African Journal of Biotechnology Vol. 10(84), pp. 19505-19513, 26 December, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1715
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

An analysis of the genetic diversity and genetic structure of *Eucommia ulmoides* using inter-simple sequence repeat (ISSR) markers

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Accepted 28 September, 2011

Scientific approaches to conservation of threatened species depend on a good understanding of the genetic information of wild and artificial population. The genetic diversity and structure analysis of 10 *Eucommia ulmoides* population was analyzed using inter-simple sequence repeat (ISSR) markers in this paper. A low genetic diversity at the population level (PPB=36.99%) and a high genetic diversity at the species level (PPB=85.54%) were revealed. Analysis of molecular variance (AMOVA) also detected a significant differentiation among the populations. Unweighted pair-group method with arithmetic averaging (UPGMA) clustered the 10 population into 3 groups, and no significant correlation was found between the genetic distance and the corresponding geographic distance among the populations, which is consistent with the results of principal coordinate analysis (PCoA). The genetic variation between the artificial and wild populations revealed a decreased genetic diversity and aggravated genetic differentiation in the artificial populations. A conservation strategy of *Eucommia ulmoides* is discussed based on the information of the population genetic structure and variation.

Key words: *Eucommia ulmoides*, inter-simple sequence repeat (ISSR), genetic diversity, genetic structure, conservation.

INTRODUCTION

Eucommia ulmoides Oliv, the only representative in the genus *Eucommia* in the Eucommiaceae, is a long-lived dioecious perennial tree (Tippo, 1940) endemic to China. *E. ulmoides* is a tertiary relict species with a unique systematic status. As one of the tertiary species surviving only in China, *E. ulmoides* have been spared from the direct impact of the pleistocene glaciations in some parts of the country. It is generally believed that *E. ulmoides* originated from the Qinling Mountain range, which is hypothesized to have served as potential refugia for deciduous trees (Axelrod et al., 1998). *E. ulmoides* has important medicinal and economic values, the bark has been used as a traditional Chinese medicine for more than 2,000 years (Tippo, 1940), and it is reported to elicit pharmacological effects on coronary blood flow, pain relief, diuresis, blood pressure and lipid metabolism (Kawasaki et al., 2000). *E. ulmoides* was also planted as an ornamental tree and used for timber and *E. ulmoides* gum (gutta-percha or balata) (Zhang, 1992). With the growth of commercial demands in recent years, it is critically endangered in the wild and was designated a second-category state-protected endangered plant in China (Fu, 1992).

Even though it has an endangered status in the wild, there is no clear recovery plan for this species, and the widespread artificial populations are cultivated mainly for their economic benefit but not for conservation. Scientific approaches to the conservation and utilization of plant genetic resources require the accurate assessment of the amount and distribution of the genetic variation within

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Abbreviations: ISSR, Inter-simple sequence repeat; **AMOVA**, averaging. analysis of molecular variance; **UPGMA**, unweighted pair-group method with arithmetic averaging; **PCoA**, principal coordinate analysis; **RAPD**, random amplified polymorphic DNA; **PPB**, percentage of polymorphic bands; **CTAB,** cetyl trimethylammonium bromide.

Table 1. Sample details of *E. ulmoides* populations in the study.

Population codes	Location	Habitat	Longitude(N), Latitude(E)	Population size	Sample size
LY	Luoyang, Henan Province	botanical garden	N 34°41′, E 112°27′	8	8
NY	Nanyang, Henan Province	cultivation base	N 33°01′, E 112°30′	>200	8
SY	Shiyan, Hubei Province	cultivation base	N 32°40′, E 110°47′	>200	8
LG	Langao, Shanxi Province	cultivation base	N 32°19′, E 108°54′	>200	8
SQ	Shiquan, Shanxi Province	cultivation base	N 33°02′, E 108°14′	>100	8
LV	Lveyang, Shanxi Province	agricultural field	N 33°19′, E 106°08′	23	8
BJ	Baoji, Shanxi Province	field border	N 34°22′, E 107°20′	17	8
ZZ	Zhouzhi, Shanxi Province	cultivation base	N 34°09′, E 108°12′	>1000	7
XA	Xi'an, Shanxi Province	botanical garden	N 34°15′, E 108°52′	12	8
NJ	Nanjing, Jiangsu Province	botanical garden	N 32°03′, E 118°46′	7	7

