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

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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 rareness 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.

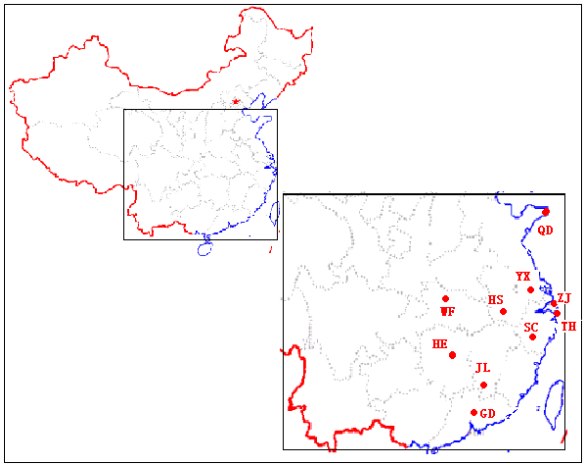

Figure 1. Distribution of natural populations of *Machilus thunbergii* natural populations in east China. QD = Qingdao, Shandong; YX = Yixing, Jiangsu; ZJ = Zhujiajian, Zhejiang; TH = Taohuadao, Zhejiang; SC = Suichang, Zhejiang; HS = Huangshan, Anhui; JL = Jiulianshan, Jiangxi; GD = Huizhou, Guangdong; 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 Mg^{2+}, 1 ng/L template DNA, 0.3 mM deoxyribonucleotide triphosphates, 0.5 μM primer, and 1X PCR buffer. The follow-

ing PCR procedure was used: pre-denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 30 s, annealing at melting temperature for 35 s, and extension at 72°C for 35 s; final extension at 72°C for 7 min; and storage at 4°C. The PCRs were performed on a TP600 PCR Thermal Cycler Dice (TaKaRa Bio, Japan). Amplification products were analyzed using 2% agarose gel electrophoresis, and a digital image was recorded with the Image Acquisition and Analysis System (Bio-Rad).

Table 1. ISSR primers used to analyze genetic diversity of *Machilus thunbergii*.

Primer	Sequence	Tm (°C)
UBC835	$(AG)_8$YC	55.0
UBC830	$(TG)_8$G	59.0
UBC815	$(CT)_8$T	53.8
UBC823	$(TC)_8$C	53.5
UBC856	$(AC)_8$YA	58.0
UBC860	$(TG)_8$RA	51.0
UBC874	$(CCCT)_4$	61.2
UBC899	CATGGTGTTGGTCATTGTTCCA	62.0

R = (A, G), Y = (C, T). Tm = melting temperature.

Statistical analysis

ISSRs are dominant markers, and bands with consistent electrophoretic mobility in the amplification products of the same primer are considered to show homology. In the present study, every band in an electrophoretogram was considered a marker and representative of a binding site of a primer. Statistical analysis was conducted based on the presence or absence of DNA bands at each specific position on the gel; for each sample, ISSR bands were scored as 1 (present) or 0 (absent). Only amplified bands that were clearly resolved and had a length of 200~1800 bp were recorded, and a 0/1 data matrix was established. Using the POPGENE version 1.3.1 software (Yeh et al., 1999), we calculated the following parameters: percentage of polymorphic loci (PPL), Shannon information diversity index (I), Nei's genetic diversity index (H_E), total gene diversity, within-population genetic diversity (H_S), coefficient of genetic differentiation (G_{ST}), gene flow (N_m), and Nei's genetic distance. Based on Nei's (1978) unbiased genetic distance between populations as implemented in the POPGENE software, unweighted pair-group method using arithmetic averages (UPGMA) clustering analysis was conducted on the genetic distance estimates. Output files (distance, group, and population files) acquired through calculation of the Euclidean distance between individuals with the AMOVA-PREP software (Miller, 1998) were used as input files. Molecular variance analysis was conducted with the WINAMOVA version 1.55 software (Excoffier et al., 1992), and using population as the unit, we analyzed the correlation between the genetic and geographical distances among the 10 populations with a Mantel test.

RESULTS

Genetic diversity of *M. thunbergii* populations

PCR amplification was conducted on genomic DNA isolated from a total of 205 indi-

viduals 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.

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

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.

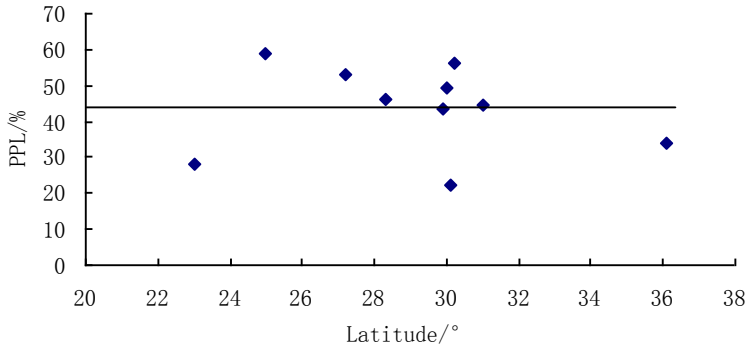

Figure 2. Percentage of polymorphic loci (PPL) for each *Machilus thunbergii* population from different latitudes.

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 3. Coefficients of genetic differentiation for the *Machilus thunbergii* populations sampled.

Total genetic diversity (H_T)	Genetic diversity within populations (H_S)	Genetic diversity between populations (D_{ST})	Coefficient of gene differentiation (G_{ST})	Estimate of gene flow from G_{ST} (N_m)
0.2139	0.1410	0.0729	0.4118	0.7141

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 4. Analysis of molecular variance for the *Machilus thunbergii* populations sampled.

Source of variance	d.f.	SD	MS	Variation component	Total variation	P
Between populations	9	1195.863	132.874	5.747	46%	<0.01
Within populations	210	1417.437	6.750	6.750	54%	<0.01
Total	219	2613.300				

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

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 (Zhujiajian, 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).

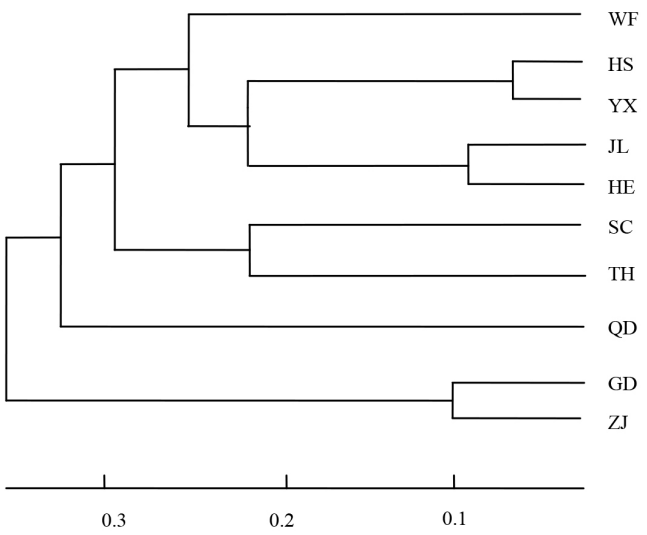

Figure 3. UPGMA dendrogram for populations of *Machilus thunbergii* based on Nei's (1978) genetic distance. For abbreviations, see legend to Figure 1.

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,

2004). 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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