

## **Isolation and Characterization of 20 Polymorphic Microsatellite Markers for *Juglans mandshurica* (Juglandaceae)**

Authors: Chen, Chang-Mei, Han, Shi-Jie, Yuan, Shu-Sheng, Wang, Chun-Jing, and Yu, Jing-Hua

Source: Applications in Plant Sciences, 1(7)

Published By: Botanical Society of America

URL: <https://doi.org/10.3732/apps.1200009>

---

BioOne Complete ([complete.BioOne.org](https://complete.BioOne.org)) is a full-text database of 200 subscribed and open-access titles in the biological, ecological, and environmental sciences published by nonprofit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Complete website, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at [www.bioone.org/terms-of-use](https://www.bioone.org/terms-of-use).

Usage of BioOne Complete content is strictly limited to personal, educational, and non - commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

---

BioOne sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

## ISOLATION AND CHARACTERIZATION OF 20 POLYMORPHIC MICROSATELLITE MARKERS FOR *JUGLANS MANDSHURICA* (JUGLANDACEAE)<sup>1</sup>

CHANG-MEI CHEN<sup>2</sup>, SHI-JIE HAN<sup>3</sup>, SHU-SHENG YUAN<sup>2</sup>, CHUN-JING WANG<sup>2</sup>, AND JING-HUA YU<sup>2,4</sup>

<sup>2</sup>State Engineering Laboratory of Bio-Resource Eco-Utilization (Heilongjiang), Northeast Forestry University, Harbin 150040, People's Republic of China; and <sup>3</sup>Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, Liaoning, People's Republic of China

- *Premise of the study:* Fifty microsatellite loci were developed for the endangered species *Juglans mandshurica* to investigate its genetic diversity and population structure.
- *Methods and Results:* In all, 50 microsatellite markers were isolated from *J. mandshurica*, using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol. Twenty of these polymorphic markers were assessed in samples collected from 98 individuals among five populations in northeastern China. Across all of the *J. mandshurica* samples, the number of alleles per locus ranged from one to 17.
- *Conclusions:* These new microsatellite loci will be useful for conservation genetics studies of *J. mandshurica*.

**Key words:** genetic conservation; Juglandaceae; *Juglans mandshurica*; microsatellite marker; simple sequence repeat.

*Juglans mandshurica* Maxim. (Juglandaceae) is a deciduous tree that is widely distributed in northeastern China, as well as in some areas of Korea and the Russian Far East (Lu, 1982). However, the habitat of *J. mandshurica* is now seriously threatened by human activities (Wang et al., 2011). *Juglans mandshurica* has been used in folk medicine for many years, particularly in China and India (Xu et al., 2010). Its leaves, fruits, roots, stem bark, and seeds have also been used as traditional medicine for cancer treatment in Asia and Europe (Kim et al., 1998; Sun, 2004). The therapeutic benefits of *J. mandshurica* have been ascribed to its naphthoquinone content. Naphthoquinone is known to have various physiological properties including the induction of apoptosis effect (Kang et al., 2001).

To date, there have been only a few reports on microsatellites in *J. mandshurica* (Qi et al., 2011). The molecular genetic diversity studies of *J. mandshurica* are limited to the work by Woeste et al. (2002) using dominant markers. Here, we used an improved technique for the isolation of codominant compound microsatellite markers (Lian et al., 2006) to isolate 20 polymorphic microsatellite loci from *J. mandshurica*. These polymorphic markers can be useful for the development of effective conservation programs of the species.

<sup>1</sup>Manuscript received 4 December 2012; revision accepted 26 January 2013.