both natural and artificial populations. To date, previous studies have mainly focused on the resource distribution, morphological characteristics, dynamics and pharmacological properties (Wang and Zhang, 2004; Du, 1997), and there are few studies about the genetic variation of *E. ulmoides* populations. RAPD markers have previously been applied to *E. ulmoides* for the genetic diversity analysis of artificial populations and have revealed a high genetic variation (Wang et al., 2006). However, random amplified polymorphic DNA (RAPD) has also some limitations including uncertain locus homology and especially sensitivity to the reaction conditions. In recent years, as a microsatellite-based technique, inter-simple sequence repeat (ISSR) amplification (Zietkiewicz et al., 1994) has been applied as an alternative to genetic analysis. Because of the higher annealing temperature and longer sequence of the ISSR primers, ISSR analysis overcomes many of the technical limitations of RAPD, and has proved to be useful in population genetic studies in a wide range of plant species (Okun et al., 2008; Bhagyawant and Srivastava, 2008).

In the present study, we used ISSR markers to investigate the genetic composition of wild and artificial *E. ulmoides* populations, mainly from the Qinling Mountain range, with the following aims: (1) to evaluate the genetic diversity at the population and species levels in the germplasm resources of *E. ulmoides*, (2) to assess the distribution of the genetic variation within and between populations and to construct a dendrogram demonstrating the genetic relationships among them and (3) to evaluate the genetic consequences of the recovery of *E. ulmoides* and develop methods for establishing genetic-diversity-based management practices for its conservation.

MATERIALS AND METHODS

Plant material and DNA extraction

Fresh young leaves were collected from 78 individuals of 10 populations during December 2007. Table 1 summarizes the characteristics of the analyzed populations. The NJ population, which is situated far from the Qinling Mountain range, and the other 9 populations were selected from different geographic sites along the Qinling Mountain range (Figure 1). The DNA was extracted from 0.3 g of young leaf material using a previously established protocol (Doyle, 1991) that was modified by adding 1% β-mercaptoethanol (v/v) and 1% PVP (w/v) to the CTAB extraction buffer. The DNA samples were diluted to 20 ng/µl and stored at -20°C for later use.

PCR amplification

A total of 67 primers were tested in 3 samples to find suitable repeats and anchors; 8 of the primers yielded clear and reproducible banding patterns and were then selected for further analysis (Table 2). The ISSR-PCR reaction mixtures (20 µl) contained the following components: 2.0 µl of 10×PCR buffer, 1.5 mM $MgCl_2$, 0.4 µM of each primer, 0.2 mM of each dNTP, 2.5% formamide, 20 ng of template genomic DNA and 1 U of Taq DNA polymerase. The amplification was performed as follows: an initial 4 min at 94°C, 38 cycles of 30 s at 94°C, 45 s at the specific annealing temperature, 90 s at 72°C, and a final 5 min extension at 72°C. Negative controls and replicates were included to verify the repeatability of the results for all of the reactions. The amplification was performed in a PTC-100 thermocycler (MJ Research), and the amplification products were separated on 2% agarose gels.

Data analysis

Only primers that gave consistent profiles across the populations as well as those that appeared to have diagnostic markers were chosen for further analysis. The presence and absence of bands were scored as 1 or 0, respectively. Bands with the same migration distance were considered homologous. A pair-wise similarity matrix was computed and analyzed using POPGENE version 1.32 (Yeh et al., 2000) assuming a Hardy-Weinberg equilibrium. The genetic diversity within and among the populations was measured by the percentage of polymorphic bands (PPB), Nei's gene diversity (H) (Nei, 1973) and Shannon's information index (I) (Lewontin, 1972). To examine the genetic relationship among the populations, Nei's unbiased genetic distances (Nei, 1978) were calculated for all of the population pairs. A Mantel test was performed to estimate a correlation between the matrices of Nei's (1978) genetic distances and of the geographical distances using NTSYS-pc version 2.1 (1,000 permutations) (Rohlf, 2000).

