



# Development and characterization of microsatellite loci in *Excentrodendron hsienmu* (Chun & How) H.T. Chang & R.H. Miao

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**ABSTRACT.** Microsatellite markers were isolated using dual-suppression-PCR for the endangered species *Excentrodendron hsienmu* (Tiliaceae) to evaluate the genetic diversity and population structure of this species. A total of 11 polymorphic microsatellite loci were characterized in *E. hsienmu*. The number of alleles per locus ranged from 2 to 9, with an average of 5.27. The expected heterozygosity value ranged from 0.053 to 0.780, with an average of 0.568 and the observed heterozygosity value ranged from 0 to 0.595, with an average of 0.268. The polymorphic information content value ranged from 0.051 to 0.740, with an average of 0.521. These newly

designed markers will be of great potential significance and profound influence in future research related to the genetic diversity, population structure, and patterns of gene flow of this species, which will contribute to the implementation of conservation and management strategies for this species.

**Key words:** Genetic diversity; Microsatellite loci; SSRs;  
*Excentrodendron hsienmu* (Chun & How) Hung T. Chang & R.H. Miao

## INTRODUCTION

*Excentrodendron hsienmu* (Chun & How) Hung T. Chang & R.H. Miao, commonly known as hsienmu or huomushu, is an evergreen tree distributed in the limestone rainforest exclusively restricted to Guangxi, Yunnan, in southeastern China, and Vietnam (Tang et al., 2007). It is one of the most economically important hardwoods prized for making chopping blocks, furniture, and fuel wood due to its extraordinary hardness and long-burning time (Tang et al., 2005). Because of anthropogenic activities, the individuals and populations of *E. hsienmu* have been in severe decline in the past years and are listed on International Union for Conservation of Nature (IUCN) Red Lists of threatened species (IUCN, 2015). While a better understanding of the genetic diversity and population structure in this species could implement effective conservation and management strategies to protect this important germplasm resource, only one such study on genetic diversity of this species has been reported that utilized inter-simple sequence repeat (ISSR) markers (Gao, 2006). However, ISSR loci are dominant markers and are difficult to use in the calculation of heterozygosity and paternity analysis, suggesting that additional polymorphic genetic markers are needed. Microsatellites or simple-sequence repeat (SSR) loci are co-dominant, highly polymorphic, multi-allelic, reproducible, locus-specific, and are abundantly dispersed throughout the plant genome (Gupta and Varshney, 2000). Here, we report the development of 11 polymorphic microsatellite markers for *E. hsienmu* that will be used in the study of genetic diversity and population structure in the future.

## MATERIAL AND METHODS

Total genomic DNA was isolated from silica dried leaves from a single *E. hsienmu* individual from the Longzhou, Guangxi population (106°56'37"N; 22°27'53"E) using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer instructions. The concentration of genomic DNA was determined by running the samples on a 1.5% agarose gel. Microsatellites were developed following the dual-suppression-PCR protocol (Lian et al., 2006). Total genomic DNA was digested with the *EcoRV* or *HaeIII* restriction enzymes (Takara, Dalian, China) in order to construct a DNA library for *E. hsienmu*. The digested fragments were ligated with a specific blunt adaptor (consisting of a 48-mer upper strand: 5'-GTAATACGACTCACTATAGGGCACGCGTGGTTCGACGGCCCGGGCTGGT-3', and an 8-mer lower strand with the 3'-end capped by the amino residue: 5'-ACCAGCCC-NH<sub>2</sub>-3') by T4 DNA ligase (Takara). The fragments were then PCR-amplified from the

