Applications in Plant Sciences 2013 1(3): 1200321

PRIMER NOTE

# DEVELOPMENT AND CHARACTERIZATION OF POLYMORPHIC MICROSATELLITE LOCI IN *PHELLODENDRON AMURENSE* (RUTACEAE)[1]

JING-HUA YU[2], CHANG-MEI CHEN[2], SHI-JIE HAN[3], XIAO-RUI GUO[2], SHU-SHENG YUAN[2], CHUN-JING WANG[2], AND YUAN-GANG ZU[2,4]

[2]State Engineering Laboratory of Bio-Resource Eco-Utilization, Northeast Forestry University, Harbin 150040, Heilongjiang, People's Republic of China; and [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 *Phellodendron amurense* to assess the genetic diversity and population structure of this plant.
- *Methods and Results:* In total, 27 microsatellite markers were developed for *P. amurense* by using an enriched genomic library and hybridization; all of these primers successfully amplified DNA fragments in *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 *P. amurense* and to inform toward the development of effective conservation programs for this species.

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

*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 *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 *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 *P. amurense*. The results of our study may contribute to the development of specific management priorities and measures to preserve genetic diversity in *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 *Eco*RI and *Hin*dIII restriction endonucleases (TaKaRa Biotechnology Co., Dalian, Liaoning, China) and was then ligated to *Eco*RI amplified fragment length polymorphism (AFLP) adapters (5′-CTCGTAGACTGCGTACC-3′ and 3′-CTGACGCATGGTTAA-5′) and *Hin*dIII 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: $(GT)_{15}$ and $(AG)_{15}$. 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),

[1]Manuscript received 24 June 2012; revision accepted 3 September 2012.

The authors thank Dr. Zhongling Guo, Dr. Wei Cao, Dr. Qinggui Wang, and Dr. Chunnan Fan for help with sample collection. This study was supported by the National Basic Research Priorities Program of the Ministry of Science and Technology of China (2007FY110400-5).

[4]Author for correspondence: zygorl@nefu.edu.cn

doi:10.3732/apps.1200321

TABLE 1. Primer sequences and characteristics of 27 microsatellite loci in *Phellodendron amurense*.