To investigate the partitioning of the genetic variation within and

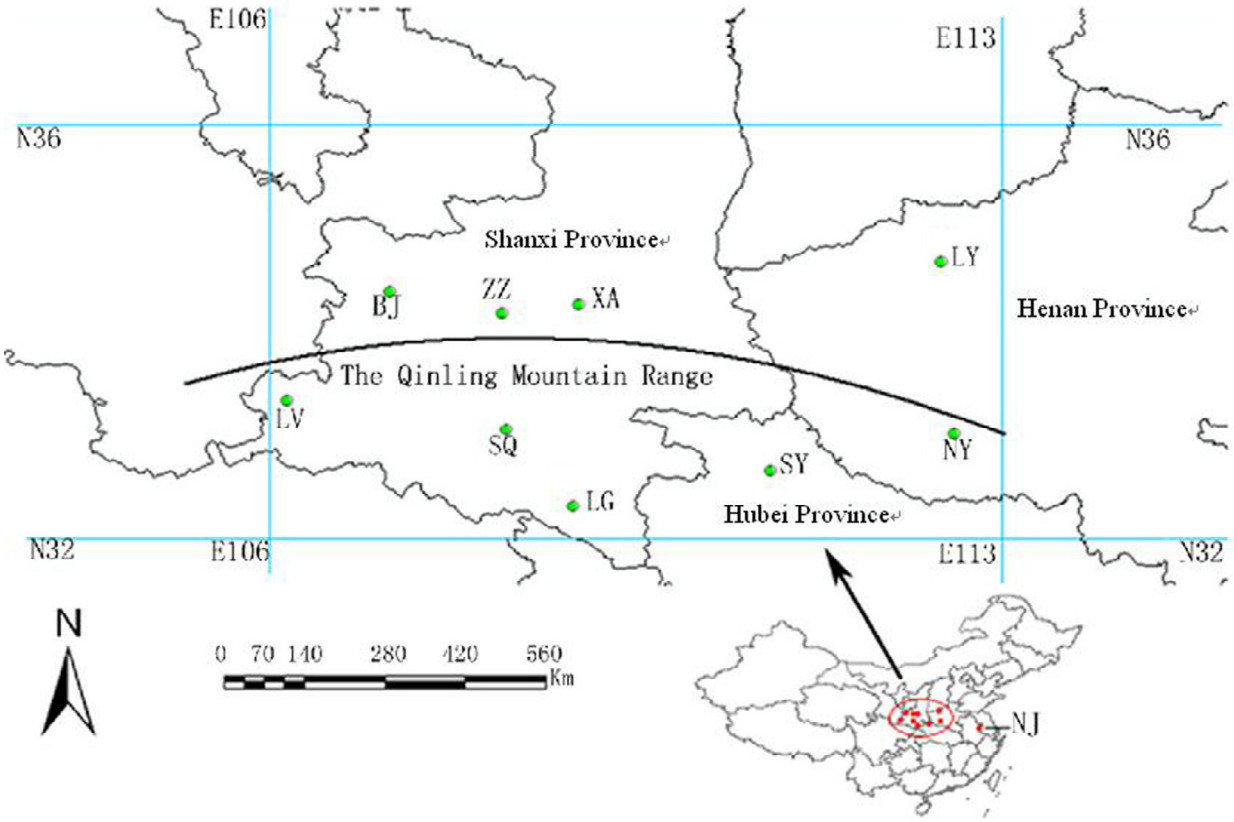

Figure 1. Distribution of populations of *E. ulmoides* in China.

Table 2. Primer code, primer sequences, number of amplified bands, number of polymorphic bands and percent polymorphic bands for each ISSR primer used.

