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Genetic diversity and population structure of *Eucommia ulmoides* Oliver, an endangered medicinal plant in China

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ABSTRACT. *Eucommia ulmoides* Oliver, one of the tertiary relict species found only in China, is the only extant species of Eucommiaceae. Using inter-simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) markers, we studied the genetic diversity and population genetic structure of 187 accessions from 17 *E. ulmoides* populations throughout its main distribution in China. A total of 65 bands were amplified using eight ISSR primers, of which 50 bands (76.9%) were polymorphic. Meanwhile, another 244 bands were observed using eight SRAP primer combinations and 163 (66.8%) of these were polymorphic. The analysis of molecular variation (AMOVA) indicated that 88.8 and 92.4% of the total variation resided within populations based on ISSR and SRAP analysis, respectively. Moreover, we found that the *E. ulmoides* populations were clustered into six distinct groups using ISSR and SRAP markers via the unweighted pair-group method (UPGMA). Furthermore, STRUCTURE analysis showed that these 17 populations could be classified into four groups using ISSR markers, but only two groups using SRAP markers. No

significant relevancy was observed between genetic and geographic distances among the sampled populations. The results of this study support the view that exchange of seeds among local farmers plays an important role in shaping the present genetic distribution pattern. "Core collection" is suggested for genetic diversity conservation of *E. ulmoides* in China.

Key words: Genetic diversity; Diversity conservation; ISSR; SRAP; *Eucommia ulmoides* Oliver

INTRODUCTION

Eucommia ulmoides Oliver, a typical elm-like deciduous woody plant, is a specific tertiary species that grows exclusively in China and is referred to as living fossil. More importantly, this plant is restricted to one species, one genus (*Eucommia*), and one family (Eucommiaceae) (Takeno et al., 2008). With the Chinese name Du-zhong, the bark of *E. ulmoides* has been applied as a traditional Chinese medicine (TCM) for more than 2000 years. The major effective constituents of *E. ulmoides* include types of lignans, iridoids, phenylpropanoids, flavonoids, polysaccharides, and terpenes (Dai et al., 2013). *E. ulmoides* possesses the potential for multiple pharmacological applications, including nourishment of the liver and kidney, strengthening of the muscles and bones, prevention of the high risk of abortion, hypertension, and diabetes (Kwan et al., 2003; National Pharmacopoeia Committee, 2010). Furthermore, Gutta-percha, an important industrial gum (Yan, 1989), can be also found in the leaves, bark, and seed shells of *E. ulmoides* (Tangpakdee et al., 1997).

As a second-category, state-protected endangered plant in China, *E. ulmoides* is situated in an endangered status in the wild. Considering both the biological conservation and the economic benefits, the Chinese government began to promote the cultivation of *E. ulmoides* in the early 1950s. Until now, a widespread artificial population has been mainly distributed in the Guizhou, Hubei, Henan, and Shaanxi provinces of China. *E. ulmoides* has rich genetic resources; however, previous studies have mainly focused on cultivation (Sun et al., 2013), Gutta-percha application (Suzuki et al., 2012), contents of active medicinal components (Dai et al., 2013), and pharmacological properties (Deyama et al., 2001). Thus, exploring the genetic diversity and population structure of *E. ulmoides* is very useful and important for its cultivation and production.

Using molecular markers to reveal polymorphism at the DNA level has been considered a powerful tool for evaluating the characteristics of plant genetic diversity. In previous studies, various molecular markers, such as inter-simple sequence repeats (ISSR) (Wu et al., 2011), random amplified polymorphic DNA (Wang et al., 2006), amplified fragment length polymorphism (Yao et al., 2012), and microsatellites (SSR) (Zhang et al., 2013) were used to detect the genetic diversity of *E. ulmoides*. Due to its superior reliability and reproducibility of bands, ISSR is regarded as a simple and efficient marker among these. Sequence-related amplified polymorphism (SRAP) markers, which specifically target open reading frames, have been widely used in population genetic studies of various plant species, including many medicinal plants (Song et al., 2010; Wang et al., 2012). However, to the best of our knowledge, SRAP markers have never been used in the genetic diversity analysis of *E. ulmoides*.