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

<sup>4</sup>Author for correspondence: yujh@mail.nefu.edu.cn

doi:10.3732/apps.1200009

## METHODS AND RESULTS

Total genomic DNA was extracted from silica gel-dried leaves of *J. mandshurica* using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle, 1991). After digestion with *EcoRI* and *HindIII* (TaKaRa Biotechnology Co., Dalian, Liaoning, China) at 37°C for 4 h, a fraction containing 200–1000-bp fragments was isolated from total genomic DNA (250 ng) and ligated with *EcoRI* adapters (5'-CTCGTAGACTGCGTACC-3' and 3'-CTGACGCATGGTTAA-5') and *HindIII* adapters (5'-GACGATGAGTCCTGAG-3' and 3'-TACTCAGGACTCTCGA-5') using T4 DNA ligase (Biomantbio, Shanghai, China) at 4°C overnight. Ligated fragments were subsequently PCR amplified with adapter-specific primers. To enrich the fragments containing microsatellite repeats, PCR products were hybridized with biotin-labeled probes, (AG)<sub>15</sub> and (GT)<sub>15</sub>. Streptavidin-coated magnetic beads (Promega Biotech, Beijing, China) were then used to separate and capture DNA fragments that were hybridized to the probes, according to the manufacturer's instructions. DNA fragments containing simple sequence repeats were eluted from streptavidin-coated magnetic beads in a 1.5-mL tube and put in a freeze-drying machine at -40°C for 3 h. Microsatellite-enriched fragments in eluted solutions were PCR amplified in 20-μL reaction mixtures, consisting of 8.6 μL of DNA template, 10 μL of 2× PCR Mix (Boyouxinchuang Biotech, Beijing, China), 0.3 μL of E00 (10 μM), 0.3 μL of H00 (10 μM), and 0.2 μL of *Taq* polymerase. PCR conditions were as follows: five cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 45 s; and final extension at 72°C for 10 min. The PCR products were ligated into the pMD-18T vector (TaKaRa Biotechnology Co.) and then transformed into *Escherichia coli* DH5α competent cells to generate the microsatellite sequence-enriched library. Recombinant colonies were selected by white/blue screening on Luria-Bertani agar plates containing ampicillin (60 μg/mL).

Positive clones were then screened using the universal primers of pMD-18T (M13-47 and RV-M, 5'-CGCCAGGGTTTCCAGTCACGAC-3' and 5'-GAGCGGATAACAATTCACACAGG-3', respectively), as well as the (AG)<sub>9</sub>/(GT)<sub>9</sub> tandem repeat primers. PCR amplifications were performed in a 15-μL reaction mixture containing DNA template (single colonies), 7.5 μL of 2× PCR Mix (Boyouxinchuang Biotech), 0.1 μL of M13-47 (0.2 μM), 0.1 μL of RV-M (0.2 μM), 0.1 μL of (AG)<sub>9</sub>/(GT)<sub>9</sub> (0.2 μM), 0.2 μL of *Taq* polymerase, and 7 μL of ddH<sub>2</sub>O. PCR amplification was performed in the GeneAmp PCR System 9600 Thermal Cycler (Applied Biosystems, Foster City, California, USA) under the following conditions: initial denaturation at 94°C for 10 min; 33 cycles of denaturation

TABLE 1. Primer sequences and characteristics of 50 microsatellite loci in *Juglans mandshurica*.