*EcoRV* or *HaeIII* DNA library using the compound SSR primer (AC)<sub>6</sub>(AG)<sub>5</sub> or (TC)<sub>6</sub>(AC)<sub>5</sub> and an AP<sub>2</sub> adaptor (5'-CTATAGGGCACGCGTGGT-3'). Each 50- $\mu$ L PCR volume contained 25-50 ng genomic DNA, 1X PCR buffer with MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.5 U *ExTaq* polymerase (Takara), and 0.5 mM each compound SSR and AP<sub>2</sub> primers. The PCR amplification conditions were as follows: 1 cycle of 10 min at 94°C, 30 s at 62°C, and 1 min at 72°C; 5 cycles each of 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; and a final cycle of 30 s at 94°C, 30 s at 60°C, and 9 min at 72°C. The PCR products were purified using a DNA clean-up kit (Axygen, Union, CA, USA), and ligated into pMD 19-T vector (Takara), and transformed into DH5 $\alpha$  competent cells (Takara). A single clone was confirmed using the M13 universal primers (M13 forward primer: 5'-TGTAACGACGGCCAGT-3'; M13 reverse primer: 5'-CAGGAAACAGCTATGACC-3'). Positive clones were sent to Life Technologies for sequencing 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 using Primer Premier ver. 5.0 (Clarke and Gorley, 2001).

The primer pairs of the designed gene and compound SSR primer were used to amplify microsatellite repeats from 37 individuals from two *E. hsienmu* populations: the Longzhou (LZ), Guangxi population and the Hekou (HK), Yunnan population (103°55'45"N; 22°40'40"E). Voucher specimens for the sampled populations have been deposited in the Guangxi Key Laboratory of Superior Timber Trees Resource Cultivation, Guangxi Forestry Research Institute. In order to examine their effectiveness and levels of polymorphism, the 5'-end of the compound SSR primers were labeled with fluorescent dyes (FAM or HEX). The amplified products were analyzed using 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 3.0 (Kalinowski et al., 2007) was used to calculate  $N_A$  (number of alleles per locus),  $H_o$  (observed heterozygosity),  $H_e$  (expected heterozygosity), and PIC (polymorphic information content). Genepop (<http://genepop.curtin.edu.au/>) was employed to test the Hardy-Weinberg equilibrium (HWE) and linkage disequilibria (LD).

## RESULTS

In total, 11 microsatellite loci were identified (Table 1) from the tested plant material. All 11 loci were successfully amplified in the 32 individuals collected from the two *E. hsienmu* populations. The  $N_A$  ranged from 2 to 9 alleles, with an average of 5.27. Individually, the LZ population  $N_A$  ranged from 2 to 7, with an average of 4.4; in the HK population the  $N_A$  ranged from 1 to 8, with an average of 4.1. The  $H_o$  and  $H_e$  values ranged from 0 to 0.595, with an average of 0.268 (0 to 0.682, with an average of 0.298 in the LZ population; 0 to 0.533, with an average of 0.224 in the HK population), and ranged from 0.053 to 0.780, with an average of 0.568 (0.045 to 0.741, with an average of 0.539 in the LZ population; 0 to 0.893, with an average of 0.580 in the HK population), respectively. The PIC values were from 0.051 to 0.740, with an average of 0.521 (0.043 to 0.679, with an average of 0.486 in the LZ population; 0 to 0.786, with an average of 0.517 in the HK population) (Tables 1 and 2). Most of the microsatellite loci deviated significantly from the HWE ( $P < 0.001$ ). The pairwise LD between the 11 pairs of loci was not significant ( $P > 0.001$ ).