The current prevailing hypothesis is that there are low levels of genetic differentiation among *E. ulmoides* populations, but each population may represent high genetic diversity. Yao et al. (2012) and Zhang et al. (2013) ascribed it to a high level of historical gene flow mediated by human activities. To test this hypothesis, more appropriate molecular makers are needed to investigate genetic variation of the more extant populations of *E. ulmoides*. Thus, we combined ISSR and SRAP markers to investigate the genetic composition of 187 accessions from 17 *E. ulmoides* populations across their main distribution. The aim of this study is to assess the genetic diversity and population structure of this endangered species, and to speculate a "core collection" that captures the most available genetic diversity of the species for conservation and breeding progress in the future.

MATERIAL AND METHODS

Plant material

We sampled 17 populations dispersed throughout the main distribution of *E. ulmoides* in China (Figure 1). The ages of individuals sampled ranged from 15 to 30 years. We collected over 1000 seeds from each of twenty trees selected in every population [twelve trees in the Ankang (AK) population]. The seeds of the same population were mixed together and seeded in the medicinal botanical garden at Northwest A&F University, China. Fresh leaves of seedlings were collected for DNA extraction. Each population was positioned using a GPS and the details of the studied populations are described in Table 1.

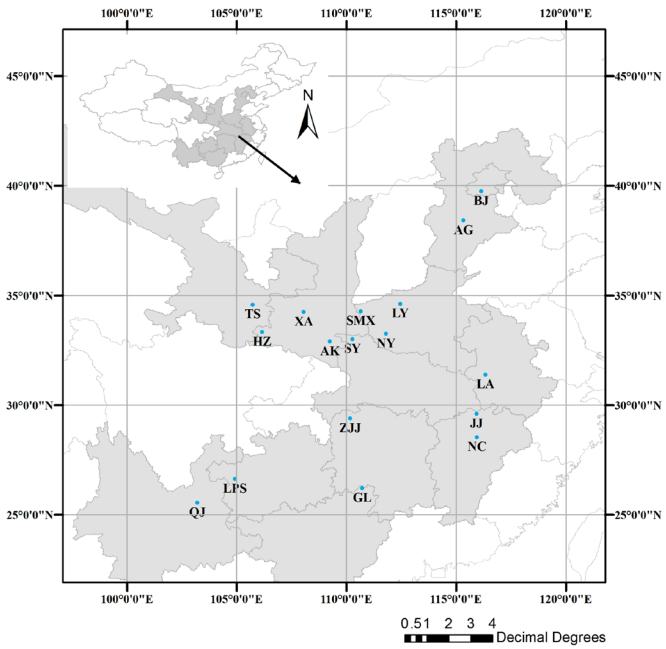

Figure 1. Geographical distribution of the 17 *Eucommia ulmoides* populations sampled in this study. Population abbreviations are provided in Table 1.

Table 1. Details of the germplasm sources of *Eucommia ulmoides*.

Population code	Location	Latitude (°N)	Longitude (°E)	Altitude (m)	Population size	Sample size
LY	Luoyang, Henan Province	34°37'	112°27'	143	200	14
SMX	Sanmenxia, Henan Province	34°17'	110°39'	926	2000	11
HZ	Hanzhong, Shaanxi Province	33°20'	106°10'	687	>4000	11
XA	Xi'an Xian, Shaanxi Province	34°15'	108°03'	448	200	12
BJ	Beijing	39°45'	116°08'	51	200	13
ZJJ	Zhangjiajie, Hunan Province	29°24'	110°10'	389	900	9
GL	Guilin, Guangxi Province	26°13'	110°43'	427	500	13
SY	Shiyan, Hubei Province	33°01'	110°16'	698	1000	11
LA	Liu'an, Anhui Province	31°24'	116°20'	124	900	13
AG	Anguo, Hebei Province	38°25'	115°20'	32	1200	9
TS	Tianshui, Gansu Province	34°35'	105°43'	1171	1500	9
LPS	Liupanshui, Guizhou Province	26°39'	104°55'	1951	2000	14
NC	Nanchang, Jiangxi Province	28°33'	115°57'	20	1000	8
QJ	Qujing, Yunnan Province	25°33'	103°12'	2050	500	9
NY	Nanyang, Henan Province	33°03'	111°15'	417	1200	9
JJ	Jiujiang, Jiangxi Province	29°36'	115°56'	137	500	10
AK	Ankang, Shaanxi Province	32°54'	109°14'	269	12	12

DNA extraction

Total genomic DNA was extracted from approximately 100 mg fresh leaves using the NuClean PlantGen DNA Kit (KangWei, China). The DNA was then suspended in TE buffer. DNA concentration and quality were evaluated by electrophoresis on a 1.0% agarose gel. DNA was stored at -20°C.