| Locus | Primer sequences (5'–3')                                 | Repeat motif                             | Fragment size (bp) | $T_a$ (°C) | GenBank accession no. |
|-------|--|--|--------------------|------------|-----------------------|
| P1*   | F: CCAAGGGAATACAAGGTCT<br>R: GTTGCCTGAACATCACAGAT        | (AG) <sub>11</sub>                       | 186                | 58         | JQ618127              |
| P2    | F: GGTC AAGGCTCTCTGCCTCAA<br>R: TAAAATCACCCTCCACTC       | (TC) <sub>15</sub>                       | 249                | 57         | JQ618128              |
| P3*   | F: GGAGTTTCGGGTAGGGTTGA<br>R: TAATGGTTGGAGGAATGGAG       | (TC) <sub>15</sub>                       | 184                | 58         | JQ618129              |
| P4    | F: TAGGAGATATTTTCAAGAGGA<br>R: TTGTGTGAGCATGAGTTTGTAG    | (AC) <sub>8</sub>                        | 136                | 57         | JQ618130              |
| P5*   | F: TATTTTCATGCC AAGACCAGG<br>R: GTCCTAAAAGAGTGATTTGTGTGT | (CA) <sub>8</sub> (AT) <sub>5</sub> (AG) | 164                | 57         | JQ618131              |
| P6    | F: AAACAGCATCTGAAACCCACA<br>R: GCTTTTGCCTCCATAATTAG      | (CT) <sub>10</sub> (CA) <sub>8</sub> T   | 288                | 56         | JQ618132              |
| P7    | F: AAAAGAGGTGTTGAGGATGG<br>R: TTATTTTCACTTGCTTTGCC       | (GA) <sub>13</sub>                       | 113                | 55         | JQ618133              |
| P8*   | F: CTGAAAAGTGGGCAAGCA<br>R: GGGAGACATACCCGACAAGG         | (GA) <sub>14</sub>                       | 280                | 59         | JQ618133              |
| P9    | F: CACGACGGCACAAC TAAAGG<br>R: CACTGAAGGCACACCCAAGA      | (AG) <sub>20</sub>                       | 144                | 57         | JQ618134              |
| P10   | F: AAGATTGTTCTAAGTTGTGTC<br>R: AATGTGTAGGTCAATAGAGG      | (AT) <sub>5</sub> (AG) <sub>15</sub>     | 136                | 58         | JQ618135              |
| P11   | F: TCCACCGTAAAAGATTGTT<br>R: GCTTCATAGAGATTTCCCAT        | (AG) <sub>16</sub>                       | 108                | 56         | JQ618136              |
| P12*  | F: AAACCCTATCTCCGCGA<br>R: GATGGAGAGCTAAGGAGTCG          | (CT) <sub>11</sub>                       | 108                | 57         | JQ618137              |
| P13   | F: GGTTAGAGTGAGCGAGAGTTG<br>R: ATCCTTAGAGTTGAATGGGC      | (AG) <sub>15</sub>                       | 186                | 58         | JQ618138              |
| P14*  | F: AAAAAACCTTGCACCAAG<br>R: CCACTAAAAACACTCCATCA         | (GA) <sub>26</sub>                       | 211                | 56         | JQ618139              |
| P15   | F: CTCTCTCGCAAAACCTCTCG<br>R: CGTGCATGCTAGGAAACTTA       | (CT) <sub>26</sub>                       | 171                | 58         | JQ618140              |
| P16*  | F: CTGCTGATGTGGTGGTAAG<br>R: GATCTGGTTGTGGAGGGAA         | (AG) <sub>9</sub>                        | 194                | 59         | JQ618141              |
| P17   | F: TGAGTACAGAAC TGGCATG<br>R: ACTGGGGTGGGCTAAAAAG        | (TG) <sub>18</sub>                       | 187                | 55         | JQ618142              |