Primer codes	Primer sequences (5'-3')	Number of amplified bands	Number of polymorphic bands	Polymorphic bands (%)
UBC7	GAGGAGGAGGAGGC	8	7	87.5
UBC11	AGAGAGAGAGAGAGAGTA	9	7	77.8
UBC13	GACAGACAGACAGACA	13	13	100.0
UBC811	GAGAGAGAGAGAGAGAC	8	6	75.0
UBC835	AGAGAGAGAGAGAGAGYC	11	10	90.9
UBC866	CTCCTCCTCCTCCTC	10	8	80.0
UBC876	GATAGATAGACAGACA	11	9	81.8
UBC881	GGGTGGGGTGGGGTG	13	11	84.6

among the populations, WINAMOVA software (version 1.55) (Excoffier et al., 1992) was used to carry out an analysis of molecular variance (AMOVA). The genetic differentiation values (Φ_{ST}) between pairs of the populations were calculated, which are analogous to traditional F statistics. Permutation procedures (1000 replicates) for the significance testing of the variance components were performed, and the gene flow between pairs of the populations ($N_m=[1-F_{ST}]/4\ F_{ST}$) was calculated from the Φ_{ST} values (Wright, 1951).An unweighted pair-group method with arithmetic averaging (UPGMA) dendrogram was constructed from Nei's genetic distance with 1,000 permutations of bootstrapping to display graphically the relative divergence among the populations sampled. PCoA was also conducted to visualize the dispersion of the individuals in relation to the first two principal axes of variation. Both analyses were performed using the NTSYS software (Rohlf, 2000).

RESULTS

The selected 8 primers amplified a total of 83 bands, ranging from 200 to 2,500 bp, of which 71 bands (85.54%) were polymorphic (Table 3). The, percentage of

Table 3. Genetic diversity estimates of *E. ulmoides* populations.

Population	PPB	H	I
LY	39.76	0.1329	0.2000
NY	36.14	0.1256	0.1874
SY	34.94	0.1303	0.1920
LG	27.71	0.1095	0.1607
SQ	20.48	0.0736	0.1106
LV	50.60	0.1842	0.2744
BJ	44.58	0.1637	0.2428
ZZ	19.28	0.0770	0.1122
XA	46.99	0.1739	0.2568
NJ	49.40	0.1642	0.2473
Population average	36.99	0.1335	0.1984
Species-level value	85.54	0.2465	0.3814

Table 4. Analysis of molecular variance.

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage of variation	Fixation indices (F_{ST})	*P*-value
Among populations	9	314.57	3.70	37.76%	0.3776	＜0.001
Within populations	68	414.78	6.10	62.24%		＜0.001

polymorphic bands (PPB) at the population level varied from 19.28% to 50.60%, with an average of 36.99%. The mean gene variations of the 10 populations estimated by H and I were 0.1335 and 0.1984, respectively. When calculated at the species level, the PPB, H and I values equaled 85.54%, 0.2465 and 0.3814, respectively. Among the 10 populations investigated, the highest and lowest levels of genetic variability occurred in populations LV and ZZ, respectively.

The individuals of the LY, LV, BJ, XA and NJ populations were obtained from botany gardens or nature protection regions, which have been less influenced by human impact; the other 5 populations were obtained from plantations. In the 10 populations, the wild population, LV, possessed the highest diversity (PPB=50.60%, H=0.1842, I=0.2744), whereas the cultivation population, ZZ, possessed the lowest diversity (PPB=19.28%, H=0.0770, I=0.1122). Only considering the 5 cultivated populations, the mean values of PPB, H and I were 27.71%, 0.10 and 0.15, which were significantly lower than those of the wild populations (PPB=46.27%, H=0.1638, I=0.2443).

The AMOVA analysis using the ISSR data revealed that the most variation (62.24%) was within populations, but a significant proportion (37.76%) was observed among the populations in the present study (Table 4). The significance tests of the variance components among and within the populations, as determined by 1000 random permutations, showed a significant difference ($P<0.001$). The values of the Φ_{ST} that represented the genetic differentiation between the pairs of the populations varied from 0.0014 to 0.6452 (Table 5). The value of the Φ_{ST} of the artificial population pairs based on the ISSR analysis ranged from 0.2225 to 0.6452, with a mean of 0.48, which is twice the mean value of the Φ_{ST} of the wild populations (0.27). The average number of individuals exchanged between the populations per generation (N_m) was 0.412, which suggested that the gene flow in *E. ulmoides* was low.

