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Isolation and characterization of polymorphic microsatellite loci from *Zelkova schneideriana* Hand.-Mazz.

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ABSTRACT. *Zelkova schneideriana* is a highly valued hardwood species. An improved technique for isolating codominant compound microsatellite markers was used to develop simple sequence repeat markers for *Z. schneideriana*. A total of 12 microsatellite loci were identified. Overall, the number of alleles per locus ranged from 8-19, with an average of 11.75. Observed heterozygosity and expected heterozygosity values ranged from 0.109-0.709 and 0.832-0.929, respectively. Polymorphic information content is from 0.803-0.915, with an average of 0.854. These markers will be very important for future research related to the genetic diversity, population structure, patterns of gene flow, and mating system of this species.

Key words: Genetic diversity; Microsatellite loci; Simple sequence repeat; *Zelkova schneideriana* Hand.-Mazz. SSRs

INTRODUCTION

Zelkova schneideriana Hand.-Mazz. is the second-most state-protected plant species; it is a deciduous hardwood tree that belongs to the genus *Ulmus*. *Zelkova* produces excellent timber and shows massive, hard, sheeny, and durable features (Zhang et al., 2011). *Z. schneideriana* is also one of the most important landscape species because of its large crown and with paler green in spring and orange or rusty red leaves in the autumn (Cao et al., 2005). This species is highly valued in China, Korea, Japan, and other East Asian countries (Lo et al., 1995). However, it has become a rare and endangered species in China because of uncontrolled commercial logging and the lack of effective propagation methods (Fu and Jin, 1992). Currently, most of the efforts have been focused on the breeding and cultivation of this species. Studies of the genetic diversity, population ecology, and conservation of *Z. schneideriana* are insufficient and limited (Liu et al., 2005), making the development of genetic markers in *Z. schneideriana* very important.

Microsatellite markers are highly polymorphic, abundant, and relatively evenly distributed throughout eukaryotic genomes, and thus can be used as co-dominant markers. The popularity of these markers is related to their ease of amplification by polymerase chain reaction (PCR), their co-dominant nature, and their typically high levels of allelic diversity at different loci (Arif et al., 2011). The dual-suppression-PCR technique was recently developed. By applying this technique, Lian et al. (2006) developed simple sequence repeat (SSR) markers for more than 30 species. This technique for isolating codominant compound microsatellite markers was used to develop SSR markers for *Z. schneideriana* in this study.

MATERIAL AND METHODS

Three diploid populations of *Z. schneideriana* plants, including Anhui population (AH), Jiangxi population (JX), and United States population (US) were collected. Voucher specimens for the sampled populations were deposited in the herbarium from Forestry Research Institute of Guangxi Zhuang Autonomous Region. Genomic DNA was extracted from silica-gel-dried leaves using a modified hexadecyltrimethylammonium bromide method (Doyle, 1991). One individual from the AH population was selected for digestion with the *Eco*RV restriction enzyme (Takara, Dalian, China) for construction of a DNA library for *Z. schneideriana*. After digestion, the fragments were ligated to a specific unequal-length adaptor (consisting of an upper strand 5'-GTA ATA CGA CTC ACT ATA GGG CAC GCG TGG TCG ACG GCC CGG GCT GGT-3' and a lower strand, with the 3' end-capped with an amino residue: 5'-ACC AGC CC-3') by T4 DNA ligase (Takara). Subsequently, the fragments were PCR-amplified from the *Eco*RV DNA library using the compound SSR primer $(AC)_6(AG)_5$ or $(TC)_6(AC)_5$ and an AP_2 adaptor (5'-CTA TAG GGC ACG CGT GGT-3'). Each 50-μL PCR contained 30-50 ng genomic DNA, of 1X PCR buffer with $MgCl_2$, 0.2 mM of each dNTP, 0.5 U *Taq* polymerase (Takara), and 0.5 mM of each compound SSR primer and AP_2. The PCR amplification conditions were as follows: 1 cycle for 9 min at 94°C, 30 s at 62°C, and 1 min at 72°C; 5 cycles each for 30 s at 94°C, 30 s at 62°C, and 1 min at 72°C; 35 cycles each of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C; ending with a final cycle of 30 s at 94°C, 30 s at 60°C, and 9 min at 72°C. The products were purified using a DNA clean-up kit (Axygen, Union City, CA, USA) and transformed into competent JM109 cells (Takara) after ligation with the pMD 18-T vector (Takara). A single clone was evaluated using the $M13^+$ (5'-TGT AAA ACG