| P18*  | F: TAGAAACCTCGTGACTTG<br>R: ACTGCTAGAGCCTATGGAA          | (AC) <sub>10</sub>                       | 214                | 58         | JQ618143              |
| P19   | F: CCGAATGAGGAAGGAAGG<br>R: ACACGGCTTAGGGCCATAAA         | (GT) <sub>22</sub>                       | 126                | 56         | JQ618144              |
| P20   | F: CAGCCACCCATTACCATC<br>R: CACAACCTCACA AAAACCAACAAC    | (CT) <sub>9</sub> (CA) <sub>7</sub>      | 283                | 57         | JQ618145              |
| P21*  | F: TGTACTCTGTTGGGTCGT<br>R: CTGGTGTAGCAGTTCATTT          | (GA) <sub>11</sub> (TA) <sub>4</sub>     | 196                | 58         | JQ618146              |
| P22   | F: CCTTCTGCTCAGACAACA<br>R: CTAGACCAAAGACCACA ACTAT      | (AG) <sub>13</sub>                       | 241                | 56         | JQ618147              |
| P23*  | F: CAGGACAGACAACCCCAT<br>R: TAACACTCCACGCACGCAC          | (AG) <sub>5</sub> (AG) <sub>10</sub>     | 200                | 59         | JQ618148              |
| P24   | F: TATATTGTGGGAGGTGGGT<br>R: TTAAGGGAGTTGTTGAAGC         | (CT) <sub>9</sub>                        | 316                | 57         | JQ618149              |
| P25   | F: GGGAGAA TGA AATGACGG<br>R: GAAACGAAGGAAAAATGAGG       | (AG) <sub>19</sub>                       | 124                | 57         | JQ618150              |
| P26*  | F: CTTCAAATAATGGAACGGT<br>R: TAAAGAGATGGAGTACGCT         | (CT) <sub>17</sub>                       | 195                | 56         | JQ618151              |
| P27   | F: TTCATAGCACATAACAGTTC<br>R: TCCGTAACATCAATCATTC        | (CT) <sub>11</sub>                       | 283                | 58         | JQ618152              |
| P28*  | F: GCTTTTGTACTTTGTGCC<br>R: GAGCTGAATTTTTTACCTGA         | (CT) <sub>11</sub>                       | 196                | 57         | JQ618153              |
| P29*  | F: GAATAAAAGAAGTTTGAC<br>R: TCTGACCAAAATCCATAG           | (CT) <sub>10</sub>                       | 191                | 58         | JQ618154              |
| P30*  | F: ATGGGAATCACAGGTGAC<br>R: TGGGAATATCTTCGAGAG           | (AG) <sub>13</sub>                       | 252                | 57         | JQ618155              |
| P31*  | F: CCTTCAACCACTCAATA<br>R: GAGACACACGCACAAAACC           | (CT) <sub>13</sub>                       | 244                | 55         | JQ618156              |
| P32   | F: AAGTAAAACCTAAGTCC<br>R: AAAATCCAAACTCAAGCCC           | (CT) <sub>14</sub>                       | 241                | 57         | JQ618157              |
| P33   | F: CCAAGGCACCAACAAT<br>R: CCCATGCAATAACA AAACCA          | (TC) <sub>15</sub>                       | 266                | 56         | JQ618158              |
| P34   | F: TTCCCACTCTCAAATCTG<br>R: TTCTCGTGTAAAGTACCCC          | (GA) <sub>17</sub>                       | 159                | 58         | JQ618159              |
| P35*  | F: TGCTTTTACCTTCCTCT<br>R: GTCCAACCCAAGTTCTCTCC          | (AG) <sub>18</sub>                       | 296                | 59         | JQ618160              |