PCR amplification

A set of 100 ISSR primers (Isshiki et al., 2008) was synthesized according to the sequences obtained from the University of British Columbia, Canada. All 100 primers were screened for their amplification efficiency using six representative samples. According to the amplification efficiency and reproducibility, eight primer pairs (Table 2) were selected to test all populations. All samples were amplified at least three times if the initial amplification failed.

Table 2. List of the inter-simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) primers used in this study of *Eucommia ulmoides*.

ISSR primers sequences	Sequences (5'→3')	SRAP primer combinations	Forward primer sequences	Reverse primer sequences
UBC825	$(AC)_8T$	Me2Em8	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTAGC
UBC826	$(AC)_8C$	Me5Em8	TGAGTCCAAACCGGAAG	GACTGCGTACGAATTAGC
UBC828	$(TG)_8A$	Me7Em4	TGAGTCCAAACCGGTCC	GACTGCGTACGAATTTGA
UBC848	$(CA)_8RG$	Me8Em4	TGAGTCCAAACCGGTGC	GACTGCGTACGAATTTGA
UBC851	$(GT)_8YG$	Me8Em9	TGAGTCCAAACCGGTGC	GACTGCGTACGAATTACG
UBC856	$(AC)_8YT$	Me9Em5	TGAGTCCAAACCGGACA	GACTGCGTACGAATTAAC
UBC857	$(AC)_8TA$	Me10Em5	TGAGTCCAAACCGGAGA	GACTGCGTACGAATTAAC
UBC881	$(GGGTG)_3$	Me10Em9	TGAGTCCAAACCGGAGA	GACTGCGTACGAATTACG

The PCR mixtures (20 µL total volume) contained 2.0 µL 10X PCR buffer, 2.0 mM $MgCl_2$, 200 mM dNTPs, 1 mM ISSR primers, 1 U Taq DNA polymerase (KangWei), and approximately 10 ng template DNA. PCR amplification was performed on an AB Applied Biosystem (Gene Company Limited, USA), using the following PCR program: 5 min of dena-

turing at 94°C, 40 cycles of three steps: 30 s of denaturing at 94°C, 30 s of annealing at 58°C, and 1 min of elongation at 72°C, with a final elongation step of 10 min at 72°C. The PCR products were separated by electrophoresis on a 1.5% agarose gel using 1X TBE buffer, pH 8.0, at room temperature, using a 5000-bp molecular size standard (KangWei). The gel was visualized using ethidium bromide staining.

SRAP analysis was carried out according to previously established protocols described by Li and Quiros (2001). SRAP primer combinations (100 pairs) were initially screened using six representative samples. Primer combinations were excluded if their banding patterns were difficult to score or if they failed to amplify consistently in all lines. Of these 100 SRAP primer pairs, eight primer combinations that consistently produced clear and diverse amplified bands were selected (Table 2). All samples were amplified at least three times if the initial amplification failed.

The PCR mixture (20 μL total volume) contained 2.0 μL 10X PCR buffer, 2.0 mM $MgCl_2$, 200 mM dNTPs, 0.5 mM forward primer, 0.5 mM reverse primer, 1 U Taq DNA polymerase (KangWei), and approximately 20 ng template DNA. PCR amplification was performed on an AB applied Biosystem (Gene Company Limited) using the following PCR program: 5 min of denaturing at 94°C, 5 cycles of three steps: 1 min of denaturing at 94°C, 1 min of annealing at 35°C, and 2 min of elongation at 72°C. In the following 35 cycles, the annealing temperature was increased to 50°C, with a final elongation step of 8 min at 72°C. The PCR products were separated on a 6% non-denaturing polyacrylamide gel and SRAP bands were stained using silver sequence DNA staining reagents, using a 5000-bp molecular size standard (KangWei).

