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Efficient development of polymorphic microsatellite loci for *Pteroceltis tatarinowii* (Ulmaceae)

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ABSTRACT. *Pteroceltis tatarinowii* (Ulmaceae) is a scientifically and economically important temperate deciduous tree that is endemic to China. In the present study, 12 *P. tatarinowii* polymorphic microsatellite loci were developed using the tailed primer-M13-simple sequence repeats (TP-M13-SSR) biotin-capture method. The number of alleles per locus ranged from 2 to 10, with an average of 6.58. The observed and expected heterozygosity ranged from 0.208 to 0.958 and from 0.198 to 0.858, with average values of 0.703 and 0.710, respectively. The markers isolated in this study represent a favorable tool for further analyses of the population genetic structure and evolutionary history of this relic tree.

Key words: *Pteroceltis tatarinowii*; Microsatellite; Population genetics; Conservation

INTRODUCTION

Pteroceltis tatarinowii is a temperate deciduous tree endemic to China. The species belongs to the sole monotypic genus of *Pteroceltis* (Ulmaceae), and is also a tertiary relic plant. Hence, the species harbors important implications for delimiting systematic and paleontological puzzles. Because of its unique taxonomic status and fragmented distribution, it is considered a "rare endangered" species by the Red Data Book of Chinese Plants (Fu, 1992). *P. tatarinowii* is widely distributed across the mainland of China, and it characteristically grows on bare limestone mountains. The bark (phloem fiber) is the sole raw material for the manufacture of Chinese traditional Xuan paper (Fu, 1992; Cao, 1993), which has been listed in World Intangible Cultural Heritage. Moreover, due to strong endurance during drought and in poor soil, *P. tatarinowii* is an ideal mountain tree for water and soil maintenance in reconstructed vegetation, and it has been successfully employed in Shitai County of Anhui Province (Chen, 1994).

In our previous study, non-coding regions of the chloroplast genome and inter-simple sequence repeats(ISSR) in the nuclear genome were investigated to detect *P. tatarinowii* genetic diversity and population history (Li et al., 2012, 2013). Considering the discriminative limitation of cytoplasmic DNA inheritance and the dominant nature of inter-simple sequence repeats (ISSR), it is necessary to employ co-dominant markers to reappraise the genetic variation and phylogeographic patterns of this economic fiber tree. Therefore, the development of a suite of polymorphic microsatellite markers would utilize one of the most common and efficient measures currently used in molecular genetic analysis (Seino et al., 2014; Wang et al., 2015). Here, we used a modified biotin-capture with M13-tailed method (Schuelke, 2000) to isolate target microsatellite loci.

MATERIAL AND METHODS

Microsatellites were isolated from an enriched library that was constructed using a modified biotin-capture with a tailed M13 primer (Schuelke, 2000; Bloor et al., 2001; Zane et al., 2002). Using the modified CTAB (cetyltrimethylammonium bromide) protocol of Doyle (1991), total genomic DNA was extracted from silica-gel dried leaves of 72 individuals collected from Gansu (N = 24), Anhui (N = 24), and Guangdong Province (N = 24) populations. Approximately 4 µg mixed DNA of three individuals was digested with the *Sau*3A I restriction enzyme (TaKaRa, Dalian, Liaoning, China). The obtained size fragments, ranging from 300 to 1000 bp, were then recovered from 1.5% agarose gels using a gel extraction column kit (TaKaRa). The retrieved and purified fragments were ligated to the corresponding adaptor pair of *Sau*L A (5'-GGCCAGAGACCCCAAGCTTCG-3') and *Sau*L B (5'-PO4-GAT CCGAAGCTTGGGGTCTCTGGCC-3') linkers (Hamilton et al., 1999). The ligated fragments were amplified using PCR with *Sau*L A as a primer. The 400-1000 bp amplified fragments were recovered from 1.5% agarose gels using a gel column extraction kit (TaKaRa). To enrich the fragments harboring microsatellite repeats, the retrieved fragments were hybridized with 5-biotinylated $(AC)_{12}$/$(AG)_{12}$ oligonucleotide probes, and were then captured with streptavidin-coated magnetic beads (Roche, Mannheim, Germany). The above microsatellite-enriched DNA fragments were PCR-amplified using SauL A as the primer. The PCR products were purified and subsequently ligated into the plasmid PMD-19T vector (TaKaRa), and were then transformed into DH5α competent cells. Transformed cells grew at 37°C for 8 h on LB/ampicillin/IPTG/X-gal plates for blue and white selection. Positive colonies yielding two or more bands were screened for the