TABLE 1. Continued.

| Locus | Primer sequences (5'–3')                            | Repeat motif                             | Fragment size (bp) | T <sub>a</sub> (°C) | GenBank accession no. |
|-------|---|--|--------------------|---------------------|-----------------------|
| P36   | F: ACCAAACGAGAACGAGTAA<br>R: AGGGGATAGATTGTGATAC    | (GA) <sub>13</sub> (GT) <sub>4</sub>     | 240                | 57                  | JQ618161              |
| P37   | F: CGTTTGTAGTTTCTGCCT<br>R: CACACACTACATGGATGTC     | (GA) <sub>33</sub>                       | 291                | 58                  | JQ618162              |
| P38   | F: ATGTGTAACCAGATAAGG<br>R: ACAAATTGGTCATCTCTAGA    | (GA) <sub>18</sub>                       | 158                | 57                  | JQ618163              |
| P39   | F: GATCTGAAGAGCCTGCCT<br>R: ATCCACCAAAAACCTAAAA     | (AG) <sub>30</sub>                       | 198                | 56                  | JQ618164              |
| P40   | F: CCTCGTCTACTCTCCCTT<br>R: ATGTGGGTCGTGGGTTTGTGTC  | (CT) <sub>24</sub>                       | 170                | 58                  | JQ618165              |
| P41*  | F: AGCATACTTCAATGGAT<br>R: CGCAGAATACACGCCAAATAG    | (AG) <sub>18</sub>                       | 206                | 59                  | JQ618166              |
| P42   | F: ACAGAATGTGAGGTTTACTACG<br>R: GTGCTACTCTTTTGTGGAT | (CT) <sub>10</sub>                       | 250                | 57                  | JQ618167              |
| P43   | F: TACAGCACACCCCTGAAAT<br>R: AACGACGCCGACCAACAAC    | (AG) <sub>18</sub>                       | 234                | 58                  | JQ618168              |
| P44*  | F: AAGCATCATCTCTATTTCTC<br>R: ACTTTGTGGGTGTTTCTAT   | (CT) <sub>11</sub>                       | 200                | 57                  | JQ618169              |
| P45*  | F: TTGATGCCTGTAGTGAAATG<br>R: TAATGCTATGGAAGTATGGA  | (CT) <sub>10</sub> (CA) <sub>13</sub>    | 252                | 57                  | JQ618170              |
| P46   | F: ATTCTAATGCCCGCACTTG<br>R: AGGATCTGTTGACACATACAT  | (ACC) <sub>3</sub> (AC) <sub>8</sub>     | 234                | 58                  | JQ618171              |
| P47*  | F: TGTGAAAAGGACTTCACAT<br>R: GGATCTCAAGACTGGCTAG    | (AT) <sub>4</sub> (AG) <sub>2</sub> (AC) | 217                | 57                  | JQ618172              |
| P48   | F: TTATTATGATGGGTCTTTG<br>R: TTGATACCAACTGTAACGC    | (TG) <sub>20</sub>                       | 198                | 56                  | JQ618173              |
| P49   | F: CAAAGCAGAGGCTGAGATA<br>R: CTAAGCAAAGGAACGAAAG    | (GC) <sub>4</sub> (AC) <sub>5</sub>      | 180                | 57                  | JQ618174              |
| P50   | F: GGGGAAACAGCAACAATAG<br>R: TCCAGGAAGTTAGGGTGTGAG  | (AC) <sub>18</sub>                       | 268                | 58                  | JQ618175              |

Note: T<sub>a</sub> = annealing temperature when run individually.

\* Indicates polymorphic primers; all other loci were monomorphic.

at 94°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 45 s; and final extension at 72°C for 10 min. A total of 90 colonies were randomly selected and sequenced on an ABI Prism ABI 3730xL automated DNA sequencer (Applied Biosystems); 82 of these contained repeats. Primer Premier 5 software (PREMIER Biosoft International, Palo Alto, California, USA) was used to design primer pairs for 50 of the 82 repeat-containing samples. The forward polymorphic primer of each pair was also labeled with a fluorochrome, 6-FAM (Applied Biosystems).

These 50 primer pairs were then tested for amplification in 20 samples from five different populations (four individuals each) from the mountains of north-eastern China (Appendix 1). PCR products were electrophoresed on 8% denaturing polyacrylamide gels and visualized by silver staining. We found that all 50 primer pairs successfully amplified products after PCR optimization (denaturation at 94°C for 45 s, at 55–60°C for 45 s, and 32 cycles at 72°C for 45 s, with a final extension of 10 min at 72°C) (Table 1), and that 20 of these primer pairs amplified high-quality, polymorphic PCR products. These 20 polymorphic primer pairs were characterized in 98 individuals among five natural populations of *J. mandshurica* (Appendix 1). The distances between these individuals were more than 100 m. PCR products were analyzed by capillary electrophoresis, visualized using the ABI 3730xL sequencer with a GeneScan 500 ROX Size Standard (Applied Biosystems), and scored using GeneMarker (SoftGenetics, State College, Pennsylvania, USA).