**Table 1.** Characterization of 11 polymorphic microsatellite loci developed for *Excentrodendron hsienmu*.

Locus	Primers sequences (5'-3')	Repeat motif	Ta (°C)	Allele size (bp)	N <sub>A</sub>	H <sub>O</sub>	H <sub>E</sub>	PIC	Accession No.
XmSSR1	F: ACACACACACAGAGAGAGAG R: ACCCTGCAATAATAAATAACC	(AC) <sub>n</sub> (AG) <sub>3</sub> ...(AGGG) <sub>3</sub>	56	92	4	0.324	0.701	0.638	KU064726
XmSSR2	F: ACACACACACAGAGAGAGAG R: TTCAGGGTTCAGCATTCAAAGT	(AC) <sub>n</sub> (AG) <sub>3</sub>	56	192	5	0.270	0.577	0.522	KU064727
XmSSR3	F: ACACACACACAGAGAGAGAG R: TAACCACCAAAGTCCAACGAAA	(AC) <sub>n</sub> (AG) <sub>7</sub>	56	139	4	0.324	0.679	0.613	KU064728
XmSSR4	F: ACACACACACAGAGAGAGAG R: TTGGAGGGATAGAAAGTTTACG	(AC) <sub>n</sub> (AG) <sub>3</sub>	56	100	8	0.216	0.625	0.595	KU064729
XmSSR5	F: ACACACACACAGAGAGAGAG R: CATAGTCCACGACATCATAATCC	(AC) <sub>n</sub> (AG) <sub>3</sub>	56	122	2	0.000	0.053	0.051	KU064730
XmSSR6	F: ACACACACACAGAGAGAGAG R: ACATTCTGAGATGCCATAGTTT	(AC) <sub>n</sub> (AG) <sub>3</sub>	56	149	5	0.243	0.662	0.585	KU064731
XmSSR7	F: ACACACACACAGAGAGAGAG R: GGTTCATTTGATCGACGAGAC	(AC) <sub>n</sub> (AG) <sub>8</sub>	56	162	5	0.432	0.671	0.598	KU064732
XmSSR8	F: ACACACACACAGAGAGAGAG R: GTGGTTTGCTCACATCGGACTC	(AC) <sub>n</sub> (AG) <sub>6</sub>	56	134	7	0.351	0.745	0.691	KU064733
XmSSR9	F: ACACACACACAGAGAGAGAG R: AGTGAAAGAAACGAGCAATGAA	(AC) <sub>n</sub> (AG) <sub>6</sub>	56	140	9	0.135	0.780	0.740	KU064734
XmSSR10	F: TCTCTCTCTCACACACACAC R: ACATCACCACCTTCGCATCAG	(TC) <sub>n</sub> (AC) <sub>3</sub>	56	116	3	0.054	0.054	0.052	KU064735
XmSSR11	F: TCTCTCTCTCACACACACAC R: ATTCGTTGGATCCATGATTAA	(TC) <sub>n</sub> (AC) <sub>3</sub>	56	94	6	0.595	0.699	0.647	KU064736

Ta = annealing temperature used in PCR; N<sub>A</sub> = number of alleles; H<sub>O</sub> = observed heterozygosity; H<sub>E</sub> = expected heterozygosity; PIC = polymorphic information content.

**Table 2.** Genetic diversity of 11 microsatellite loci in two populations of *Excentrodendron hsienmu*.

Locus	Longzhou, Guangxi (N = 22) 106°56'37"N; 22°27'53"E				Hekou, Yunnan (N = 15) 103°55'45"N; 22°40'40"E			
	N <sub>A</sub>	H <sub>O</sub>	H <sub>E</sub>	PIC	N <sub>A</sub>	H <sub>O</sub>	H <sub>E</sub>	PIC
XmSSR1	4	0.364	0.701	0.628***	4	0.267	0.671	0.586***
XmSSR2	5	0.318	0.561	0.510***	4	0.200	0.609	0.528***
XmSSR3	4	0.455	0.728	0.658***	3	0.133	0.579	0.499***
XmSSR4	5	0.182	0.520	0.483***	6	0.267	0.726	0.664***
XmSSR5	2	0.000	0.089	0.083*	1	0.000	0.000	0.000
XmSSR6	5	0.318	0.707	0.629***	3	0.133	0.570	0.456***
XmSSR7	3	0.500	0.587	0.494 n.s.	5	0.333	0.756	0.685***
XmSSR8	7	0.227	0.741	0.679***	4	0.533	0.756	0.683***
XmSSR9	5	0.182	0.682	0.631***	8	0.067	0.839	0.786***
XmSSR10	2	0.045	0.045	0.043	2	0.067	0.067	0.062
XmSSR11	6	0.682	0.569	0.504 n.s.	5	0.467	0.802	0.739***
Mean	4.4	0.298	0.539	0.486	4.1	0.224	0.580	0.517

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

The 11 novel microsatellite loci developed in *E. hsienmu* in this study was evaluated in two populations. The mean N<sub>A</sub>, H<sub>O</sub>, H<sub>E</sub> and PIC values were 5.27, 0.268, 0.568 and 0.521, respectively, which show high levels of polymorphism, indicating that these newly designed markers will be of great potential significance and profound influence in future research related to the genetic diversity, population structure, and patterns of gene flow of this species, which will contribute to implement the conservation and management strategies of this species.

## Conflicts of interest

The authors declare no conflict of interest.

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