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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF 11 POLYMORPHIC MICROSATELLITE MARKERS IN *TAPISCIA SINENSIS* (STAPHYLEACEAE)[1]

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- *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.

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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 *Mse*I (New England Biolabs, Beverly, Massachusetts, USA) and ligated with *Mse*I 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., *Mse*I-N) for 18 cycles in a total volume of 20 µL. 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 $(AC)_{15}$ probes in 250 µL of hybridization buffer containing saline sodium citrate (SSC) 4.7× 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 µL 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_{1000} [10 mM Tris–HCl, 1 mM EDTA, 1 M NaCl]) and high stringency buffer (SSC 0.2× and 0.1% SDS) to remove the nonrepetitive DNA. Captured DNA fragments were released from the complex by incubating for 5 min

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

Locus	Repeat motif	Primer sequences (5′–3′)	T_a (°C)	Size range (bp)	GenBank accession no.
TS1	$(TG)_{12}(AG)_{10}$	F: TGGGCATGTGGAACAAAGTA R: CCCACAAACTCAGCCTCTTC	55	220–240	KC996734
TS2	$(TG)_{14}(AG)_9$	F: GTGCACTGAGGTGAGGAACA R: CTGCATGCGACACTTCCTT	58	149–223	KC996735
TS3	$(TG)_{12}(AG)_9$	F: GAAAGTTGCAGGCCTTACCA R: CCACTTACGGCTACTCAAAACC	58	143–235	KC996736
TS4	$(TC)_{11}$	F: CCACAGCCTTGGTTATGGAT R: GCAGAAATGGCCGGAATTAT	58	234–258	KC996737
TS5	$(TG)_9$	F: TGGAAGCAAAACATGGAGAG R: TCCACCTGATACATCCCTCA	58	180–186	KC996738
TS6	$(TC)_6(AC)_5$	F: $(TC)_6(AC)_5$ R: GCTGCTTTTCGTGAGAGCTT	56	157–165	KC996739
TS7	$(TC)_6(AC)_7$	F: $(TC)_6(AC)_5$ R: AGAACCCCTTCCTTTCTGGA	57	206–212	KC996740
TS8	$(TC)_6(AC)_{14}$	F: $(TC)_6(AC)_5$ R: TTGCTTCCTATGACCAAACTTACA	60	234–244	KC996741
TS9	$(TC)_6(AC)_6$	F: $(TC)_6(AC)_5$ R: AGTGGGGAAGTGAGCATTTG	56	187–197	KC996742
TS10	$(TC)_6(AC)_9$	F: $(TC)_6(AC)_5$ R: AAGAGACACATTCGGCCTGT	57	202–218	KC996743
TS11	$(TC)_6(AC)_5$	F: $(TC)_6(AC)_5$ R: ATCAAATCCACGGGCTCA	56	188–206	KC996744

Note: T_a = 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′-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3′ and an 8-mer with the 3′-end capped by an amino residue: 5′-ACCAGCCC-NH_2-3′) 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 $(TC)_6(AC)_5$, and an adapter primer AP2 (5′-CTATAGGGCACGCGTGGT-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 $MgCl_2$, 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*.

	Lushan (n = 36)			Shunhuangshan (n = 34)			Wuyishan (n = 32)			
Locus	A	H_e	H_o	A	H_e	H_o	A	H_e	H_o	P_{HWE}
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_e = expected heterozygosity; H_o = 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*.

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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.