



## Development and characterization of microsatellite markers for *Ulmus chenmoui* (Ulmaceae), an endangered tree endemic to eastern China

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**ABSTRACT.** *Ulmus chenmoui* (Ulmaceae) is an endangered tree found on Langya Mountain, eastern China. To better understand the population genetics of *U. chenmoui* and conserve the species, we developed microsatellite markers. Using a suppression-polymerase chain reaction technique, 74 compound microsatellite primer pairs were designed. Twelve microsatellite markers were polymorphic in 39 individuals, and the number of alleles per locus ranged from 3 to 9. The observed and expected heterozygosities ranged from 0.051 to 0.769 and from 0.533 to 0.768, respectively. Significant linkage disequilibrium was detected for three pairs of loci ( $P < 0.01$ ), which may be due to

a recent population bottleneck and the small population size. Nine of the 12 loci deviated from the Hardy-Weinberg equilibrium ( $P < 0.01$ ), which could be explained by significant inbreeding rather than the presence of null alleles. These markers will provide a solid basis for future efforts in population genetic studies of *U. chenmoui*, which in turn will contribute to species conservation.

**Key words:** Conservation; Microsatellite marker; Population genetics; Ulmaceae; *Ulmus chenmoui*

## INTRODUCTION

*Ulmus chenmoui* W.C. Cheng (Ulmaceae) is a small deciduous tree that is mainly found on Langya Mountain, Anhui Province, China below 200 m (Fu and Jin, 1992). The species is listed as endangered (third class protection) in the China Red Data Book, based on International Union for Conservation of Nature criteria (Fu and Jin, 1992). The continuous reduction in the range and population size of this species may result in a rapid degradation of the natural habitat (Fu, 1995; Sun, 2009).

To date, most research has focused on the biology and cultivation of *U. chenmoui* (Fu and Huang, 2002; Sun, 2009; Han et al., 2010), and our knowledge of its population genetics and reproductive characteristics is insufficient. Therefore, it is necessary to develop molecular markers to provide genetic information. Microsatellites, such as simple sequence repeats (SSRs), are highly polymorphic, co-dominant, and exhibit Mendelian inheritance, and have been widely used in studies of population genetics (Geng et al., 2008; Abdul-Muneer, 2014; Islam et al., 2014; Nakajima et al., 2014; Lai et al., 2015). In this study, we identified 12 codominant compound SSR markers from *U. chenmoui* using the suppression-polymerase chain reaction (PCR) technique (Lian et al., 2006) to investigate the spatial genetic structure, interpopulation diversity, mating system, and gene flow of *U. chenmoui*, which will provide essential information in developing conservation strategies for this endangered species.

## MATERIAL AND METHODS

### Sample collection and DNA extraction

The population on Langya Mountain, Chuzhou, Anhui Province, China (32°16.5506'N, 118°16.5685'E) consists of fewer than 150 individuals occupying an area of 2.5 ha. Leaves were randomly selected from 39 trees. Young leaves were dried in silica gel and stored at room temperature until use. Total genomic DNA was extracted from dried leaves using a modified cetyltrimethyl ammonium bromide method (Zhou et al., 1999).

### Isolation of microsatellite markers

Microsatellite markers were isolated using a suppression-PCR technique for isolating