Genetic diversity statistics, including the number of alleles, observed heterozygosity, and expected heterozygosity, were calculated based on 98 samples using the software POPGENE32 (version 1.31; Yeh et al., 1999). The number of alleles ranged from one to 17 (mean = 6), whereas the expected and observed levels of heterozygosity were 0–0.925 and 0–1.000 (mean = 0.358 and 0.627), respectively. The large number of alleles per locus indicates the potential usefulness of these primers to characterize the population genetic structure of *J. mandshurica*. Moreover, nine loci (P8, P12, P18, P23, P26, P30, P41, P44, P45) in *J. mandshurica* were found to show significant deviations from Hardy–Weinberg equilibrium ( $P < 0.01$  in  $\chi^2$  test) (Table 2) due to heterozygote deficiency. No significant linkage disequilibrium was detected between any pairs of loci.

## CONCLUSIONS

Twenty polymorphic microsatellite markers were identified in *J. mandshurica* and used to investigate its genetic diversity and population structure. The microsatellite loci described here (including both monomorphic and polymorphic primers) can be useful for conservation genetic studies of *J. mandshurica*.

## LITERATURE CITED

- DOYLE, J. J. 1991. DNA protocols for plants: CTAB total DNA isolation. In G. M. Hewitt and A. Johnston [eds.], *Molecular techniques in taxonomy*, 283–293. Springer-Verlag, Berlin, Germany.
- KANG, K. H., K. H. LEE, M. Y. KIM, AND K. H. CHOI. 2001. Caspase-3-mediated cleavage of the NF- $\kappa$ B subunit p65 at the NH<sub>2</sub> terminus potentiates naphthoquinone analog-induced apoptosis. *Journal of Biological Chemistry* 276: 24638–24644.
- KIM, S. H., K. S. LEE, J. K. SON, G. H. JE, J. S. LEE, C. H. LEE, AND C. J. CHEONG. 1998. Cytotoxic compounds from the roots of *Juglans mandshurica*. *Journal of Natural Products* 61: 643–645.
- LIAN, C. L., M. A. WADUD, Q. F. GENG, K. SHIMATANI, AND T. HOGETSU. 2006. An improved technique for isolating codominant compound microsatellite markers. *Journal of Plant Research* 119: 415–417.
- LU, A. M. 1982. The geographical dispersal of Juglandaceae. *Acta Phytotaxonomica Sinica* 20: 257–274.
- QI, J. X., Y. B. HAO, Y. ZHU, C. L. WU, W. X. WANG, AND P. LENG. 2011. Studies on germplasm of *Juglans* by EST-SSR markers. *Acta Horticulturae Sinica* 38: 441–448.

TABLE 2. Results of PCR screening of 20 polymorphic microsatellite loci in *Juglans mandshurica*.<sup>a</sup>

