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Short Note: Isolation and Characterization of 12 Polymorphic Microsatellite Markers in *Engelhardia roxburghiana* (Juglandaceae)

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Abstract

Engelhardia roxburghiana is a common half evergreen tree with a wide distribution in southeast Asia. Despite its ecological and pharmaceutical values, its genetic diversity is poorly studied. Our objective was to develop nuclear microsatellite markers to investigate the level of genetic diversity within and among populations in the future. Using the microsatellite-enriched library and PCR-based screening method, 12 microsatellite markers were developed and showed polymorphism in a population. The number of alleles per locus for these 12 microsatellites ranged from four to 15. The observed and expected heterozygosities ranged from 0.358 to 0.897 and from 0.369 to 0.886, respectively. The

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developed microsatellites will be useful for studying genetic diversity and population structure in *E. roxburghiana*.

Key words: DHS plot, DNA enriched libraries, genetic marker, genetic variation, Hardy-Weinberg equilibrium, linkage disequilibrium, marker development, microsatellite, population genetics, spatial genetic structure, subtropical China.

Engelhardia roxburghiana Wallich, belonging to Juglandaceae family, is a common half evergreen tree with a wide distribution in southeast Asia from Pakistan east to south China and Indonesia (Lu et al., 1999). It is a pioneer species, can grow in various soil types and withstand drought. Therefore it is an important tree used for reforestation and site restoration programs (Manos and Stone, 2001). *Engelhardia roxburghiana* has unisexual flowers and wind dispersed pollens. Its fruit consist of a single-seeded nut with relatively large trilobate wing formed by bract, that serves to disseminate the seed by wind. *Engelhardia roxburghiana* is also an important medical plant. Its leaf, stem or root extractions are useful for the treatment of several ailments like obesity, fever, tuberculosis, inflammation, tumour (Wu et al., 2007; Wu et al., 2012; Xin et al., 2012). However, no markers for the genetic analysis of this species and relatives have been reported in spite of its ecological and

Table 1. – Characteristics of 12 microsatellite primers developed in *Engelhardia roxburghiana* T_a: annealing temperature; N_a: number of alleles.

Locus	Repeat motifs	Primer sequences (5'-3')	Size range (bp)	T_a(℃)	5'-fluorescence label	EMBL Accession No.
HQ-23	$(CT)_{11}(CA)_{19}$	F: GGTGAAATATCTTGGCACCAC R: CCATGCCACACAAACAAAAC	145-183	56	FAM	HG421129
HQ-29	$(GT)_{11}$	F: TTCCTATTTTTATCAAGTCCATGC R: ATCACATCTGCACCTTGACG	170-178	56	FAM	HG421130
HQ-36	$(TG)_{10}$	F: CTCCCATTACAGGCCACAAC R: CAACACACACTCCCCAAAAG	219-229	56	HEX	HG421131
HQ-49	$(TTG)_6(TTA)_3$	F: AAAAGCAAGAAAAGGCTTGG R: CCACCCATCAACTTCACAAG	149-168	56	FAM	HG421132
HQ-53	$(AC)_4$ GC$(AC)_3$AT$(AC)_5$ GC$(AC)_3$AT$(AC)_4$	F: CAACTAAGAGACGCCACCAA R: CTGCCTGCTGATTGATTGAA	190-220	58	HEX	HG421133
HQ-54	$(TG)_{14}(TTTG)_4(TG)_2$	F: ATTCAATCTAACTAGCTCGTG R: TTAGATATATTCGTTGGCATC	147-183	52	FAM	HG421134
HQ-89	$(GA)_{15}GG(GA)_5$	F: CACCCATGGGAGAATCAAAA R: TCGTTCACCGATGATGATGT	155-189	56	FAM	HG421135
HQ-149	$(CT)_8(CA)_6$ CG$(CA)_3$	F: CCACCATAACGTGACCAACA R: CACGAAACGAAAATGAGCTG	212-232	52	HEX	HG421136
HQ-156	$(AC)_{17}$	F: TTGAATCAGCATCCCTCTCC R: ATGTTGCGCGTTCTTAGCTT	219-247	52	HEX	HG421137
HQ-20(AG)	$(AG)_8AAA(AG)_7$	F: TTGCTGGCTGCAGTTTTATG R: GTCTCCCTCATCCCCAATTT	234-252	56	HEX	HG421138
HQ-23(AG)	$(GA)_4GG(GA)_{11}$ C$(GA)_3$	F: CAGACCTCTGCACCTTCTCC R: GCAGCTCTTTCTGCTTCCAC	142-192	52	FAM	HG421139
HQ-51(AG)	$(GA)_{13}$	F: GGAGAAGGAGGGTGAAGAGG R: GGCAAGTACAAGGTGCCATT	184-222	52	HEX	HG421140

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Table 2. – Genetic diversity of 12 loci in 486 *Engelhardia roxburghiana* individuals in DHS Plot.