presence of microsatellite motifs via PCR using SauL A and $(AC)_{12}$ or $(AG)_{12}$ oligonucleotides as primers (Wu et al., 2008). In total, 152 positive clones were selected and sequenced on an automated ABI 3700 DNA sequencer. Seventy-two primer pairs were designed using Primer Premier 5.0 software (http://www.pre-mierbiosoft.com/), and 19 primers sets that yielded consistent and specific targeted products were chosen for subsequent allelic polymorphism testing in 72 individuals from the three populations. Nested PCR reactions were performed using single tube reactions with three oligos (oligo1: Foreword SSR primer with M13 at the 5′-end; oligo2: Reverse SSR primer; oligo3: universal fluorescent tag (FAM, HEX, TAMRA) labeled M13 primer (note that M13 primers were synthesized following the original published 5'-TGT AAA ACG ACG GCC AGT-3' sequence) (Schuelke, 2000). The PCR reactions were performed in 15 µL mixture volumes containing 1.5 µL 10X buffer (Mg^{2+} free), 2.5 mmol dNTP mixture, 30-50 ng DNA, 0.4 µmol oligo1 and oligo2, 0.1 µmol oligo3, 0.5 units *Taq* DNA polymerase (TaKaRa), and 1.2 µL 25 µmol $MgCl_2$. The PCR conditions were 5 min at 94°C for initial denaturizing followed by 27 cycles of 30 s at 94°C, 30 s at optimized annealing temperatures (Table 1), and 30 s at 72°C. The procedure was then switched to 10 cycles of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C, with a final extension time of 8 min at 72°C. PCR products were screened and identified on 1.5% agarose gels, and the final PCR product was sent to the Crystal Biological Technology Co., LTD. (Shanghai) for genotyping.

Tests of significant deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were conducted using GENEPOP version 3.4 (Raymond and Rousset, 1995). Micro-Checker version 2.2.3 was used to determine null alleles (van Oosterhout et al., 2004). Cervus 2.0 software (Belkhir et al., 1996) was selected to identify the number of alleles per locus (N_A), observed and expected heterozygosity per locus (H_O/H_E), and polymorphic information content (PIC). The significance values for all tests were corrected using the sequential Bonferroni procedure (Rice, 1989).

RESULTS

In total, 19 microsatellite loci were identified, and 12 of these (PTTA8, PTTA9, PTTA11, PTTA12, PTTA16, PTTA17, PTTA31, PTTA37, PTTA42b, PTTA45b, PTTA53, and PTTA60) were sufficiently polymorphic and stable for amplification of the 72 *P. tatarinowii* individuals collected from the three populations. The remaining seven loci (PTTA18b, PTTA26, PTTA42, PTTA46, PTTA65r, PTTA78, and PTTA91) may be useful in other populations (Table 1). The number of alleles per locus ranged from 2 to 10, with an average of 6.58, which is indicative of high allelic diversity. The observed and expected heterozygosity values varied from 0.208 to 0.958 and from 0.198 to 0.858, with averages of 0.703 and 0.710, respectively. The PIC values for individual loci ranged from 0.187 to 0.823 with an average of 0.655. Of the 12 loci, no significant deviations from Hardy-Weinberg equilibrium(HWE) were detected by the Bonferroni adjustment with $P < 0.0042$ (Table 2), including a heterozygote deficit. Across the three populations, significant linkage disequilibrium was not detected between any pair of loci. However, some pairs displayed linkage in a single population (Gansu population: PTTA45 and PTTA53; Guangdong population: PTTA8 and PTTA9; PTTA9 and PTTA31). The null alleles were present at loci PTTA17 and PTTA31 in the Gansu population and at PTTA11 in the Guangdong population. Hence, rather than allelic sites, the respective linkage disequilibrium may be attributed to null alleles and small population effects.