**Table 1.** Characteristics of 12 microsatellite loci isolated from *Ulmus chenmoui*.

Locus	Primer sequence (5'-3')	Repeat motif	$T_m$ (°C)	Size range (bp)	$N_A$	$H_o$	$H_E$	$F_{IS}$	Dye	Accession No.
Ulmc2	F: ATTGCAATTCCTCGTACAAACGTTGAA R: ACACACACACACACTCTCTC	(AC) <sub>6</sub> (TC) <sub>7</sub>	56	152-182	7	0.385	0.560*	<b>0.325</b>	VIC	KP872848
Ulmc3	F: TAAGATCGTTGGAAAGTTCAGTTCCA R: ACACACACACACACTCTCTC	(AC) <sub>6</sub> (TC) <sub>7</sub>	56	121-139	9	0.154	0.726*	<b>0.793</b>	VIC	KP872849
Ulmc6	F: ACCCTAAATAGATTTCATAAAGATT R: ACACACACACACACTCTCTC	(AC) <sub>6</sub> (TC) <sub>9</sub>	51	192-196	3	0.513	0.575	0.121	FAM	KP872850
Ulmc9	F: AATCAITGTTTAGTTGGACAGGATT R: ACACACACACACACTCTCTC	(AC) <sub>6</sub> (TC) <sub>9</sub>	51	209-235	6	0.231	0.756*	<b>0.701</b>	FAM	KP872851
Ulmc20	F: AGTTAGCCGAAGTGTAGGTGATTA R: TCTCTCTCTCTCACACAC	(TC) <sub>6</sub> (AC) <sub>8</sub>	56	174-192	4	0.500	0.639	0.230	VIC	KP872852
Ulmc22	F: AATTACAGGACATGCATGTAAGTT R: TCTCTCTCTCTCACACAC	(TC) <sub>6</sub> (AC) <sub>8</sub>	51	181-199	6	0.769	0.768	0.012	FAM	KP872853
Ulmc24	F: TCCCTCTCTGAAATGCTGAGCAA R: TCTCTCTCTCTCACACAC	(TC) <sub>6</sub> (AC) <sub>7</sub>	56	176-180	4	0.263	0.577*	<b>0.553</b>	FAM	KP872854
Ulmc47	F: TATCGAAACAAAGCCTTAGCCCAA R: ACACACACACACACTCTCTC	(AC) <sub>6</sub> (TC) <sub>5</sub>	56	139-143	3	0.368	0.577*	<b>0.373</b>	PET	KP872855
Ulmc50	F: ATCTAATGCAATTTGTTCTTTCAA R: TCTCTCTCTCTCACACAC	(TC) <sub>6</sub> (AC) <sub>5</sub>	54	127-133	4	0.333	0.595*	<b>0.451</b>	PET	KP872856
Ulmc52	F: TCGTAAATACCGTAGGGCTTATAA R: TCTCTCTCTCTCACACAC	(TC) <sub>6</sub> (AC) <sub>5</sub>	56	137-143	4	0.459	0.680*	<b>0.337</b>	VIC	KP872857
Ulmc57	F: TTGAFAATTTAGTGTGCAATTAA R: TCTCTCTCTCTCACACAC	(TC) <sub>6</sub> (AC) <sub>5</sub>	53	120-146	6	0.051	0.533*	<b>0.906</b>	PET	KP872858
Ulmc65	F: GAAAGTAGTCGGAATGACCTACCT R: ACACACACACACACAGAGAG	(AC) <sub>6</sub> (AG) <sub>5</sub>	57	118-128	4	0.211	0.610*	<b>0.662</b>	VIC	KP872859
Mean					5	0.353	0.633	<b>0.453</b>		

$T_m$ : annealing temperature of the primer pair;  $N_A$ : number of alleles;  $H_o$ : observed heterozygosity;  $H_E$ : expected heterozygosity;  $F_{IS}$ : inbreeding coefficient;  $F_{IS}$  in bold indicates significant deviation from zero.

codominant compound SSR markers (Lian et al., 2001, 2006). Briefly, genomic DNA was separately digested with six blunt-end restriction enzymes, i.e., *AfaI*, *AluI*, *EcoRV*, *HaeIII*, *HincII*, and *SspI*, and the restricted fragments were then separately ligated with a specific blunt adaptor (48-mer: 5'-GTAATACGACTCACTATAGGGCACGCGTGGTTCGACGGCCCGGGCTGGT-3' and 8-mer with the 3'-end capped with an amino residue: 5'-ACCAGCCC-NH<sub>2</sub>-3') using a DNA ligation kit (Takara Bio Inc., Shiga, Japan). The ligated fragments were treated with ddGTP using AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Carlsbad, CA, USA) to block the polymerase-catalyzed extension of the 8-mer adapter strand.

DNA fragments were amplified using a compound SSR primer [(AC)<sub>6</sub>(TC)<sub>5</sub>, (TC)<sub>6</sub>(AC)<sub>5</sub>, (AC)<sub>6</sub>(AG)<sub>5</sub>, (AG)<sub>6</sub>(AC)<sub>5</sub>, or (TC)<sub>6</sub>(TG)<sub>5</sub>] and an adaptor primer (5'-CTATAGGGCACGCGTGGT-3') from the adapter-ligated DNA library (i.e., *AfaI*, *AluI*, *EcoRV*, and *HaeIII*) of *U. chenmoui*. The amplified, smeared fragments were cloned into a pEASY®-T1 Cloning Vector System (Beijing Transgen Biotech, Beijing, China) according to the manufacturer instructions. Recombinant clones were identified by blue/white screening on lysogeny-broth agar plates containing ampicillin, X-gal, and isopropyl β-D-1-thiogalactopyranoside. The cloned fragments were amplified using M13 forward and reverse primers from the plasmid DNA of insert-positive clones. After electrophoresis on a 1.5% agarose gel, amplified fragments longer than 400 bp were sequenced using a M13 primer (Beijing Genomics Institute, Shenzhen, China). For each fragment containing compound SSR sequences [(AC)<sub>6</sub>(TC)<sub>n</sub>, (TC)<sub>6</sub>(AC)<sub>n</sub>, (AC)<sub>6</sub>(AG)<sub>5</sub>, (AG)<sub>6</sub>(AC)<sub>5</sub>, or (TC)<sub>6</sub>(TG)<sub>5</sub>] at one end, a specific primer was designed using PRIMER version 5.0 (Clarke and Gorley, 2001) flanking the compound SSR. The specific primer and corresponding compound SSR primer were used as a compound SSR marker.