| Locus   | Xiaoxing'anling<br>(N = 19) |                |                | Zhangguangcailing<br>(N = 20) |                |                | Changbai Mountain<br>(N = 20) |                |                | Laoyeling<br>(N = 19) |                |                | Wanda Mountain<br>(N = 20) |                |                | HWE P value <sup>b</sup> |
|---------|-----------------------------|----------------|----------------|-------------------------------|----------------|----------------|-------------------------------|----------------|----------------|-----------------------|----------------|----------------|----------------------------|----------------|----------------|--------------------------|
|         | A                           | H <sub>o</sub> | H <sub>e</sub> | A                             | H <sub>o</sub> | H <sub>e</sub> | A                             | H <sub>o</sub> | H <sub>e</sub> | A                     | H <sub>o</sub> | H <sub>e</sub> | A                          | H <sub>o</sub> | H <sub>e</sub> |                          |
| P1      | 5                           | 0.313          | 0.720          | 6                             | 0.688          | 0.470          | 6                             | 0.250          | 0.728          | 5                     | 0.250          | 0.754          | 6                          | 0.125          | 0.758          | 0.128                    |
| P3      | 5                           | 0.250          | 0.780          | 5                             | 0.625          | 0.474          | 7                             | 0.375          | 0.798          | 5                     | 0.438          | 0.760          | 6                          | 0.250          | 0.841          | 0.012                    |
| P5      | 5                           | 0.375          | 0.726          | 6                             | 0.000          | 0.712          | 8                             | 0.125          | 0.758          | 5                     | 0.188          | 0.601          | 6                          | 0.125          | 0.677          | 0.011                    |
| P8      | 7                           | 0.438          | 0.798          | 4                             | 0.188          | 0.591          | 8                             | 0.250          | 0.768          | 6                     | 0.063          | 0.750          | 7                          | 0.313          | 0.802          | 0.000                    |
| P12     | 5                           | 0.500          | 0.595          | 3                             | 0.063          | 0.619          | 7                             | 0.563          | 0.633          | 6                     | 0.313          | 0.534          | 3                          | 0.625          | 0.542          | 0.000                    |
| P14     | 12                          | 0.400          | 0.869          | 8                             | 0.267          | 0.770          | 9                             | 0.438          | 0.605          | 8                     | 0.125          | 0.794          | 9                          | 0.077          | 0.846          | 0.010                    |
| P16     | 5                           | 0.188          | 0.565          | 3                             | 0.063          | 0.542          | 5                             | 0.500          | 0.559          | 4                     | 0.438          | 0.569          | 4                          | 0.375          | 0.595          | 0.113                    |
| P18     | 5                           | 0.688          | 0.750          | 4                             | 0.688          | 0.466          | 7                             | 0.750          | 0.651          | 5                     | 0.438          | 0.655          | 7                          | 0.375          | 0.813          | 0.000                    |
| P21     | 7                           | 0.125          | 0.802          | 6                             | 0.500          | 0.629          | 11                            | 0.125          | 0.865          | 8                     | 0.188          | 0.728          | 7                          | 0.250          | 0.710          | 0.011                    |
| P23     | 3                           | 0.563          | 0.365          | 4                             | 0.063          | 0.625          | 2                             | 0.625          | 0.315          | 3                     | 0.688          | 0.365          | 2                          | 0.438          | 0.466          | 0.000                    |
| P26     | 8                           | 0.188          | 0.843          | 5                             | 0.125          | 0.649          | 6                             | 0.063          | 0.790          | 5                     | 0.000          | 0.665          | 7                          | 0.000          | 0.702          | 0.000                    |
| P28     | 7                           | 0.313          | 0.633          | 4                             | 0.813          | 0.236          | 6                             | 0.125          | 0.748          | 4                     | 0.625          | 0.375          | 5                          | 0.313          | 0.712          | 0.081                    |
| P29     | 4                           | 0.250          | 0.579          | 4                             | 0.313          | 0.591          | 3                             | 0.500          | 0.486          | 4                     | 0.250          | 0.659          | 4                          | 0.813          | 0.236          | 0.046                    |
| P30     | 7                           | 0.500          | 0.768          | 5                             | 0.188          | 0.623          | 17                            | 0.125          | 0.925          | 6                     | 0.250          | 0.750          | 11                         | 0.375          | 0.857          | 0.000                    |
| P31     | 2                           | 0.938          | 0.063          | 2                             | 0.875          | 0.121          | 2                             | 0.938          | 0.063          | 1                     | 1.000          | 0.000          | 2                          | 0.938          | 0.063          | 0.066                    |
| P35     | 6                           | 0.375          | 0.786          | 7                             | 0.063          | 0.702          | 6                             | 0.313          | 0.714          | 7                     | 0.188          | 0.756          | 6                          | 0.375          | 0.655          | 0.794                    |
| P41     | 6                           | 0.375          | 0.849          | 7                             | 0.063          | 0.726          | 6                             | 0.250          | 0.843          | 6                     | 0.125          | 0.827          | 8                          | 0.250          | 0.798          | 0.000                    |
| P44     | 5                           | 0.000          | 0.768          | 4                             | 0.000          | 0.599          | 5                             | 0.125          | 0.730          | 5                     | 0.000          | 0.726          | 5                          | 0.438          | 0.508          | 0.000                    |
| P45     | 6                           | 0.133          | 0.821          | 6                             | 0.286          | 0.725          | 8                             | 0.063          | 0.819          | 5                     | 0.438          | 0.736          | 6                          | 0.273          | 0.823          | 0.000                    |
| P47     | 2                           | 0.875          | 0.315          | 2                             | 0.813          | 0.272          | 3                             | 0.688          | 0.401          | 3                     | 0.625          | 0.325          | 3                          | 0.750          | 0.232          | 0.316                    |
| Average | 6                           | 0.389          | 0.670          | 5                             | 0.334          | 0.557          | 6.6                           | 0.359          | 0.660          | 5                     | 0.331          | 0.616          | 6                          | 0.374          | 0.632          |                          |

