Genetic diversity of natural populations of *Machilus thunbergii*, an endangered tree species in eastern China, determined with ISSR analysis

J. Liu, H.G. Sun, J.M. Jiang, W.H. Shao and Q.F. Luan

Research Institute of Subtropical Forestry, Chinese Academy of Forestry, Fuyang, Zhejiang, China

Corresponding author: J.M. Jiang
E-mail: jiang_jingmin@163.com

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**ABSTRACT.** The genetic diversity of 10 *Machilus thunbergii* populations in eastern China was analyzed using inter-simple sequence repeat markers. The populations showed high genetic diversity, with an overall population genetic diversity of 0.2343. Genetic diversity varied largely among populations, and populations with the highest genetic diversity were mainly from the eastern and western parts of the natural distribution area. Small populations, lack of effective gene flow, and fragmentation of habitats have led to greater genetic differentiation among populations, with 41.18% of genetic variation existing among populations. Unweighted pair-group method with arithmetic mean cluster analysis indicated that populations distributed between latitudes 25° and 31°N were clustered together and should be prioritized for *in situ* conservation. Northern, eastern, and southern populations were located in peripheral areas of the distribution range and were clustered separately. Collection of distinctive germplasm from peripheral populations should be promoted and *ex situ* conservation of elite germplasm should be implemented.

**Key words:** *Machilus thunbergii*; Population; Genetic diversity; ISSRs; Genetic differentiation
INTRODUCTION

Genetic diversity is the product of the long-term evolution of a species and is both the basis for its reproduction and adaptation to environmental change and an important component of broader biological diversity (Yuan et al., 2007). The adaptation of populations to various environmental conditions is, to a great extent, influenced by their inherent genetic diversity, which partly governs the evolutionary trend of the populations (Liu et al., 2008). Recent studies have suggested that a lack of genetic diversity has unfavorable impacts on the long-term survival of a species (He et al., 2009). Research on the genetic diversity of endangered plants can help not only to understand the evolutionary history of such species and the mechanisms responsible for their rarity or endangerment but also to determine whether scientifically effective measures can be applied to protect endangered species. Thus, such research is a core focus of conservation biology studies (Zhao et al., 2003).

*Machilus thunbergii* is an evergreen broad-leaved tree species that belongs to the Lauraceae. It is listed as a tertiary state-protected rare tree species and is well known for its high-value wood (Xie, 2005). However, human disturbance (such as seed harvesting, transplanting of understory seedlings, and felling of mature trees) and fire have led to habitat destruction, serious habitat fragmentation, and a reduction in the number of natural populations, and therefore, the species has been listed as vulnerable (Jiang and Yu, 2001). Comparatively more research has been conducted on *M. thunbergii* in Japan and Korea, which has addressed mainly the extraction and use of active substances, community structure (Maesako, 1985), and genetic variation (Kim et al., 1992). Previous studies of *M. thunbergii* in China have focused mainly on seedling propagation (Shao et al., 2007), community characteristics (Zeng, 1999), and morphological variation of seeds within and between populations (Chen et al., 2007). Studies have rarely considered the conservation biology of *M. thunbergii*—for example, its genetic diversity—which is central to understanding its endangerment and devising conservation strategies. For effective conservation of the germplasm resources of *M. thunbergii*, studies on characteristics such as genetic diversity are of great importance.

Inter-simple sequence repeats (ISSRs) are microsatellite molecular markers first described by Zietkiewicz et al. (1994) that are now applied in genetic map construction (Bornet and Branchard, 2001; Sankar and Moore, 2001), gene mapping (Ratnaparkhe et al., 1998), genetic diversity analysis (Luan et al., 2006), and identification of germplasm resources (Escandón et al., 2007). ISSR molecular markers have been applied successfully in studies of the genetic diversity and genetic structure of endangered tree species such as *Ginkgo biloba* (Ge et al., 2003), *Camellia nitidissima* (Bin et al., 2005), and *Paeonia delavayi* (Yang et al., 2005). Therefore, in the present study, the ISSR molecular marker technique was applied to analyze the genetic diversity among 10 natural populations within the main distribution area of *M. thunbergii* in China. The objectives were to reveal the genetic structure and genetic diversity of the populations as well as determine genetic differentiation, estimate gene flow between populations, and explore, from a molecular perspective, the mechanisms contributing to its endangerment. The overall aims were to obtain a clear picture of the survival potential and extent of endangerment of *M. thunbergii* and to provide a scientific basis for formulating and implementing comprehensive strategies for the conservation of its germplasm resources.
MATERIAL AND METHODS