To examine polymorphisms of the isolated compound SSR markers, we performed a PCR assay in a 10-μL reaction mixture containing approximately 10 ng of template DNA, 5 μL 1X AmpliTaq Gold® 360 Master Mix (Applied Biosystems), 0.4 μL 360 GC Enhancer, and 0.25 μL 0.5 μM of each specific primer and fluorescent dye-labeled compound SSR primer (6-FAM, VIC, NED, or PET; Applied Biosystems). The PCRs were performed in a Veriti® Thermal Cycler (Applied Biosystems). The PCR cycling conditions were as follows: 10 min at 95°C; 38 cycles of 30 s at 95°C, 30 s at the annealing temperature of each designed specific primer, and 1 min at 72°C; with a 7-min extension at 72°C in the final cycle. The PCR products were genotyped on an ABI 3730 genetic analyzer with the GeneScan™-500 LIZ® Size Standard (Applied Biosystems), and allele sizes were determined using GeneMapper™ software version 4.0 (Applied Biosystems).

## Data analysis

GenAIEx 6.5 (Peakall and Smouse, 2006) was used to calculate the number of alleles per locus ( $N_A$ ), observed heterozygosity ( $H_O$ ), and expected heterozygosity ( $H_E$ ). Micro-Checker version 2.2.3 was used to assess null allele frequencies by the Van Oosterhout algorithm method (Van Oosterhout et al., 2004), and GENEPOP version 4.2 (Raymond and Rousset, 1995; Rousset, 2008) was used to test the Hardy-Weinberg equilibrium (HWE) for each locus and genotypic linkage disequilibrium (LD) between pairs of microsatellites (66 pairs). The P values for HWE and LD were corrected for multiple comparisons with sequential Bonferroni tests (Rice, 1989). The program FSTAT version 2.9.3 was used

to calculate the inbreeding coefficient ( $F_{IS}$ ) (Goudet, 1995). Significance levels were determined by sequential Bonferroni tests (Rice, 1989). BOTTLENECK version 1.2.02 (Piry et al., 1999) was used to test for recent population bottlenecks. Deviations from the mutation-drift equilibrium were calculated under three different mutation models: infinite allele model (IAM), stepwise mutation model (SMM), and the two-phase model (TPM; 30% IAM and 70% SMM). The significance of the deviations was tested using the Wilcoxon signed-rank test with 1000 iterations.

## RESULTS AND DISCUSSION

A total of 111 clones with positive inserts were chosen and sequenced. Ninety-six sequences were found to contain compound SSR motifs, of which 22 were discarded because the repeat was too close to one end of the insert to design a suitable primer or because the sequence was identical to that of another insert(s). All of the successfully sequenced fragments were flanked by a compound SSR sequence at one end. Finally, 74 sequences [(AC)<sub>6</sub>(TC)<sub>n</sub> (17), (TC)<sub>6</sub>(AC)<sub>n</sub> (29), and (AC)<sub>6</sub>(AG)<sub>n</sub> (28)] with the appropriate microsatellites and sufficient flanking regions were selected for locus-specific primer design. With these primer pairs, 24 loci were successfully amplified and yielded a single band of the right size using four individuals. Twelve of 24 loci were polymorphic in 39 individuals.

The number of alleles per locus ranged from three to nine, with an average of five (Table 1). The observed and expected heterozygosities ranged from 0.051 to 0.769 and from 0.533 to 0.768, respectively (Table 1). Significant LD was found in three pairwise comparisons of the polymorphic loci (Ulmc47 and Ulmc57, Ulmc50 and Ulmc57, and Ulmc57 and Ulmc65;  $P < 0.01$ ). A recent bottleneck was detected in the *U. chenmoui* population under IAM, SMM, and TPM ( $P < 0.05$ ), which may have caused the significant LD and HWE deviations at these loci. Therefore, the LD observed in the *U. chenmoui* population may be due to a recent population bottleneck and the small population size (Sun et al., 2014), not because of a linkage relationship between these loci.

Nine significant deviations from the HWE were detected after Bonferroni corrections ( $P < 0.01$ , Table 1). Significant  $F_{IS}$ , as well as the existence of null alleles, was also detected in the nine loci. Most of the departures from the HWE were caused by significant inbreeding rather than the presence of null alleles, because the presence of null alleles was inferred from the Micro-Check (Islam et al., 2014). Other factors, such as a recent bottleneck, small population size, and genetic drifting could also have resulted in deviations from the HWE.

In this study, we developed 74 genomic SSR primer pairs from enriched genomic SSR libraries in *U. chenmoui* using a suppression-PCR technique (Lian et al., 2006). Twelve microsatellite loci were polymorphic, and the polymorphism levels were comparable to those of microsatellite loci described previously in other elm species (Zalapa et al., 2008; Maharramova et al., 2014). The 12 microsatellite loci identified in this study will be useful for investigating the genetic diversity, spatial genetic structure, and mating system of the *U. chenmoui* population, which could facilitate plans for its conservation and management.

## Conflicts of interest

The authors declare no conflicts of interest.

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