mixture was incubated at room temperature for 30 min with constant gentle agitation. The beads-probe-DNA complex was washed with low stringency buffer (TEN<sub>1000</sub> [10 mM Tris-HCl, 1 mM EDTA, 1 M NaCl]) and high stringency buffer (SSC 0.2 $\times$  and 0.1% SDS) to remove the nonrepetitive DNA. Captured DNA fragments were released from the complex by incubating for 5 min

<sup>1</sup>Manuscript received 8 June 2013; revision accepted 16 August 2013.

The authors thank Zuozhou Li, Puxin Gao, and Mingke Yan for help in collecting samples. This work was supported in part by the Key Project of the Chinese Academy of Sciences (KSCXZ-EW-J-20) and the Sciences Foundation of Jiangxi Province (2010GQN0114).

<sup>6</sup>These authors contributed equally to this work.

<sup>7</sup>Author for correspondence: jinjvzhang@163.com

doi:10.3732/apps.1300051

TABLE 1. Characterization of 11 polymorphic microsatellite loci developed in *Tapiscia sinensis*.

| Locus | Repeat motif                          | Primer sequences (5'–3')                                              | T <sub>a</sub> (°C) | Size range (bp) | GenBank accession no. |
|-------|---------------------------------------|-----------------------------------------------------------------------|---------------------|-----------------|-----------------------|
| TS1   | (TG) <sub>12</sub> (AG) <sub>10</sub> | F: TGGGCATGTGGAACAAAGTA<br>R: CCCACAACTCAGCCTCTTC                     | 55                  | 220–240         | KC996734              |
| TS2   | (TG) <sub>14</sub> (AG) <sub>9</sub>  | F: GTGCACTGAGGTGAGGAACA<br>R: CTGCATGCGACACTTCCTT                     | 58                  | 149–223         | KC996735              |
| TS3   | (TG) <sub>12</sub> (AG) <sub>9</sub>  | F: GAAAGTTGCGAGGCCTTACCA<br>R: CCACTTACGGCTACTCAAACC                  | 58                  | 143–235         | KC996736              |
| TS4   | (TC) <sub>11</sub>                    | F: CCACAGCCTTGGTTATGGAT<br>R: GCAGAAATGGCCGGAATTAT                    | 58                  | 234–258         | KC996737              |
| TS5   | (TG) <sub>9</sub>                     | F: TGAAGCAAACATGGAGAG<br>R: TCCACCTGATACATCCCTCA                      | 58                  | 180–186         | KC996738              |
| TS6   | (TC) <sub>6</sub> (AC) <sub>5</sub>   | F: (TC) <sub>6</sub> (AC) <sub>5</sub><br>R: GCTGCTTTTCGTGAGAGCTT     | 56                  | 157–165         | KC996739              |
| TS7   | (TC) <sub>6</sub> (AC) <sub>7</sub>   | F: (TC) <sub>6</sub> (AC) <sub>5</sub><br>R: AGAACCCCTTCCTTTCTGGA     | 57                  | 206–212         | KC996740              |
| TS8   | (TC) <sub>6</sub> (AC) <sub>14</sub>  | F: (TC) <sub>6</sub> (AC) <sub>5</sub><br>R: TTGCTTCCTATGACCAAACCTACA | 60                  | 234–244         | KC996741              |
| TS9   | (TC) <sub>6</sub> (AC) <sub>6</sub>   | F: (TC) <sub>6</sub> (AC) <sub>5</sub><br>R: AGTGGGGAAGTGAGCATTTG     | 56                  | 187–197         | KC996742              |
| TS10  | (TC) <sub>6</sub> (AC) <sub>9</sub>   | F: (TC) <sub>6</sub> (AC) <sub>5</sub><br>R: AAGAGACACATTCGGCCTGT     | 57                  | 202–218         | KC996743              |
| TS11  | (TC) <sub>6</sub> (AC) <sub>5</sub>   | F: (TC) <sub>6</sub> (AC) <sub>5</sub><br>R: ATCAAATCCACGGGCTCA       | 56                  | 188–206         | KC996744              |

Note: T<sub>a</sub> = annealing temperature.

at 95°C in 50 µL of TE (Tris–HCl 10 mM, EDTA 1.0 mM [pH 8.0]). Recovered DNA fragments were amplified for 35 cycles using *Mse*I-N primers. PCR products were ligated into pMD 18-T plasmid vector (TaKaRa Biotechnology Co.) and transformed into JM109 competent cells (Promega Corporation). Four hundred and twenty clones were amplified using M13 forward and reverse primers and visualized by agarose gel electrophoresis. Of these, 220 identified positive clones with a size range of 400–700 bp were sequenced with an ABI BigDye Terminator Cycle Sequencing Kit in an ABI PRISM 3770 automated sequencer (Applied Biosystems, Foster City, California, USA).