Material

Samples were collected from 10 natural populations of *M. thunbergii* across its geographic range in eastern China (Figure 1). The communities in which *M. thunbergii* primarily occurs are mainly young and semi-mature evergreen broad-leaved forests. In each population, we randomly selected 20-30 individuals (205 individuals total) with a distance of more than 50 m between individuals. Young, healthy leaves were collected from each individual and stored in freezer bags, quickly transported to the laboratory, and stored at -70°C until use.

Figure 1. Distribution of natural populations of *Machilus thunbergii* natural populations in east China. QD = Qingdao, Shandong; YX = Yixing, Jiangsu; ZJ = Zhujiadjian, Zhejiang; TH = Taohuadao, Zhejiang; SC = Suichang, Zhejiang; HS = Huangshan, Anhui; JL = Jiulianshan, Jiangxi; GD = Huizhou, Guangdong; HE = Hengshan, Hunan; WF = Wufeng, Hubei.

Extraction of genomic DNA

Genomic DNA was extracted from collected leaves using an improved cetyltrimethylammonium bromide method (Jiang et al., 2011). The purified DNA was analyzed with 0.8% agarose gel electrophoresis, photographed with a Gel Documentation and Analysis System (Bio-Rad, USA), diluted to a concentration of 20 ng/μL, and stored at -20°C until use.

Establishment of the ISSR-polymerase chain reaction (PCR) system

The ISSR-PCR primers used composed UBC primer set no. 9, which was synthesized by Beijing Aoke Bioengineering (China). The eight primers that showed the best repeatability and polymorphism were selected from among 100 primers (Table 1). The reactions were performed in a 20-μL volume that contained 0.05 U/μL Taq, 2.0 mM Mg²⁺, 1 ng/L template DNA, 0.3 mM deoxyribonucleotide triphosphates, 0.5 μM primer, and 1X PCR buffer. The follow-
PCR amplification was conducted on genomic DNA isolated from a total of 205 indi-
Individuals from 10 natural populations of *M. thunbergii* using eight ISSR primers (Table 2). The number of loci amplified by each primer ranged from 6 to 11, and the band size was 300–1800 bp. A total of 92 loci were detected, of which 84 were polymorphic and the PPL was 91.30%. The PPL varied greatly among the 10 populations, ranging from 22.35 to 58.82% (mean 43.65%). Among populations, JL (Jiulianshan, Jiangxi) showed the highest PPL (58.82%), followed by WF (Wufeng, Hubei) (56.47%). The HS (Huangshan, Anhui) population contained the lowest PPL (22.35%). The highest PPL was 2.5-fold that of the lowest PPL. Nei’s ($H_e$) values for the populations ranged from 0.0847 to 0.1662, and $I$ values ranged from 0.1258 to 0.2557; both measures of genetic diversity showed trends in variation among populations similar to those revealed by PPL data. The values of each average genetic diversity parameter at the population level (PPL = 43.65%, $H_e = 0.1410$, $I = 0.2139$; see Table 2) were lower than those values at the species level (PPL = 91.30%, $H_e = 0.2343$, $I = 0.3697$).