Table 1. Characteristics of 19 microsatellite primers developed in *Pteroceltis tatarinowii.*

Primer	Primer sequence (5'-3')	Repeat motif	Ta (ºC)	Size range (bp)	Accession No.
PTTA8*	F: CATATTTCCTCTTCCCCTAA				
	R: ACAGCTCACCCATACCTTC	(CT)12A (TCT)5	55	219-243	KR005831
PTTA9*	F: CACCTTTGCTTACTCCCTG				
	R: AATGTACTCGCTAATGAACC	(GA)13 …(GT)8	56	203-241	KR005832
PTTA11*	F: AGCGACTGAGGGTTTCATG				
	R: GCTTCTGCTCCGCCTTTCT	(GA)5…(AG)13…(GT)8	62	245-269	KR005833
PTTA12*	F: CAGGGCACTCCAATAGAATAG				
	R: ATGGTGCTGGGATGGGAAG	(AG)13	60	251-273	KR005834
PTTA16*	F: CATTTGGATACACCAGGAAGG				
	R: CAGCCATTGATGCTTAGTCC	(CT)11	60	165-185	KR005835
PTTA17*	F: TCTAGGCTGTATAAAGGGAC				
	R: GATGAAGTAAATGGGGAATC	(TC)5…(TC)12…(TC)12	54	221-249	KR005836
PTTA31*	F: CATGTCACCATTACCGAAC				
	R: ACACAGTAAGAAAACACACC	(TC)19	55	140-166	KR005837
PTTA37*	F: TGGCGATGTGAAGCCCTAAG				
	R: TCATTTCAACGGTCAAGATTAC	(AG)10	56	259-279	KR005838
PTTA42b*	F: CAATAATAGCCTTGCATCTC				
	R: CTCCCTTTGAACAAACCTC	(TG)6…(TG)10	56	279-317	KR005839
PTTA45b*	F: CCTGTCCAGCTACTAATTTG				
	R: GTCTGCGATGGTATCTGTT	(AT)6(GT)18	56	176-190	KR005840
PTTA53*	F: CAGGTCCAGAGGGAGAAAC				
	R: CCCAGGGTCAAATAGGTAAT	(AG)12	60	288-312	KR005841
PTTA60*	F: GCTTCCTTGGGTCTCATCC				
	R: TCCACAGACGAGTAGTTCTCC	(GA)9…(AG)6	56	343-373	KR005842
PTTA18b	F: GCGGTGATTAGAAACTTGTAC				
	R: GCACATTTAGCCATTTTCAC	(TG)12	56	186-196	NO
PTTA26	F: GCGAAGCATTCTGAGGTAC				
	R: CTGGTATTGTCTCCATCGTC	(TG)9	57	217-231	NO
PTTA42	F: CTAACCAAAGTGCTGTTTGAG				
	R: CTTTCTCACCCCATCCTAAT	(CT)7…(CA)9(AAAC)5	56	197-215	NO
PTTA46	F: TCTCCCTTCCATGCACAAT				
	R: AGTGCTTGTGGTTGTAGCG	(CT)12	53	136-152	NO
PTTA65r	F: CATCTTAACTGTGGCACTAC				
	R: GTCTATGGTGGAGATGAAC	(AC)22	56	239-269	NO
PTTA78	F: ACCTACGCAGAATAATCGC				
	R: GATAACTTCACGGTTCCAG	(GA)12	56	126-158	NO
PTTA91	F: TGACCCATTAAGATGTGGTGG				
	R: GTGGCCCTTGACTTTGTGG	(AC)8	57	262-288	NO

The details of repeat motifs, forward (F) and reverse (R) primer sequences, optimal temperatures (T_A), and allele size ranges are given. The asterisk (*) represents the polymorphic and stable primers chosen for further population genetic analyses. “NO” represents primers without accession numbers.