| Locus | Primer sequences (5′–3′) | Repeat motif | Size (bp) | $T_a$ (°C) | GenBank accession no. |
|---|---|---|---|---|---|
| P1* | F: TTAACCCCAGTGTTTCCCTT | $(TC)_9$ | 198 | 56 | JQ613286 |
| | R: CATCCTTTGTGGCGTCTATT | | | | |
| P10* | F: GATTCACACAATTTGGTTCT | $(AC)_9$ | 118 | 57 | JQ613295 |
| | R: AACGCATGAATATAATGTAC | | | | |
| P11* | F: ACCACAACCGTCACACACTC | $(AC)_{11}$ | 205 | 56 | JQ613296 |
| | R: TGCCGCTTACCTTATCACTT | | | | |
| P13* | F: AAATGTTGGTGTTTGGTGAC | $(TG)_{10}$ | 198 | 59 | JQ613298 |
| | R: TATTTCAAAGGAACAGGCAT | | | | |
| P17 | F: TTAAAAGTAACATTTACCTGTC | $(GA)_{22}(GA)_6(GA)_5$ | 187 | 55 | JQ613302 |
| | R: TTAATACCCTTTTCCCTTT | | | | |
| P21* | F: AACTTTCCATCTAGACCCGA | $(GA)_{11}$ | 368 | 59 | JQ613306 |
| | R: ATTTCCAAACAGAGCATCCG | | | | |
| P22* | F: TCGGCTTCCCCCATTTTCC | $(TC)_{14}$ | 192 | 58 | JQ613307 |
| | R: TTCAAGTGGTTGGTGCTCC | | | | |
| P23* | F: GGTCTTGAGCCCAATAACG | $(CT)_{19}$ | 331 | 59 | JQ613308 |
| | R: TGGCACCATCTCACTAGCA | | | | |
| P24* | F: CTTGGTTTCAGGTGTAGTT | $(TC)_8(TC)_3$ | 262 | 58 | JQ613309 |
| | R: GAAAGATGTAGGTGTTGCT | | | | |
| P3* | F: TTAACCTTGAATTGCTGACC | $(TG)_{21}$ | 182 | 57 | JQ613288 |
| | R: GTAACCCTCGACCCTTGTAT | | | | |
| P4* | F: TGAGTTTTTGAACCCTATC | $(GA)_{18}$ | 87 | 56 | JQ613289 |
| | R: AGTATGTATTGACCTTTGCT | | | | |
| P40* | F: AGATGCGTGGATGGAAAAC | $(GA)_{18}$ | 211 | 56 | JQ613325 |
| | R: CGTAGCACACAATGAGTGG | | | | |
| P47* | F: CCTTGTAGCAATAGTACGG | $(TC)_{11}$ | 297 | 58 | JQ613332 |
| | R: ACTTGATGTGTCAGGGGTG | | | | |
| P7* | F: ACCCCACTTCGTTGTTGTC | $(TC)_{32}(TTC)_8$ | 208 | 57 | JQ613292 |
| | R: GGCGTTTTGAGTTATGATTC | | | | |
| P8* | F: GCTGCCAAAATAGACAAAA | $(AG)_{11}$ | 128 | 58 | JQ613293 |
| | R: ATCTCATTCTCAAGCCCTT | | | | |
| P9* | F: GAAGCTTCTAATCAAAATTTTCG | $(GA)_{16}$ | 135 | 55 | JQ613294 |
| | R: GGGAGGTTTCATCATTCTAAC | | | | |
| P2 | F: AACATTTGTTTGACGGCTTA | $(CA)_{12}$ | 155 | 58 | JQ613287 |
| | R: TATTGACTAACAGTCATAGC | | | | |
| P5 | F: TGGTCGCTAAAAATCTAAAC | $(TC)_{26}$ | 133 | 56 | JQ613290 |
| | R: AGACGAGTGACATCAAGGA | | | | |
| P15 | F: GCTTTTCTCGCTTCACTCTC | $(CT)_{12}$ | 281 | 55 | JQ613300 |
| | R: CCATTACCTTACATCCGCTT | | | | |
| P20 | F: GAATTAGGGCACACAACCCC | $(CT)_{48}$ | 167 | 59 | JQ613305 |
| | R: TTCTTCTGAGAGACCCACCG | | | | |
| P26 | F: ACATTTCTGACGGCGGTGA | $(AG)_{26}$ | 118 | 57 | JQ613311 |
| | R: CCAGCCTGAATCAAGCGTT | | | | |
| P27 | F: GATGTAAAAGGAATGGGCT | $(GA)_{15}$ | 342 | 58 | JQ613312 |
| | R: TCTGAATCTGTGGATGGGT | | | | |
| P34 | F: AGACGGTATAAGGCATAAGC | $(AG)_{16}$ | 106 | 56 | JQ613319 |
| | R: AATGGGAGCATCTTTCTTTA | | | | |
| P35 | F: TCATCTTCTTCCTTATCC | $(TC)_{10}$ | 193 | 55 | JQ613320 |
| | R: GAGGTTTTGAGCAGTTT | | | | |
| P36 | F: CTTATCCTAGCCTTCCCCT | $(GA)_9$ | 131 | 56 | JQ613321 |
| | R: TAACCCCACCACCAACCAT | | | | |
| P42 | F: TCAAATTAGGGCACAGAAC | $(CT)_{14}$ | 308 | 58 | JQ613327 |
| | R: GAAAACTTGCACAATCAACT | | | | |
| P48 | F: GAATACCACGAACCCAGGA | $(GA)_{12}$ | 225 | 55 | JQ613333 |
| | R: GGCCATAAAGCCAAACCAT | | | | |

*Note*: $T_a$ = annealing temperature.
* Indicates polymorphic primers.

0.3 µL of H00 (10 µM), and 0.2 µL of *Taq* polymerase. Thermocycling conditions were as follows: denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 45 s for five cycles, followed by a final extension step for 10 min at 72°C. After purification using a gel extraction kit (Omega Bio-Tek, Winooski, Vermont, USA), the PCR products were ligated into the pMD-18T vector (50 ng/µL; TaKaRa Biotechnology Co.) according to the manufacturer's instructions; ligation products were then transformed into competent *Escherichia coli* DH5α cells (TaKaRa Biotechnology Co.). Transformants were identified by blue/white screening on Luria–Bertani (LB) agar plates containing ampicillin (60 µg/mL).

Positive clones were tested by PCR using the universal primers of the RV-M (5′-GAGCGGATAACAATTTCACACAGG-3′) (TaKaRa Biotechnology Co.) and the $(GT)_9$ tandem repeat primers. One hundred thirty-four positive clones were selected and sequenced on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, California, USA), and, in total, 88 clones were found to contain repeats.