ACG GCC AGT-3')/M13- (5'-CAG GAA ACA GCT ATG ACC-3') universal primers. Positive clones were obtained and sequenced on an ABI Prism 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Specific primers were designed based on sequences flanking the compound SSR primers using Primer Premier 5.0 (Clarke and Gorley, 2001).

To examine the effectiveness and polymorphism of the primers, 20 individuals of *Z. schneideriana* from the AH population, 23 individuals from the JX population, and 12 individuals from the US population were collected. To evaluate polymorphism levels, compound SSR primers were labeled with fluorescent dyes (6-FAM or HEX). Amplified products were analyzed by fluorescence capillary electrophoresis on an ABI Prism 3730 automated DNA sequencer (Applied Biosystems). The data were compiled and scored using GeneMaker 2.2.0 (Soft-Genetics, State College, PA, USA). Cervus 2.0 (Kalinowski et al., 2007) was used to calculate number of alleles per locus (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), and polymorphic information content (PIC). Genepop (http://genepop.curtin.edu.au/) was employed to test the Hardy-Weinberg equilibrium and linkage disequilibria.

RESULTS

A total of 12 polymorphic microsatellite loci were identified (Table 1). N_A per locus ranged from 6-11 in the US population, with an average of 8.58 alleles per locus. H_O ranged from 0.167-0.667, with an average of 0.341. H_E values ranged from 0.681-0.917, with an average of 0.851. The PIC of the 12 microsatellite loci ranged from 0.602-0.866, with an average of 0.794. In the JX population, N_A per locus ranged from 7-15, with an average of 10.42 alleles per locus. H_O ranged from 0.043-0.870, with an average of 0.471. H_E values ranged from 0.815-0.929, with an average of 0.879. The PIC ranged from 0.768-0.901, with an average of 0.843. N_A per locus ranged from 7-16 in the AH population, with an average of 9.25 alleles per locus. H_O ranged from 0.150-0.700, with an average of 0.417. H_E values ranged from 0.790-0.935, with an average of 0.849. The PIC ranged from 0.736-0.905, with an average of 0.805 (Table 2). Most of microsatellite loci deviated significantly from Hardy-Weinberg equilibrium ($P < 0.001$). Pairwise linkage disequilibrium between the 12 pairs of loci was not significant ($P < 0.001$).

Table 1. Characterization of 12 microsatellite loci in *Zelkova schneideriana* Hand.-Mazz.