Data analysis

Amplified bands were scored either as presence (1) or absence (0), and scored for a binary data matrix with polymorphic and reproducible bands. The matrix was then used for the following analysis: the percentage of polymorphic loci (*PPB*), the effective number of alleles, observed number of alleles, gene flow (N_m), Shannon's information index (*I*) (Lewontin, 1995), and Nei's gene diversity (*H*) (Nei, 1973) were obtained by the software package POPGENE version 3.2 (Yeh et al., 1997). Analysis of molecular variance (AMOVA) was used to estimate the variance components and their significant levels of genetic variation within and among populations using GenAlEx version 6.5 (Peakall and Smouse, 2012) based on 999 permutations. A Mantel test (Nei, 1972) for geographic and genetic distances of population pairs was also employed using the same software to determine if a relationship existed between the two data matrices with 999 permutations.

To further examine the genetic relationships among accessions, cluster analysis based on the genetic similarity matrix was performed with the UPGMA (unweighted pair group method with arithmetic mean) method (Sneath and Sokal, 1973), using the SHAN function of NTSYS-pc version 2.1 (Rohlf, 2000). Population structure was determined using the model-based program, STRUCTURE (Pritchard et al., 2000). To identify the number of populations (*K*) capturing the major structure in the data, we used a burn-in period of 50,000 Markov Chain Monte Carlo iterations and 100,000 runs, with an admixture model following Hardy-Weinberg equilibrium, correlated allele frequencies, and independent loci for each run. Twenty independent runs were performed for each simulated value of *K*, ranging from 2 to 17. The

true *K* value was determined using both an estimate of the posterior probability of the data for a given *K* (Pritchard et al., 2000) and the Evanno Δ*K* (Evanno et al., 2005).

RESULTS

Genetic diversity of *E. ulmoides*

We surveyed 187 accessions from 17 *E. ulmoides* populations using eight ISSR primers (Table 3). A total of 65 bands were identified, of which 50 were polymorphic (76.9%) with a minimum of 5 (UBC851) and a maximum of 12 (UBC848) bands per primer (Table 3). Representative banding patterns detected by the primer UBC-881 are shown in Figure 2. The size of amplified fragments ranged from 200 to 2000 bp with an average of 8.1 fragments per primer. *PPB* produced by each primer ranged from 40.0 (UBC-851) to 91.7% (UBC-848). Genetic diversity (Table 4) was relatively low at the population level. *PPB* ranged from 24.6 to 60.0%, with a mean of 42.6% (Table 4). The values of genetic diversity as estimated by *H* varied from 0.085 to 0.245 and *I* ranged from 0.130 to 0.356. However, *E. ulmoides* populations had high levels of genetic diversity at the species level (*PPB* = 76.9%; *H* = 0.273; *I* = 0.403).

Table 3. Polymorphism in *Eucommia ulmoides* revealed by inter-simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) markers.

ISSR				SRAP			
Primer	Bands generated	N	*PPB* (%)	Primer combination	Bands generated	N	*PPB* (%)
UBC825	9	8	88.9	Me2Em8	15	7	46.7
UBC826	8	4	50.0	Me5Em8	46	31	67.4
UBC828	7	6	85.7	Me7Em4	23	14	60.9
UBC848	12	11	91.7	Me8Em4	27	14	51.9
UBC851	5	2	40.0	Me8Em9	18	11	61.1
UBC856	8	7	87.5	Me9Em5	36	25	69.4
UBC857	9	7	77.8	Me10Em5	35	27	77.1
UBC881	7	5	71.4	Me10Em9	44	34	77.3
Total	65	50	76.9	Total	244	163	66.8
Average	8.1	6.3		Average	30.5	20.4	

N = number of polymorphic bands; *PPB* = percentage of polymorphic bands.

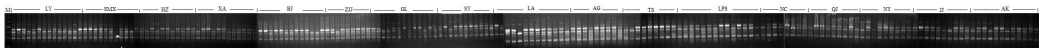

Figure 2. Representative inter-simple sequence repeat banding patterns of primer UBC881 in *Eucommia ulmoides*. Population abbreviations are provided in Table 1.