The matrix of Nei's unbiased measures of genetic distance is listed in Table 6. The largest genetic difference (0.2661) occurred between populations NJ and ZZ, whereas the smallest difference occurred between populations XA and BJ (0.0159). The result of the UPGMA cluster analysis (Figure 2) showed that the clusters were not related to the geographic distance between the populations. The results indicate that 10 populations were clustered into the following 3 groups: group I comprised the LY and NY populations from Henan Province; the 6 populations from Shanxi Province and SY from Hubei Province formed group II; and the NJ population, which is far from the Qinling Mountain Range, formed group III. The Mantel test revealed that there was no statistically significant correlation between the pairwise genetic distance and the corresponding geographic distance among the populations ($r=0.378$, $P=0.996$). The principal coordinate analysis revealed that the first 2 components explained 11.03% and 8.33% of the total variation (Figure 3). The PCoA results confirmed the results of the cluster analysis, suggesting the reliability of the UPGMA

Table 5. Values of Φ_{ST} that represented the genetic differentiation between pairs of populations calculated by AMOVA.

Parameter	LY	NY	SY	LG	SQ	LV	BJ	ZZ	XA	NJ
LY	****									
NY	0.2704	****								
SY	0.4636	0.5076	****							
LG	0.3171	0.4442	0.2225	****						
SQ	0.3631	0.4946	0.4422	0.3571	****					
LV	0.2753	0.3720	0.2955	0.2701	0.2763	****				
BJ	0.3382	0.3923	0.3421	0.3182	0.3345	0.1917	****			
ZZ	0.5126	0.5300	0.5786	0.5747	0.6452	0.4297	0.2994	****		
XA	0.2949	0.4168	0.3664	0.3207	0.3210	0.1749	0.0014	0.2919	****	
NJ	0.4007	0.4846	0.4362	0.3556	0.5033	0.3310	0.3698	0.5509	0.3406	****

Table 6. Nei's unbiased measures of Nei's genetic distance among 10 populations.

Parameter	LY	NY	SY	LG	SQ	LV	BJ	ZZ	XA	NJ
LY	****									
NY	0.0779	****								
SY	0.1980	0.2128	****							
LG	0.1105	0.1672	0.0748	****						
SQ	0.1201	0.1818	0.1415	0.1023	****					
LV	0.1200	0.1366	0.1311	0.1162	0.1086	****				
BJ	0.1378	0.1471	0.1346	0.1227	0.1112	0.0853	****			
ZZ	0.2148	0.2108	0.2363	0.2187	0.2157	0.1755	0.0930	****		
XA	0.1176	0.1624	0.1625	0.1197	0.1069	0.0861	0.0159	0.0977	****	
NJ	0.1805	0.2157	0.1988	0.1239	0.2147	0.1539	0.1702	0.2661	0.1596	****

dendrogram.

DISCUSSION

Genetic diversity

There is evidence that a low genetic diversity is one of the main reasons for the rarity of endemic plant species (Lacerda et al., 2001; Neel and Ellstrand, 2003). Decreased genetic variation would affect the population viability by reducing the fitness of individuals, and it has dramatic effects on the evolutionary potential of species (Reed and Frankham, 2003). However, many recent studies have revealed high levels of genetic polymorphism in rare or narrowly distributed species (Cao et al., 2006; Ci et al., 2008; Mohd Arif et al., 2009). Our molecular survey revealed that the genetic diversity of *E. ulmoides* was lower than that of *Davidia involucrate* (97.03%) (Song and Bao, 2004) and *Metasequoia glyptostroboides* (93.1%) (Li et al., 2005). However, it is relatively high compared with other plants that are endangered and endemic to China, such as *Torreya jackii* (60.48%) (Li and Jin, 2007) and *Pinus squamata* (12.3%) (Zhang et al., 2005). The relatively high genetic diversity at the species level can be explained by several reasons. Firstly, *E. ulmoides* was once widely distributed in the world and harbored an abundant gene pool before the fourth glaciation period. Although, the recent glaciations dramatically narrowed the distribution range of *E. ulmoides*, some of the individuals survived in refugia and preserved their abundant genetic diversity from their ancestors (Axelrod et al., 1998). The mating system and life history is another factor that affects genetic diversity (Hamrick and Godt, 1990; Nybom, 2004). The outcrossing and long-lived woody perennial species, such as *E. ulmoides*, commonly have considerably higher levels of genetic diversity (Hamrick and Godt, 1996). In addition, *E. ulmoides* is distributed in a wide range of climatic and geographic conditions in China during the cultivation history of more than 2,000 years (Zhang, 1992) and rich vitality and high adaptability accumulated through the long-term history. As a conclusion, the variability of morphological traits observed in *E. ulmoides* (Du, 1997) is in accordance with the high degree of genetic variation revealed in the present study.

