DEVELOPMENT AND CHARACTERIZATION OF POLYMORPHIC MICROSATTELITE LOCI IN \textit{Phellodendron amurense} (Rutaceae)\textsuperscript{1}

JING-HUA YU\textsuperscript{2}, CHANG-MEI CHEN\textsuperscript{2}, SHI-JIE HAN\textsuperscript{3}, XIAO-RUI GUO\textsuperscript{2}, SHU-SHENG YUAN\textsuperscript{2}, CHUN-JING WANG\textsuperscript{2}, and YUAN-GANG ZU\textsuperscript{2,4}

\textsuperscript{2}State Engineering Laboratory of Bio-Resource Eco-Utilization, Northeast Forestry University, Harbin 150040, Heilongjiang, People’s Republic of China; and \textsuperscript{3}Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, Liaoning, People’s Republic of China.

- **Premise of the study:** Microsatellite markers were developed for the rare species \textit{Phellodendron amurense} to assess the genetic diversity and population structure of this plant.
- **Methods and Results:** In total, 27 microsatellite markers were developed for \textit{P. amurense} by using an enriched genomic library and hybridization; all of these primers successfully amplified DNA fragments in \textit{P. amurense}. These markers were screened in 74 individuals from four populations in China; 15 loci were found to be polymorphic, with the number of alleles per locus ranging from one to nine.
- **Conclusions:** The microsatellite markers developed here represent a useful tool for studying the population genetic structure of \textit{P. amurense} and to inform toward the development of effective conservation programs for this species.

**Key words:** genetic conservation; microsatellite marker; \textit{Phellodendron amurense}; Rutaceae; simple sequence repeat markers.

\textit{Phellodendron amurense} Rupr. (Rutaceae) is a plant native to northern China, Korea, and Japan (Azad et al., 2005). It is a relic of the ancient tropical flora from the Tertiary period and is now widely distributed throughout Changbai Mountain, Wanda Mountain, and Xiaoxing’anling (Yan et al., 2006). However, because this plant is frequently used in Chinese medicine, the population of \textit{P. amurense} in the wild has declined sharply in China (Yan et al., 2008), and it is on the verge of extinction because of excessive and illegal harvesting (Jie et al., 2012). Owing to the destruction and fragmentation of habitats, many species have been forced into small and isolated populations, which face further risk from the effects of environmental variation, demographic stochasticity, and reduced genetic diversity (Meffe and Carroll, 1997). Hence, the key to the protection of this endangered plant is protection of its genetic diversity.

Simple sequence repeat (SSR) markers have been used to assess genetic diversity and population structure in citrus (Barkley et al., 2006) and related species. However, the distribution of these repetitive sequences in the genomic DNA is highly variable among members of the Rutaceae family (Matsuyama et al., 2001). At present, few studies have reported on resource protection and genetic diversity in \textit{P. amurense} (Qin et al., 2006); yet, such studies are crucial for the protection of this species. Here, we developed polymorphic, codominant microsatellite markers for \textit{P. amurense}. The results of our study may contribute to the development of specific management priorities and measures to preserve genetic diversity in \textit{P. amurense} populations.

**METHODS AND RESULTS**

Genomic DNA was extracted from silica gel–dried leaves (sampled from the Xiaoxing’anling population; Appendix 1) using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). Genomic DNA (300 ng) was digested with EcoRI and HindIII restriction endonucleases (TaKaRa Biotechnology Co., Dalian, Liaoning, China) and was then ligated to EcoRI amplified fragment length polymorphism (AFLP) adapters (5’-CTCGTAGACTGCGTACC-3’ and 3’-CTGAGCGATGGTTAA-5’) and HindIII AFLP adapters (5’-GACGATGAGTCCTGAG-3’ and 3’-TACTCAGGACTCTCGA-5’) with T4 DNA ligase (Shanghai Baoman Biological Technology Co. Ltd., Shanghai, China) at 4°C overnight. Adapter-ligated DNA was amplified by PCR, using the following cycling conditions: 94°C for 5 min; followed by 30 cycles each consisting of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min; and a final extension step of 72°C for 10 min. The amplified DNA fragments were denatured in boiling water for 5 min and were then hybridized with two types of 5’-biotinylated probes: (GT)\textsubscript{10} and (AG)\textsubscript{10}. Streptavidin-coated magnetic beads (Promega Biotech, Beijing, China) were prepared by gentle shaking of the vial to obtain a homogeneous slurry, then the streptavidin-coated magnetic beads were added to the hybridized DNA mixture and incubated for 10 min at room temperature to capture the DNA fragments hybridized to the probes. After enrichment of microsatellite-containing fragments on the magnetic streptavidin beads, the beads were washed four times with 300 μL of 0.1× saline sodium citrate (SSC) at room temperature, and once with 300 μL of 0.08× SSC. The separated single-stranded DNA fragments were then amplified in 20-μL reaction volumes containing 8.6 μL of DNA template, 10 μL of 2× PCR Mix (Boyouxinchuang Biotech, Beijing, China), 0.3 μL of E00 (10 μM), and 0.08× SSC. The separated single-stranded DNA fragments were then amplified in 20-μL reaction volumes containing 8.6 μL of DNA template, 10 μL of 2× PCR Mix (Boyouxinchuang Biotech, Beijing, China), 0.3 μL of E00 (10 μM), and 0.08× SSC.