Locus	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Repeat motif	TA (°C)	Size (bp)	N_A	H_O	H_E	PIC	Accession No.
ZsSSR1	$(AC)_6(AG)_5$	GGTGATAAAGGCAAGTAATAGA	$(AC)_6(AG)_5$	51.7	161	9	0.545	0.837	0.809	KJ749829
ZsSSR2	$(AC)_6(AG)_5$	ATCAGGGAACTCAGCCAC	$(AC)_6(AG)_5$	51.9	109	11	0.491	0.874	0.852	KJ749830
ZsSSR3	$(AC)_6(AG)_5$	GCTTAGGGCAGACTCATA	$(AC)_6(AG)_{16}$	47.6	260	14	0.273	0.917	0.902	KJ749831
ZsSSR4	$(AC)_6(AG)_5$	CGGGGCTGGTATCTTGTA	$(AC)_6(AG)_{27}$	53.8	125	12	0.618	0.869	0.846	KJ749832
ZsSSR5	$(AC)_6(AG)_5$	CAGCCATGAGCCAGAAAT	$(AC)_6(AG)_6$	53.6	254	19	0.709	0.929	0.915	KJ749833
ZsSSR6	$(AC)_6(AG)_5$	AAAGGTGATAAAGGCAAG	$(AC)_6(AG)_5$	47.3	164	16	0.564	0.923	0.908	KJ749834
ZsSSR7	$(AC)_6(AG)_5$	CGTCCAAGAAATCTCACA	$(AC)_6(AG)_5$	48.3	135	11	0.400	0.893	0.874	KJ749835
ZsSSR8	$(AC)_6(AG)_5$	AAGGGAAGATGAAGTGGAGA	$(AC)_6(AG)_{12}$	53.4	182	12	0.309	0.865	0.842	KJ749836
ZsSSR9	$(AC)_6(AG)_5$	CAAGTTGCACATTCATTCAG	$(AC)_6(AG)_5$	51.8	131	10	0.327	0.859	0.834	KJ749837
ZsSSR10	$(AC)_6(AG)_5$	CTTATTCAAGCTGGAGCA	$(AC)_6(AG)_9$	48.8	273	9	0.109	0.871	0.848	KJ749838
ZsSSR11	$(AC)_6(AG)_5$	CCAAACAAATCCACCTAAA	$(AC)_6(AG)_5$	50.9	162	8	0.509	0.841	0.814	KJ749839
ZsSSR12	$(AC)_6(AG)_5$	TGAGTTGTCAGAAGAGGG	$(AC)_6(AG)_7$ $(AC)_5(AC)_5$ $(AG)_5$	47	113	10	0.218	0.832	0.803	KJ749840

TA annealing temperature.

Table 2. Results of initial primer screening in 3 populations of *Zelkova schneideriana* Hand.-Mazz.

Locus	United States (N = 12)				Jiangxi (N = 23)				Anhui (N = 20)			
	N_A	H_O	H_E	PIC	N_A	H_O	H_E	PIC	N_A	H_O	H_E	PIC
ZsSSR1	8	0.333	0.837	0.778***	9	0.565	0.861	0.823***	7	0.650	0.823	0.777$^{n.s.}$
ZsSSR2	8	0.417	0.859	0.801*	9	0.522	0.897	0.864***	9	0.500	0.827	0.781***
ZsSSR3	10	0.167	0.906	0.855***	13	0.217	0.907	0.878***	10	0.400	0.910	0.877***
ZsSSR4	9	0.500	0.895	0.842*	12	0.870	0.901	0.871*	7	0.400	0.790	0.736***
ZsSSR5	10	0.667	0.917	0.866***	15	0.739	0.929	0.901*	16	0.700	0.935	0.905**
ZsSSR6	8	0.417	0.830	0.768***	13	0.652	0.925	0.897**	14	0.550	0.919	0.888***
ZsSSR7	8	0.167	0.866	0.809***	9	0.478	0.883	0.848***	9	0.450	0.883	0.846***
ZsSSR8	11	0.417	0.891	0.841***	11	0.391	0.887	0.855***	8	0.150	0.821	0.773***
ZsSSR9	8	0.167	0.855	0.797***	9	0.435	0.834	0.792***	9	0.300	0.804	0.754***
ZsSSR10	9	0.167	0.906	0.854***	8	0.043	0.867	0.829***	7	0.150	0.794	0.742***
ZsSSR11	6	0.417	0.681	0.602*	7	0.522	0.815	0.768***	7	0.550	0.841	0.795**
ZsSSR12	8	0.250	0.768	0.712***	10	0.217	0.836	0.794***	8	0.200	0.836	0.790***
Mean	8.58	0.341	0.851	0.794	10.42	0.471	0.879	0.843	9.25	0.417	0.849	0.805

N sample size for each population. *,** and ***Significant departures from Hardy-Weinberg equilibrium at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively. n.s. = not significant.

DISCUSSION

All novel microsatellite loci identified in *Z. schneideriana* in the present study showed high levels of polymorphism, indicating that these markers will significantly influence future studies related to the genetic diversity, population structure, patterns of gene flow, and mating system of this species.

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