Compared with the high genetic diversity at the species level, a relatively low genetic diversity was revealed within the populations. The same results of a low genetic diversity at the population level and a high genetic

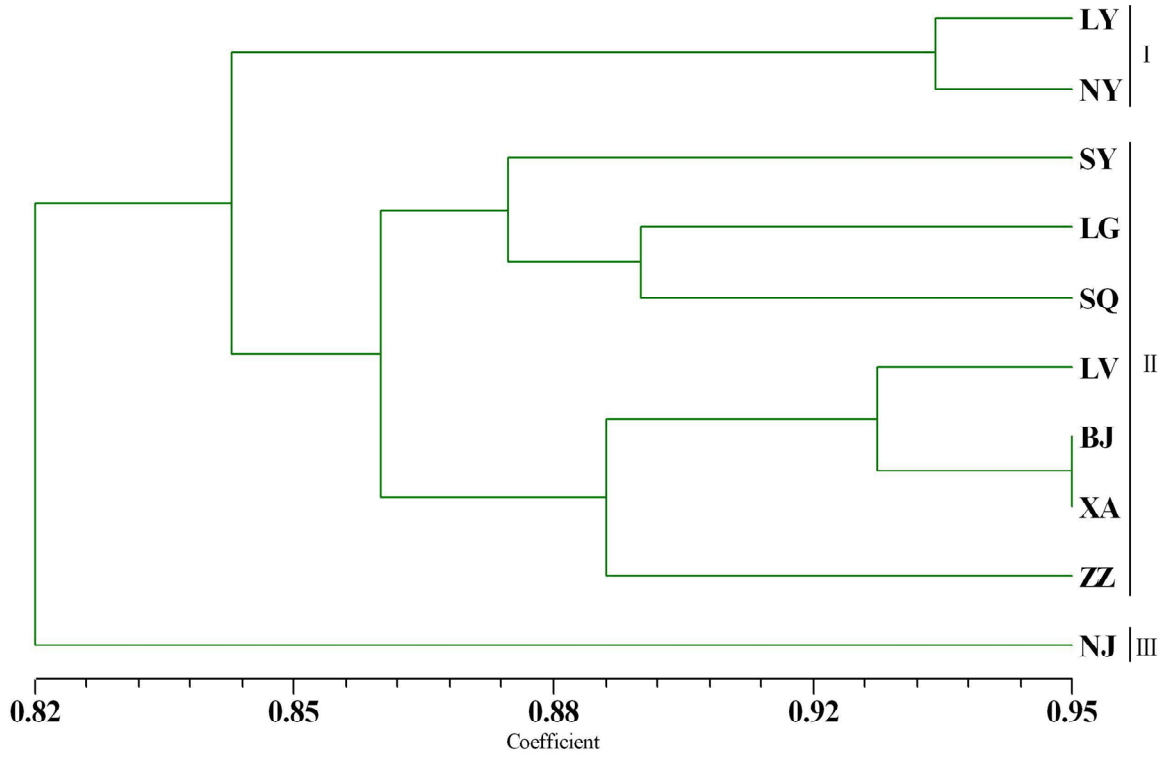

Figure 2. UPGMA dendrogram based on Nei's (1978) genetic distance

diversity at the species level were also found in other endangered and endemic angiosperms (Cao et al., 2006; Ci et al., 2008) using ISSR analysis. A critical factor affecting the within-population diversity is most likely the small population sizes and local extinction due of habitat destruction and excessive exploitation (Maki, 2003). In our research, we found that the population sizes were small in the wild, comprising individuals from a few up to 23 in number, and wild *E. ulmoides* populations had almost disappeared because of excessive logging, grazing and agriculture. Theoretically, reductions in the population size and local extinction cause genetic bottlenecks and enhance genetic drift, which, in turn, has led to a further loss of genetic diversity (Frankham et al., 2002). In our study, the genetic diversity of the cultivated *E. ulmoides* populations was significantly lower than that of the wild populations. Indeed, a decreased genetic diversity in cultivated populations has also been observed in other plant species (Hong et al., 2001) such as *Ginkgo biloba* L., another famous living fossil endemic to China. It is a common assumption that the genetic diversity will be notably lower in cultivars than in their wild relatives (Fan et al., 2004).

Genetic structure

Several complimentary measures (PCoA, Mantel Test and AMOVA) were chosen to describe the genetic diversity and degree of genetic differentiation in *E. ulmoides*, as their use allowed the maximum amount of information to be obtained from a dominant marker data set (Kothera et al., 2007). A relatively higher level of genetic differentiation was detected among the populations. The level of population differentiation (Φ_{ST}=0.3776) was higher than the mean values for other outcrossing plants (Φ_{ST}=0.28) and most long-lived perennial (Φ_{ST}=0.25) species (Nybom and Bartish, 2000; Bussell, 1999), as based on an RAPD analysis. The differentiation of the populations at the loci level is a result of historical population processes, such as natural selection, mutation, isolation and genetic drift (Reed and Frankham, 2001). In the case of *E. ulmoides*, the distribution and population size were dramatically decreased during the glaciations. If populations are small and isolated from one another, genetic drift could be capable of reducing the variation within the populations and increasing the differentiation among the populations (Ellstrand and Elam, 1993). Anthropogenic influences also contribute to the pattern of genetic variation. Humans have transformed most of the habitats of *E. ulmoides* into paddy fields or residential areas, and *E. ulmoides* has suffered from habitat fragmentation. Furthermore, a decreased population size and increased isolation strengthened the effects of genetic drift and inbreeding (Li et al., 2005). In the present study, the effective gene flow per generation for *E. ulmoides* (N_m=0.412) was lower than one successful migrant per