PCR primers for the specific amplification of each locus were designed using Primer Premier 5 software (PREMIER Biosoft International, Palo Alto, California, USA). Forty-eight SSR primer pairs were designed and tested for polymorphism in nine *P. amurense* individuals originating from four different

TABLE 2. Results for primer screening of polymorphic loci in *Phellodendron amurense*.[a]

| | Xiaoxing'anling ($N$ = 22) | | | Zhangguangcailing ($N$ = 22) | | | Changbai Mountain ($N$ = 11) | | | Laoyeling ($N$ = 19) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Locus | $A$ | $H_o$ | $H_e$ | $A$ | $H_o$ | $H_e$ | $A$ | $H_o$ | $H_e$ | $A$ | $H_o$ | $H_e$ | HWE[b] |
| P1 | 2 | 0.320 | 0.497 | 2 | 0.160 | 0.507 | 2 | 0.273 | 0.520 | 2 | 0.895 | 0.341 | 0.038 |
| P3 | 8 | 0.240 | 0.743 | 6 | 0.160 | 0.757 | 5 | 0.091 | 0.736 | 3 | 0.000 | 0.599 | 0.001 |
| P4 | 6 | 0.160 | 0.762 | 6 | 0.360 | 0.731 | 5 | 0.364 | 0.680 | 2 | 0.790 | 0.194 | 0.00 |
| P7 | 2 | 0.720 | 0.458 | 2 | 0.560 | 0.481 | 2 | 0.727 | 0.524 | 2 | 0.684 | 0.273 | 0.029 |
| P8 | 7 | 0.417 | 0.695 | 6 | 0.417 | 0.528 | 5 | 0.636 | 0.407 | 4 | 0.333 | 0.557 | 0.006 |
| P9 | 7 | 0.320 | 0.745 | 8 | 0.280 | 0.838 | 5 | 0.455 | 0.719 | 3 | 0.158 | 0.541 | 0.00 |
| P10 | 6 | 0.480 | 0.682 | 7 | 0.160 | 0.629 | 6 | 0.455 | 0.706 | 4 | 0.368 | 0.494 | 0.00 |
| P11 | 4 | 0.760 | 0.291 | 2 | 0.640 | 0.301 | 4 | 0.546 | 0.662 | 2 | 0.842 | 0.149 | 0.00 |
| P13 | 2 | 0.500 | 0.508 | 2 | 0.087 | 0.507 | 2 | 0.727 | 0.455 | 2 | 0.824 | 0.166 | 0.719 |
| P21 | 6 | 0.280 | 0.763 | 6 | 0.080 | 0.728 | 6 | 0.000 | 0.849 | 3 | 0.790 | 0.240 | 0.00 |
| P22 | 3 | 0.440 | 0.473 | 2 | 0.520 | 0.411 | 3 | 0.636 | 0.325 | 2 | 1.000 | 0.000 | 0.791 |
| P23 | 5 | 0.400 | 0.649 | 4 | 0.360 | 0.522 | 4 | 0.273 | 0.658 | 2 | 0.000 | 0.514 | 0.022 |
| P24 | 3 | 0.400 | 0.545 | 3 | 0.240 | 0.656 | 3 | 0.636 | 0.680 | 2 | 0.263 | 0.478 | 0.069 |
| P40 | 8 | 0.200 | 0.833 | 9 | 0.360 | 0.864 | 4 | 0.091 | 0.645 | 6 | 0.211 | 0.634 | 0.00 |
| P47 | 6 | 0.708 | 0.303 | 3 | 0.913 | 0.127 | 3 | 0.364 | 0.515 | 2 | 0.790 | 0.194 | 0.153 |
| Average | 5 | 0.423 | 0.596 | 5 | 0.353 | 0.572 | 4 | 0.418 | 0.605 | 3 | 0.530 | 0.358 | |

*Note*: $A$ = number of alleles; $H_e$ = expected heterozygosity; $H_o$ = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; $N$ = population size.

[a] All values are based on 74 samples located in Xiaoxing'anling, Zhangguangcailing, Changbai Mountain, and Laoyeling ($N$ = 11–22 for each).

[b] Deviation from HWE in $\chi^2$ test results ($P < 0.01$).

populations (two from Zhangguangcailing, two from Changbai Mountain, two from Laoyeling, and three from Xiaoxing'anling; Appendix 1). These PCR products were assessed on 8% polyacrylamide denaturing gels. In the individuals tested, 15 of the 27 loci were identified as polymorphic and generated consistent amplification products of the expected size range; the other 12 primer pairs amplified monomorphic loci even after PCR optimization (denaturation at 94°C for 45 s, 45 s at 55–60°C, and 32 cycles at 72°C for 45 s, with a final extension of 10 min at 72°C) (Table 1). Polymorphic SSR primers were labeled with 6-FAM fluorochromes (Applied Biosystems) and used to characterize the polymorphic microsatellite loci in 74 *P. amurense* individuals sampled from four populations (Appendix 1). PCR products were visualized by capillary electrophoresis using an ABI 3730xl Sequencer with a ROX 500 (Applied Biosystems) size standard. Population genetic studies were performed using POPGENE version 1.32 (Yeh et al., 1999).