The compound microsatellite marker technique based on a dual-suppression-PCR technique outlined in Lian et al. (2001) was also used to develop additional polymorphic microsatellite markers. Genomic DNA was separately digested with *Eco*RV and *Ssp*I blunt-end restriction enzymes (TaKaRa Biotechnology Co.). The restricted fragments were then ligated to a specific adapter (consisting of a 48-mer: 5'-GTAATACGACTCACTATAGGGCAGCGTGGTCGACGGCCCGGGCTGGT-3' and an 8-mer with the 3'-end capped by an amino residue: 5'-ACCAGCCC-NH<sub>2</sub>-3') using a DNA Ligation Kit (TaKaRa Biotechnology Co.). To enrich the microsatellite regions, fragments flanked by a microsatellite at one end were amplified from the constructed libraries by a compound simple sequence repeat (SSR) primer (TC)<sub>6</sub>(AC)<sub>5</sub>, and an adapter primer AP2 (5'-CTATAGGGCAGCGTGGT-3'). Subsequently, the amplified

fragments between 400 and 1000 bp were purified, ligated into a PMD18-T vector (TaKaRa Biotechnology Co.), and transformed into JM109 competent cells (Promega Corporation). A total of 103 identified positive clones were sequenced.

Of the 323 clones developed in both methods that were then sequenced, 111 (34%) of the DNA sequences contained SSRs, 36 of which were suitable for designing primers. Twelve out of 36 primer pairs were obtained from the AC-enriched genomic library, and 24 were developed from the compound microsatellite marker method. Primer pairs were designed based on the flanking regions of 36 selected loci with the Primer3 web interface program (Rozen and Skaletsky, 2000). Primers had an optimum length of 20 nucleotides (18 bp minimum, 25 bp maximum) and GC contents ranged from 40% to 60%. Polymorphism was initially assessed in eight individuals of *T. sinensis* from two natural populations, with four samples per population. PCR amplifications were performed in individual 15-µL reactions containing 1.5 µL of 10× buffer, 0.3 µL of 10 µM each primer, 0.3 µL of 10 mM dNTPs, 0.5 µL of 25 µM MgCl<sub>2</sub>, 1 U *Taq* polymerase (Fermentas, Vilnius, Lithuania), and 50 ng of genomic DNA. The amplification profiles included an initial denaturing at 95°C for 5 min, followed by 35 cycles of 50 s at 94°C, 45 s at 55–60°C depending on the primer pair (Table 1), and 60 s at 72°C, followed by a final extension for 10 min at 72°C. Amplified products were separated on 6% denatured polyacrylamide

TABLE 2. Genetic diversity of 11 SSR loci in three populations of *Tapiscia sinensis*.

| Locus | Lushan (n = 36) |                |                | Shunhuangshan (n = 34) |                |                | Wuyishan (n = 32) |                |                | P <sub>HWE</sub> |
|-------|-----------------|----------------|----------------|------------------------|----------------|----------------|-------------------|----------------|----------------|------------------|
|       | A               | H <sub>e</sub> | H <sub>o</sub> | A                      | H <sub>e</sub> | H <sub>o</sub> | A                 | H <sub>e</sub> | H <sub>o</sub> |                  |
| TS1   | 4               | 0.274          | 0.344          | 3                      | 0.195          | 0.214          | 2                 | 0.494          | 0.333          | 0.940            |
| TS2   | 4               | 0.111          | 0.208          | 1                      | 0.000          | 0.000          | 3                 | 0.415          | 0.222          | 0.003*           |
| TS3   | 9               | 0.514          | 0.483          | 2                      | 0.278          | 0.333          | 8                 | 0.688          | 0.694          | 0.273            |
| TS4   | 6               | 0.388          | 0.358          | 2                      | 0.128          | 0.138          | 5                 | 0.588          | 0.639          | 0.045*           |
| TS5   | 2               | 0.017          | 0.228          | 2                      | 0.455          | 0.033          | 1                 | 0.000          | 0.000          | 0.000*           |
| TS6   | 4               | 0.347          | 0.303          | 1                      | 0.000          | 0.000          | 4                 | 0.605          | 0.694          | 0.854            |
| TS7   | 4               | 0.442          | 0.417          | 2                      | 0.124          | 0.133          | 4                 | 0.709          | 0.750          | 0.696            |
| TS8   | 5               | 0.153          | 0.516          | 2                      | 0.499          | 0.000          | 3                 | 0.532          | 0.306          | 0.002*           |
| TS9   | 4               | 0.236          | 0.274          | 1                      | 0.000          | 0.000          | 3                 | 0.548          | 0.472          | 0.387            |
| TS10  | 5               | 0.407          | 0.496          | 2                      | 0.490          | 0.286          | 3                 | 0.502          | 0.528          | 0.027*           |
| TS11  | 6               | 0.602          | 0.303          | 3                      | 0.575          | 0.621          | 3                 | 0.511          | 0.583          | 0.854            |
| Mean  | 4.818           | 0.317          | 0.357          | 1.909                  | 0.249          | 0.160          | 3.545             | 0.508          | 0.475          | —                |

Note: A = number of alleles revealed; H<sub>e</sub> = expected heterozygosity; H<sub>o</sub> = observed heterozygosity.