**Table 2. Genetic diversity within the 10 populations of Machilus thunbergii sampled in this study.**

<table>
<thead>
<tr>
<th>Population</th>
<th>No. of individuals</th>
<th>No. of polymorphic loci</th>
<th>PPL</th>
<th>$H_e$</th>
<th>$I$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WF</td>
<td>20</td>
<td>48</td>
<td>56.47</td>
<td>0.1662</td>
<td>0.2557</td>
</tr>
<tr>
<td>HS</td>
<td>20</td>
<td>19</td>
<td>22.35</td>
<td>0.0847</td>
<td>0.1258</td>
</tr>
<tr>
<td>YX</td>
<td>20</td>
<td>38</td>
<td>44.71</td>
<td>0.1398</td>
<td>0.2155</td>
</tr>
<tr>
<td>JL</td>
<td>30</td>
<td>50</td>
<td>58.82</td>
<td>0.1605</td>
<td>0.2493</td>
</tr>
<tr>
<td>HE</td>
<td>20</td>
<td>45</td>
<td>52.94</td>
<td>0.1552</td>
<td>0.2400</td>
</tr>
<tr>
<td>SC</td>
<td>25</td>
<td>39</td>
<td>45.88</td>
<td>0.1414</td>
<td>0.2167</td>
</tr>
<tr>
<td>TH</td>
<td>20</td>
<td>37</td>
<td>43.53</td>
<td>0.1476</td>
<td>0.2234</td>
</tr>
<tr>
<td>QD</td>
<td>20</td>
<td>29</td>
<td>34.12</td>
<td>0.1346</td>
<td>0.1970</td>
</tr>
<tr>
<td>GD</td>
<td>25</td>
<td>24</td>
<td>28.24</td>
<td>0.1245</td>
<td>0.1779</td>
</tr>
<tr>
<td>ZJ</td>
<td>20</td>
<td>42</td>
<td>49.41</td>
<td>0.1558</td>
<td>0.2380</td>
</tr>
<tr>
<td>Population level</td>
<td></td>
<td></td>
<td>43.65</td>
<td>0.1410</td>
<td>0.2139</td>
</tr>
<tr>
<td>Species level</td>
<td></td>
<td></td>
<td>91.30</td>
<td>0.2343 (0.1682)</td>
<td>0.3697 (0.2240)</td>
</tr>
</tbody>
</table>

PPL = percentage of polymorphic loci; $H_e$ = expected heterozygoty; $I$ = Shannon information diversity index. For abbreviations, see legend to Figure 1. Number in parentheses is the standard deviation.

Analysis of the correlation with the longitude and latitude of the sample provenance indicated no significant geographical pattern in the variation of PPL in the *M. thunbergii* populations. However, a plot of the PPL of each population against the latitude of the populations (Figure 2) showed that populations with a high PPL were largely concentrated between latitudes 25° and 31°N.

![Figure 2. Percentage of polymorphic loci (PPL) for each Machilus thunbergii population from different latitudes.](image)

Genetic differentiation between populations

Analysis of genetic differentiation between the populations (Table 3) showed that the total genetic diversity of the M. thunbergii populations was 0.2139, and $H_s$ and genetic diversity between populations were 0.141 and 0.0729, respectively. The $G_{ST}$ was 0.4118, and the estimated gene flow was only 0.7141, which is a relatively small value. M. thunbergii is a formerly widely distributed tree species that usually existed in small populations and has now shrunk into some natural conservation areas, thus resulting in serious habitat fragmentation. This trend accounts for the high genetic differentiation between populations; 41.18% of the total genetic variation existed between populations, whereas $H_s$ accounted for 58.82% of the total variation.

<table>
<thead>
<tr>
<th>Total genetic diversity ($H_t$)</th>
<th>Genetic diversity within populations ($H_s$)</th>
<th>Genetic diversity between populations ($D_{ST}$)</th>
<th>Coefficient of gene differentiation ($G_{ST}$)</th>
<th>Estimate of gene flow from $G_{ST}$ ($N_m$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2139</td>
<td>0.1410</td>
<td>0.0729</td>
<td>0.4118</td>
<td>0.7141</td>
</tr>
</tbody>
</table>

The results of analysis of molecular variance (Table 4) revealed significant genetic variance ($P < 0.01$) both between and within M. thunbergii populations. Genetic variation between populations accounted for 46.0% of the total variation, and $H_s$ accounted for 54.0% of the variation.