The observed number of alleles per locus ranged from two to six in the Changbai Mountain and Laoyeling individuals, and from two to eight and two to nine in the Xiaoxing'anling and Zhangguangcailing populations, respectively, averaging four alleles per locus (Table 2) in the entire collection. In total, 242 alleles were found in the total sample collection of 74 individuals. The observed heterozygosities ranged from 0.000 to 1.000, with an average of 0.431, and the expected heterozygosities ranged from 0.000 to 0.864, with an average of 0.533. Eight loci (P3, P4, P8, P9, P10, P11, P13, and P17) showed significant deviation from Hardy–Weinberg equilibrium (HWE; $P < 0.01$ in $\chi^2$ tests) in *P. amurense* (Table 2), owing to heterozygote deficiency. The low HWE value may have been caused by the destruction and fragmentation of the habitat of this species. Significant linkage disequilibrium was not detected between any pair of loci.

## CONCLUSIONS

Fifteen of the 27 microsatellite markers are polymorphic. The microsatellite markers described here are potentially useful for characterizing the population genetic structure of *P. amurense*. The application of these microsatellite markers (including both monomorphic and polymorphic loci) will be useful for conservation genetic studies and further investigation of population genetics of *P. amurense*.

## LITERATURE CITED

AZAD, M. A. K., S. YOKOTA, T. OHKUBO, Y. ANDOH, S. YAHARA, AND N. YOSHIZAWA. 2005. In vitro regeneration of the medicinal woody plant *Phellodendron amurense* Rupr. through excised leaves. *Plant Cell, Tissue and Organ Culture* 80: 43–50.

BARKLEY, N. A., M. L. ROOSE, R. R. KRUEGER, AND C. T. FEDERICI. 2006. Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). *Theoretical and Applied Genetics* 112: 1519–1531.

DOYLE, J. J., AND J. L. DOYLE. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

JIE, W. G., B. Y. CAI, AND J. P. GE. 2012. Molecular detection and community analysis of arbuscular mycorrhizal fungi in the rhizosphere of *Phellodendron amurense*. *Annals of Microbiology* 62: 1769–1777.

MATSUYAMA, T., M. OMURA, AND T. AKIHAMA. 2001. Distribution of Rutaceae-specific repeated sequences isolated from citrus genomes. *Annals of Botany* 87: 845–849.

MEFFE, G. K., AND C. R. CARROLL. 1997. Principles of conservation biology. Sinauer Associates, Sunderland, Massachusetts, USA.

QIN, Y. J., Y. WANG, AND X. F. YAN. 2006. Status and sustainable utilization strategy of *Phellodendron amurense* resources in China. *Chinese Traditional and Herbal Drugs* 37: 1104–1107 (in Chinese).

YAN, Z. F., B. G. ZHANG, Z. ZHANG, AND J. L. YU. 2006. Genetic diversity in wild populations of *Phellodendron amurense*, a rare and endangered medicinal plant, detected by AFLP. *Biodiversity Science* 14: 488–497 (in Chinese with English abstract).

YAN, Z. F., B. G. ZHANG, AND Z. ZHANG. 2008. Genetic diversity evaluation of ex-situ conservation population of *Phellodendron amurense* detected by AFLP markers. *China Journal of Chinese Materia Medica* 33: 1121–1125 (in Chinese with English abstract).

YEH, F. C., R. BOYLE, R. C. YANG, Z. YE, J. X. MAO, AND D. YEH. 1999. POPGENE version 1.32. Computer program and documentation distributed by the author. Website: http://www.ualberta.ca/~fyeh/popgene.html [accessed 23 January 2013].

Appendix 1. Geographic localities and sample sizes of the *Phellodendron amurense* populations in this study. All voucher specimens have been deposited at the Herbarium of State Engineering Laboratory of Bio-Resource Eco-Utilization, Northeast Forestry University, Heilongjiang, China.

| Locality | Latitude (°N) | Longitude (°E) | Altitude (m) | Sample size | Population code; herbarium voucher accession code |
|---|---|---|---|---|---|
| Xiaoxing'anling | 48.3949 | 129.9367 | 415.0 | 22 | XXAL; paXXAL2010-Yu |
| Zhangguangcailing | 43.59923 | 127.3509 | 348.5 | 22 | ZGCL; paZGCL2010-Yu |
| Changbai Mountain | 42.0418 | 126.0089 | 630.0 | 11 | CBS; paCBS2010-Yu |
| Laoyeling | 42.5576 | 130.5273 | 38.0 | 19 | LYL; paLYL2010-Yu |