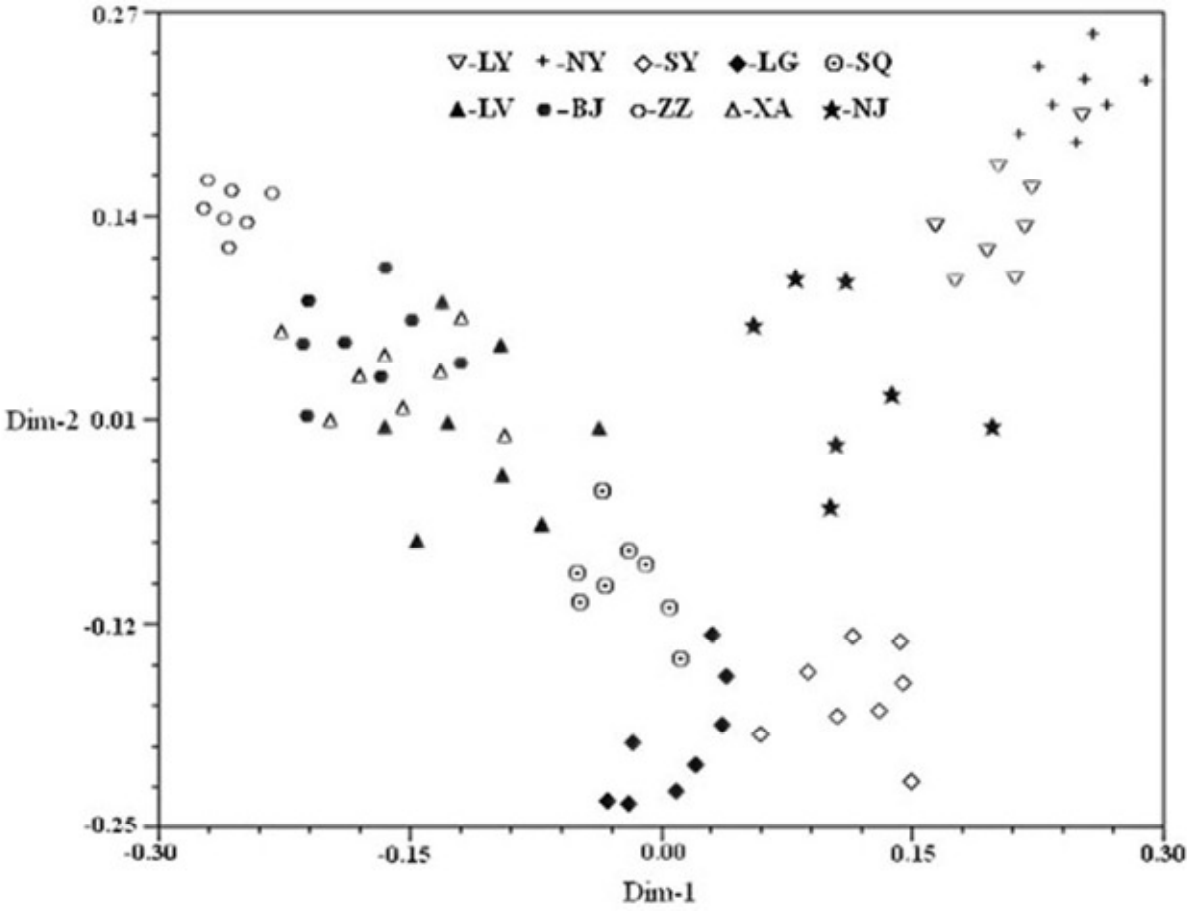

Figure 3. Two-dimensional plot of the principal coordinate analysis

generation, indicating that this level of migration will not prevent the continued divergence among the populations (Wright, 1931; Slatkin, 1987).

The genetic variation between the artificial and wild populations revealed an aggravated genetic differentiation in the artificial populations. This might be the result of biased seed collection and vegetative propagation, which indicated that the artificial selection in the inbred agricultural crops has aggravated the differentiation among the populations. Through our field survey, we found that the overall growth trend was good in the five wild populations; nevertheless, serious occurrences of pests and diseases were found widespread in the artificial populations, which suggest that the phenomenon may be caused by a loss of their genetic diversity. Together, our results indicated that although the quantity and distribution range of *E. ulmoides* have been successfully restored; artificial selection biases the genetic composition of the artificial populations.

Conservation

The successful management and preservation of populations of threatened species depend on a good understanding of the species, including both the levels and distribution of genetic variation (Francisco-Ortega et al., 2000; Wallace, 2002). A loss of genetic diversity could lead to a decrease in a species' ability to survive environmental changes and demographic fluctuations both in the short term and the long term (Reisch et al., 2003). Considering the high genetic differentiation and the limited gene flow in this species, we suggest that the conservation of *E. ulmoides* should ensure that most of the populations are preserved to conserve the vast majority of the variations. The habitats of wild *E. ulmoides*

populations should be given priority for protection and that anthropogenic destruction should be prevented to allow the species to propagate and increase in size through natural regeneration.

Given that a common goal of the reintroduction efforts of endangered species is to construct populations that maintain a genetic variability that is similar to that of the wild populations, the recovery of an endangered species is not merely restoring its numbers (Knapp and Dyer, 1998). In traditional forestry breeding, biased seed collections or asexual reproduction for desirable characteristics may not be appropriate for the conservation of the genetic variation because this material includes only a portion of the gene pool. A better collection is based on the genetic composition and on propagules that are as genetically representative as possible (McGlaughlin et al., 2002). *Ex situ* conservation can also be achieved, for example, by transplanting seedlings from different populations to increase the gene flow among populations artificially, thereby providing the maximum protection of the genetic diversity of *E. ulmoides*.

ACKNOWLEDGEMENTS

We wish to express sincere thanks to our colleagues Li Yu-mei, Fan Ying and Xie Hui for their great help during the field collecting of plant samples and molecular experiments. We are also grateful to the anonymous referees whose comments made our results more accessible to the readers.

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