Table 2. Results of initial screening of three *Pteroceltis tatarinowii* populations.

	POP-Gansu (N = 24) N34º46'E117º32'				POP-Anhui (N = 24) N24º47'E104º32'				POP-Guangdong (N = 24) N26º53'E116º55'			
Locus	N_A	H_E	H_O	PIC	N_A	H_E	H_O	PIC	N_A	H_E	H_O	PIC
PTTA8	6	0.780	0.792	0.727	6	0.793	0.708	0.746	7	0.766	0.833	0.711
PTTA9	8	0.751	0.833	0.704	8	0.732	0.708	0.683	6	0.622	0.750	0.574
PTTA11	5	0.777	0.917	0.719	7	0.802	0.792	0.758	6	0.582	0.333	0.509
PTTA12	8	0.584	0.542	0.512	7	0.807	0.875	0.760	4	0.741	0.708	0.677
PTTA16	7	0.785	0.875	0.733	6	0.803	0.750	0.754	5	0.704	0.542	0.634
PTTA17	8	0.730	0.500	0.668	5	0.501	0.542	0.432	4	0.198	0.208	0.187
PTTA31	10	0.778	0.583	0.733	7	0.781	0.875	0.730	8	0.839	0.917	0.797
PTTA37	7	0.677	0.583	0.612	2	0.422	0.333	0.328	5	0.666	0.750	0.586
PTTA42b	9	0.825	0.750	0.788	10	0.858	0.875	0.823	8	0.846	0.792	0.805
PTTA45b	3	0.675	0.625	0.587	5	0.692	0.708	0.618	3	0.441	0.542	0.392
PTTA53	9	0.804	0.708	0.760	7	0.771	0.750	0.716	6	0.725	0.792	0.657
PTTA60	10	0.723	0.667	0.676	9	0.826	0.958	0.784	6	0.759	0.875	0.706

Locus name, number of alleles per locus (NA), mean values of observed and expected heterozygosity (H_O and H_E), polymorphism information content (PIC), and P values for Hardy-Weinberg equilibrium via Bonferroni correction are shown below (P < 0.0042). Sample size (N) for each population is shown in parentheses, followed by the geographic coordinates of each sampled location.

DISCUSSION

In this study, we isolated and identified 12 *P. tatarinowii* polymorphic microsatellite sites with successful target products ranging from 126 to 373 bp using TP-M13-SSR biotin-capture method. Through the pre-screening of 72 different individuals from three far apart populations, these markers exhibited favorable stability and high degrees of polymorphism with an average of 6.58 alleles per marker. In addition, no significant departure from HWE was found among all the three populations.

P. tatarinowii is a relic and monogeneric tree endemic to China, indicative of limestone habitat. Considering the unique systematic status and economic values of the rare species, our research group had conducted continuously molecular surveys with the focus of detecting the species' population genetic diversity and tracing its evolutionary demography using non-coding regions of the chloroplast genome and ISSR markers (Li et al., 2012; Li et al., 2013). The two markers both detected still high genetic diversity harbored by this relic tree. However, the cpDNA data revealed that South China was the main refugia for *P. tatarinowii*, and a post-glacial recloniza-tion occurred in North China，which were not supported by the data based on ISSR analysis of *P. tatarinowii*. In order to further illuminate this confusion, we need more evidences especially from nuclear genome, which is dominated by seed flow and pollen flow and exhibits more genetic vari-ability. With the advantages of co-dominance, high polymorphism, well reliability and abundant ge-nomic information, microsatellite sites have been extensively used in the genetics and evolutionary studies (Provan et al., 2001; Wang et al., 2015). Herein, these 12 polymorphic microsatellite loci appear to be highly informative and are can be utilized as powerful tools to detect genetic diversity, gene flow, population evolutionary relationship and parental analysis of *P. tatarinowii*, which would further reveal the population evolutionary history of the tree, contribute understanding of deciduous trees vegetation succession in mainland of China, and aid the development of conservation and management measures for the relic, economic tree.

Conflicts of interest

The authors declare no conflict of interest.

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