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>d.f.</th>
<th>SD</th>
<th>MS</th>
<th>Variation component</th>
<th>Total variation</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between populations</td>
<td>9</td>
<td>1195.863</td>
<td>132.874</td>
<td>5.747</td>
<td>46%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Within populations</td>
<td>210</td>
<td>1417.437</td>
<td>6.750</td>
<td>6.750</td>
<td>54%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total</td>
<td>219</td>
<td>2613.300</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

d.f. = degrees of freedom; SD = standard deviation; MS = mean of square.

**UPGMA dendrogram**

Based on the estimated genetic distance between the 10 populations of M. thunbergii, a dendrogram (Figure 3) was constructed using UPGMA. The WF, HS, YX (Yixing, Jiangsu), JL (Jiulianshan, Jiangxi), and HE (Hengshan, Hunan) populations were clustered into one group; the SC (Suichang, Zhejiang) and TH (Taohuadao, Zhejiang) populations and the GD (Huizhou, Guangdong) and ZJ (Zhuijiajian, Zhejiang) populations formed two clusters, and the QD (Qingdao, Shandong) population was placed on a branch alone. Mantel tests of the genetic and geographical distances between M. thunbergii populations indicated that these distances were not significantly correlated ($r = 0.2432$, $P = 0.2965$). For example, in the UPGMA dendrogram, ZJ clustered not with TH (the geographically closest population) but with GD instead (see Figure 3).
DISCUSSION

Geographic distribution is one of the main factors that influence the genetic diversity of a plant species. Species with a wide geographic distribution tend to have a higher level of genetic diversity (Hamrick and Godt, 1990). *M. thunbergii* is an important dominant or associated species of subtropical evergreen broad-leaved forests in Asia. The $H_e$ within *M. thunbergii* (0.2343) is higher than the average genetic diversity of many plant populations (based on dominant molecular markers such as random amplified polymorphic DNA and ISSRs), according to the statistics of Nybom (2004). The results of the present study indicated that genetic diversity varied largely among the 10 sampled populations of *M. thunbergii*.

As the distribution center of *M. thunbergii*, populations between latitudes 25° and 31°N contained significantly higher genetic diversity than the average value for the populations sampled. Located on coastal and inland environments with conditions favorable for growth, populations sustain a large number of individuals adapted to various ecological conditions and thus exhibit a higher level of genetic diversity. The northern populations are located at the limit of the geographic distribution of *M. thunbergii* and either experience ecological conditions less favorable for growth and survival of the species or perhaps contain a population that migrated to these latitudes more recently and therefore has had less time for genetic diversification. Thus, these populations contain only a small number of individuals and lower genetic diversity. Therefore, differences in ecological and environmental conditions may be the main cause of the differences in genetic diversity between central populations and those in peripheral areas of the distribution range.

The $G_{st}$ among *M. thunbergii* populations (0.4118) is significantly higher than the average value of outbreeding and perennial species (0.22 and 0.19, respectively) (Nybom,
As a rare and threatened species composed of relatively small populations and subject to serious human disturbance, *M. thunbergii* is distributed widely but is scattered, mainly in statutorily protected areas. Serious fragmentation of its habitat has led to small populations and lack of effective gene flow ($N_{m} = 0.7141$) among populations, which has caused strong genetic differentiation among *M. thunbergii* populations. Cluster analysis of between-population genetic distance estimates indicated that owing to closer genetic distances, five *M. thunbergii* populations distributed between latitudes 25° and 31°N (WF, HS, YX, JL, and HE) were clustered together and showed higher genetic diversity. These populations should be prioritized for conservation. In addition to *in situ* conservation through national and provincial natural conservation areas, the area of statutorily protected natural forests of *M. thunbergii* should be extended to increase the number of individuals and population sizes and ensure preservation of forest health and structure. Northern, eastern, and southern populations are located in peripheral areas of the distribution range of this species and contain individuals adapted to different ecological and environmental conditions. Thus, germplasm collection should be increased to maximize the genetic diversity sampled, and *ex situ* conservation of elite germplasm should be implemented to provide diverse breeding resources for ornamental and timber plantation purposes.

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