\*Denotes significant departure from Hardy–Weinberg equilibrium after Bonferroni correction (P < 0.05).

gels using silver staining. A 25-bp DNA ladder (Promega Corporation) was used to identify alleles.

Of the 36 primer pairs tested, 25 primer pairs successfully amplified target fragments, and 11 loci were polymorphic, while the other 14 loci were monomorphic (Table 1). We estimated the level of genetic variability of these markers by genotyping 102 individuals derived from three extant populations (Appendix 1). We determined the number of alleles per locus ( $A$ ) and observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosities for each locus using GENEPOP (Rousset, 2008). In the Lushan population,  $A$  ranged from two to nine,  $H_e$  ranged from 0.017 to 0.602, and  $H_o$  ranged from 0.208 to 0.516. Two monomorphic loci were observed in the Shunhuangshan population; none were observed in the Wuyishan population.  $H_o$  ranged from 0 to 0.621 and  $H_e$  from 0 to 0.575 in the Shunhuangshan population. In the Wuyishan population,  $H_o$  varied from 0 to 0.750 and  $H_e$  ranged from 0 to 0.709 (Table 2). Tests for Hardy–Weinberg equilibrium (HWE) and genotypic linkage disequilibrium were performed over the whole data set using GENEPOP (Rousset, 2008). Five loci (TS2, TS4, TS5, TS8, and TS10) deviated from HWE after Bonferroni correction ( $P < 0.05$ ,  $n = 102$  individuals). The presence of null alleles was tested by using the program MICRO-CHECKER 2.2 (van Oosterhout et al., 2004); null alleles were not detected in any loci. Thus, these deviations seemed due to a deficit of heterozygotes, suggesting that the sample size was insufficient. Significant linkage disequilibrium was found in 11 pairs of loci before correction for multiple tests ( $P = 0.05$ ). However, no pairs of loci were found to be highly significant after correction for multiple tests ( $P = 0.0009$ ).

### CONCLUSIONS

This set of microsatellites will provide a useful tool for studying population structure and understanding the genetic background of extant populations across the natural range of *T. sinensis*. Information on the distribution of genetic diversity

in this species will be helpful to provide conservation strategies. We are currently using these markers to investigate the distribution of genetic diversity, gene flow, and mating system in *T. sinensis* to devise optimum management strategies for the long-term survival of *T. sinensis*.

### LITERATURE CITED

- DOYLE, J. J., AND J. L. DOYLE. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.
- FU, L. K., AND J. M. JIN. 1992. Red list of endangered plants in China. Science Press, Beijing, China.
- LIAN, C., Z. ZHOU, AND T. HOGETSU. 2001. A simple method for developing microsatellite markers using amplified fragments of inter-simple sequence repeat (ISSR). *Journal of Plant Research* 114: 381–385.
- ROUSSET, F. 2008. GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.
- ROZEN, S., AND H. SKALETSKY. 2000. 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.
- VAN OOSTERHOUT, C., W. F. HUTCHINSON, D. P. M. WILLS, AND P. SHIPLEY. 2004. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4: 535–538.
- ZANE, L., L. BARGELLONI, AND T. PATARNELLO. 2002. Strategies for microsatellite isolation: A review. *Molecular Ecology* 11: 1–16.

APPENDIX 1. List of vouchers of *Tapiscia sinensis* used in this study. All vouchers are deposited at the Wuhan Botanical Garden Herbarium (HIB), Wuhan, Hubei Province, China.

| Population code | Location                          | Altitude (m) | Latitude   | Longitude   | Voucher no.     |
|-----------------|-----------------------------------|--------------|------------|-------------|-----------------|
| SHS             | Xinning County, Hunan Province    | 1230         | 26°26'56"N | 111°1'50"E  | Z. G. Wang 1201 |
| LS              | Lushan Mountain, Jiangxi Province | 1100         | 23°8'11"N  | 113°17'57"E | Z. G. Wang 1205 |
| WYS             | Yushan County, Jiangxi Province   | 1085         | 27°52'16"N | 117°45'11"E | Z. G. Wang 1209 |

Note: LS = Lushan population; SHS = Shunhuangshan population; WYS = Wuyishan population.