The same accessions representing the 17 populations were then analyzed with 8 SRAP primer combinations (Table 3). These yielded a total of 244 fragments, of which 163 were polymorphic (66.8%), with a minimum of 15 (Me2Em8) and a maximum of 46 (Me5Em8) bands per primer combination (Table 3). Representative banding patterns detected by the primer Me10Em5 are shown in Figure 3. The size of amplified fragments ranged from 100 to 5000 bp with an average of 30.5 fragments per primer. *PPB* produced by each primer pair ranged from 46.7 (Me2Em8) to 77.3% (Me10Em9). The inter-population genetic diversity (Table 4) was low. *PPB* ranged from 27.9 to 61.1%, with a mean of 43.1% (Table 4). The

values of genetic diversity as estimated by *H* varied from 0.104 to 0.216 and *I* ranged from 0.156 to 0.320. In addition, *E. ulmoides* populations had high levels of genetic diversity at the species level (*PPB* = 66.8%; *H* = 0.228; *I* = 0.345).

Table 4. Genetic diversity of *Eucommia ulmoides* populations reveled by combined inter-simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) markers. Population abbreviations are provided in Table 1.

Population	ISSR					SRAP				
	N_A	N_E	*H*	*I*	*PPB* (%)	N_A	N_E	*H*	*I*	*PPB* (%)
HZ	1.292	1.206	0.117	0.171	29.2	1.320	1.216	0.124	0.183	32.0
LY	1.446	1.292	0.167	0.246	44.6	1.574	1.354	0.205	0.305	57.4
SMX	1.600	1.440	0.245	0.356	60.0	1.586	1.328	0.197	0.298	58.6
XA	1.338	1.234	0.133	0.196	33.8	1.352	1.248	0.140	0.206	35.2
BJ	1.523	1.393	0.217	0.314	52.3	1.611	1.363	0.213	0.320	61.1
ZJJ	1.477	1.325	0.186	0.274	47.7	1.279	1.177	0.104	0.156	27.9
GL	1.415	1.307	0.171	0.248	41.5	1.312	1.196	0.116	0.173	31.2
SY	1.554	1.395	0.222	0.322	55.4	1.574	1.373	0.216	0.319	57.4
LA	1.523	1.371	0.209	0.305	52.3	1.430	1.237	0.143	0.217	43.0
AG	1.477	1.326	0.187	0.274	47.7	1.549	1.366	0.208	0.307	54.9
TS	1.415	1.295	0.166	0.242	41.0	1.451	1.306	0.175	0.258	45.1
LPS	1.323	1.243	0.134	0.195	32.3	1.385	1.247	0.145	0.216	38.5
NC	1.400	1.268	0.155	0.228	40.0	1.316	1.200	0.118	0.176	31.6
QJ	1.323	1.219	0.126	0.186	32.3	1.307	1.205	0.118	0.175	30.7
NY	1.508	1.367	0.207	0.301	50.8	1.508	1.334	0.192	0.285	50.8
JJ	1.385	1.289	0.158	0.229	38.5	1.455	1.269	0.161	0.243	45.5
AK	1.246	1.138	0.085	0.130	24.6	1.312	1.222	0.125	0.183	31.2
Mean	1.426	1.300	0.170	0.248	42.6	1.431	1.273	0.159	0.236	43.1
At the species level	1.723	1.476	0.273	0.403	76.9	1.693	1.380	0.228	0.345	66.8

N_A = observed number of alleles; N_E = effective number of alleles; *H* = Nei's gene diversity; *I* = Shannon's Information index; *PPB* = percentage of polymorphic loci.

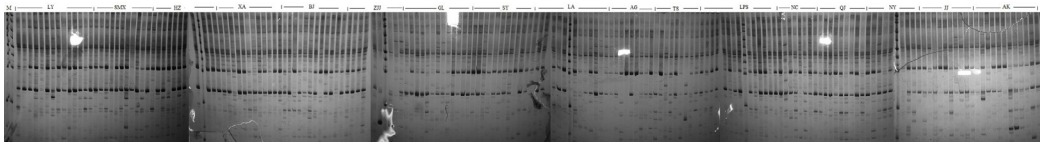

Figure 3. Representative sequence-related amplified polymorphism banding patterns of primer Me10Em5 in *Eucommia ulmoides*. Population abbreviations are provided in Table 1.