Note: A = number of alleles; H<sub>e</sub> = expected heterozygosity; H<sub>o</sub> = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; N = sample size.

<sup>a</sup>All values are based on 98 samples located in Xiaoxing'anling, Zhangguangcailing, Changbai Mountain, Laoyeling, and Wanda Mountain (Appendix 1).

<sup>b</sup>Values shown are  $\chi^2$  test results for each locus across all populations sampled.

SUN, M. L. 2004. Chemical components of manchurian walnut and the research development of its bio-activity. *Journal of Northeast Forestry University* 32: 85–86.  
 WANG, D. N., C. C. MU, Z. GAO, AND F. J. FENG. 2011. ISSR analysis of genetic diversity of *Juglans mandshurica* Maxim. populations. *Nonwood Forest Research* 29: 22–29.  
 WOESTE, K., R. BURNS, O. RHODES, AND C. MICHLER. 2002. Thirty polymorphic nuclear microsatellite loci from black walnut. *Journal of Heredity* 93: 58–60.

XU, H. L., X. F. YU, S. C. QU, R. ZHANG, X. R. QU, Y. P. CHEN, X. Y. MA, AND D. Y. SUI. 2010. Anti-proliferative effect of Juglone from *Juglans mandshurica* Maxim. on human leukemia cell HL-60 by inducing apoptosis through the mitochondria-dependent pathway. *European Journal of Pharmacology* 645: 14–22.  
 YE, F. C., R. C. YANG, AND T. BOYLE. 1999. POPGENE version 1.32: Microsoft Windows-based freeware for population genetic analysis, quick user guide. Center for International Forestry Research, University of Alberta, Edmonton, Alberta, Canada.

APPENDIX 1. Geographic localities and sample sizes of the *Juglans mandshurica* populations in this study. Voucher specimens were deposited at the Northeast Forestry University Herbarium (Heilongjiang; NEFI).

| Locality          | Latitude (°N) | Longitude (°E) | Altitude (m) | n  | Population code; herbarium voucher accession code |
|-------------------|---------------|----------------|--------------|----|---|
| Xiaoxing'anling   | 46.8927       | 128.4209       | 275.0        | 19 | XXAL; jmXXAL2010-Yu                               |
| Zhangguangcailing | 44.0133       | 127.0052       | 357.5        | 20 | ZGCL; jmZGCL2010-Yu                               |
| Changbai Mountain | 41.1009       | 126.1315       | 332.0        | 20 | CBS; jmCBS2010-Yu                                 |
| Laoyeling         | 43.0405       | 130.3383       | 364.0        | 19 | LYL; jmLYL2010-Yu                                 |
| Wanda Mountain    | 44.5802       | 131.1351       | 115.0        | 20 | WDS; jmLYL2010-Yu                                 |

Note: n = sample size.