Locus	*A*	H_o	H_e	*F*
HQ-23	15	0.897	0.886	-0.0121
HQ-29	4	0.615	0.614	-0.0018
HQ-36	5	0.358	0.369	0.0310
HQ-49	6	0.755	0.757	0.0030
HQ-53	4	0.514	0.559	0.0792*
HQ-54	15	0.816	0.827	0.0132
HQ-89	7	0.512	0.526	0.0261
HQ-149	8	0.525	0.487	-0.0785
HQ-156	13	0.794	0.782	-0.0157
HQ-20(AG)	6	0.409	0.525	0.2208*
HQ-23(AG)	11	0.383	0.412	0.0705
HQ-51(AG)	9	0.813	0.780	-0.0415

Note: *A* = number of alleles; *F*: fixation index; H_e: unbiased expected heterozygosity; H_o: observed heterozygosity.

* indicates a deviation at $P < 0.05$ from Hardy-Weinberg equilibrium after Bonferroni correction.

pharmaceutical values. To explore this, we developed 12 polymorphic microsatellite loci for *E. roxburghiana*. To our knowledge, this work is the first to report microsatellite loci in this genus.

Total genomic DNA was extracted from one dry leaf of *E. roxburghiana* from Dinghushan National Nature Reserve (112°30'39" – 112°33'41"E, 23°09"21" – 23°11'30"N) in South China using a modified CTAB method (Doyle, 1991). Approximately 250 ng genomic DNA was digested into 300 – 1000bp fragments using the restriction enzyme *Mse*I (New England Biolabs). The fragments were then ligated to *Mse*I adapters (*Mse*I F: 5'-TACTCAGGACTCAT-3' and *Mse*I R: 5'-GACGATGAGTCCTGAG-3') using T4 DNA ligase (New England Biolabs) overnight at 16 °C. The digestion-ligation mixture was subsequently diluted 10 times, and 2 µL of diluted mixture was used for PCR amplification using *Mse*I-adapter specific primers (5'-GATGAGTCCTGAGTAAN-3', i.e., *Mse*I-N). The amplified products were hybridized with 5'-biotinylated $(AG)_{15}$, $(AC)_{15}$ or $(AAC)_8$ probes. The probe-bound DNA fragments were enriched for AG, AC or AAC repeats using streptavidin-coated magnetic beads (New England Biolabs). The enriched fragments were recovered by PCR using *Mse*I-N as a primer, and then ligated into the pGEM-T plasmid vector (Promega) and transformed into the *Escherichia coli* DH5α competent cells (Takara). A PCR-based method (Lunt et al., 1999) was used to screen the recombinant clones. A total of 174 positive clones were identified and sequenced by Majorbio Biotech Co., LTD. (Shanghai, China) with M13R or M13F as primers. We detected microsatellites having at least eight AG, AC or AAC repeats in 90 sequences. We then used Primer 3 software (Rozen and Skalesky, 2000) to design primers for these sequences, and 41 of the sequences were discarded due to primer design failure: repeats close to one end of sequences or low quality of primers indicated by Primer 3 software. PCR amplifications were performed for the rest 49 sequences in a 20 µL volume containing 20 mM Tris-HCl (pH 8.4), 100 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 µM each primer, 50 ng of genomic DNA, and 1 U *Taq* polymerase (Takara). The amplification program was 95 °C for 5 min, 35 cycles of 94 °C for 30 s, optimized annealing temperature for 30 s and 72 °C for 45 s, with a final extension at 72 °C for 10 min. We checked the PCR products on 2% agarose gels and found that 32 of them could be successfully amplified with expected sizes.

Polymorphisms were initially assessed in these 32 microsatellite loci using 12 individuals that were randomly selected from 486 samples of *E. roxburghiana* [with diameter at breast height (DBH) from 1 cm to 70 cm] collected from 20-ha DHS plot in Dinghushan National Nature Reserve. PCRs were performed using the same procedure above. Electrophoresis of the products was done on ABI 3730 sequencer (Applied Biosys-

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tems, Carlsbad, California, USA), and fragment lengths were analyzed using ABI GeneMapper Software Version 4.1. Among the 32 microsatellites, 12 yielded clear and stable polymorphisms (*Table 1*). They were then assessed for allelic variation on all 486 individuals sampled (*Table 2*). To avoid biased amplification towards preferential microsatellites and possible artifacts caused by multiplex PCR, we amplified these 12 microsatellites individually.

Genetic diversity parameters for 12 loci, deviation from Hardy-Weinberg equilibrium (HWE), and genotypic linkage disequilibrium (LD) among all pairs of 12 loci were estimated using GENEPOP version 4.1.4 (Rousset, 2008). Significance levels were adjusted using the Holm's sequential Bonferroni correction (Holm, 1979). Alleles per locus varied from four to 15 (*Table 2*). The observed and expected heterozygosities ranged from 0.358 to 0.897 and from 0.369 to 0.886, respectively. Significant deviation from HWE was found in two loci [HQ-53 and HQ-20(AG)] due to heterozygote deficiency. All locus pairs showed significant deviation from LD when all 486 individuals were analysed. However, only loci HQ-23 and HQ-54 remained significant deviation from LD if only individuals with DBH ≥ 50 cm were analysed (n = 43).

Twelve microsatellites of *E. roxburghiana* were isolated and tested. Our data indicate that they are highly polymorphic. Therefore, they are useful to investigate genetic diversity, gene flow, population structure and phylogeography of the species. We also have plans to use them to study the fine-scale spatial genetic structure, mating system and pollen and seed flow in *E. roxburghiana* in 20-ha DHS plot. The results from these will provide useful information for the sustainable management of this species.

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