Genetic differentiation and gene flow in *E. ulmoides*

As revealed by the ISSR markers, the total gene diversity (*HT*) and gene diversity within populations (*HS*) were 0.271 and 0.170, respectively. The coefficient of genetic differentiation (G_{ST}) was 0.374. AMOVA analysis showed that 11.21% of the total gene diversity was found among the populations, whereas the remaining 88.79% of the total variation occurred within populations (Table 5), and N_m was 0.8357. The SRAP analysis showed that *HT* and *HS* were 0.225 and 0.159, respectively, and G_{ST} was 0.294. The AMOVA analysis indicated that 7.62% of the total gene diversity was found among the populations, whereas the remaining 92.38% of the total variation occurred within the populations (Table 5), and N_m was 1.2007.

Table 5. Analysis of molecular variance (AMOVA) for *Eucommia ulmoides* populations revealed by combined inter-simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) markers.

	SV	d.f.	SSD	MSD	VC	TVP	P
ISSR	Among Pops	16	196.66	12.291	0.983	11.21%	0.001
	Within Pops	62	482.606	7.784	7.784	88.79%	
SRAP	Among Pops	16	666.972	41.686	2.495	7.62%	0.001
	Within Pops	62	1874.825	30.239	30.239	92.38%	

SV = source of variation; d.f. = degrees of freedom; SSD = sum of squares; MSD = mean squares; VC = variance component; TVP = total variance percentage.

Population structure and cluster analysis

A dendrogram was plotted using UPGMA analysis derived from the ISSR profile data and is shown in Figure 4a. The genetic similarity (GS) coefficients among the 17 populations that were based on ISSR markers varied from 0.830 (between AK and the other populations) to 0.940 (between AK and the other populations). The 17 populations were placed into six clusters with a GS coefficient of 0.883. The first major cluster (I) comprised ten populations. Clusters II and III included two populations each. The other 3 clusters (IV, V, and VI) had only one germplasm each, that is, the populations Qujing (QJ), Nanyang (NY), and AK, respectively. Two sub-clusters (Ia, Ib) were identified within the first cluster at a GS coefficient of 0.889. The first sub-cluster (Ia) comprised populations Hanzhong (HZ) and Xi'an Xian (XA), which were in the same province. The last eight populations were assigned to the second sub-cluster (Ib).

Based on SRAP analysis, the GS coefficients between all accessions ranged from 0.870 (between population AK and other populations) to 0.980 [between population Sanmenxia (SMX) and other populations]. The SRAP-based dendrogram also showed one major cluster (I) comprising eleven populations with a GS coefficient of 0.930 (Figure 4b). Cluster II consisted of populations Tianshui (TS) and NY. The populations Liupanshui (LPS), Jiujiang (JJ), XA, and AK appeared to be distinct from all other populations, each forming a single cluster (III, IV, V, and VI), respectively. Cluster I was divided into three sub-clusters with a GS coefficient of 0.938; the first sub-cluster (Ia) comprised four populations, while six populations were assigned to the second sub-cluster (Ib). The population Zhangjiajie (ZJJ) alone formed sub-cluster Ic.

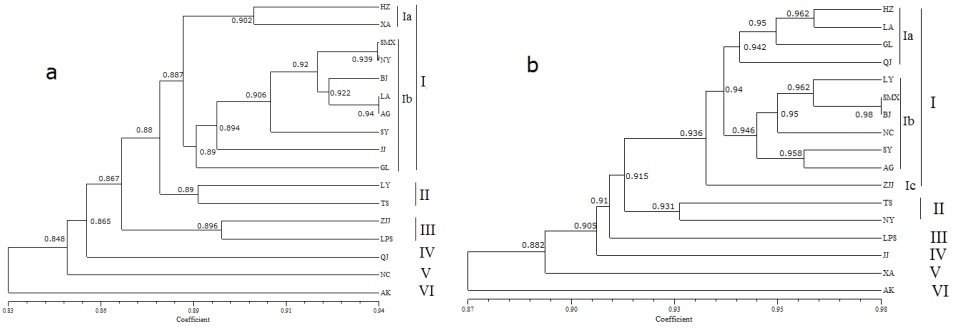

Figure 4. Dendrogram, constructed using the unweighted pair group method with arithmetic mean method, of the 17 *Eucommia ulmoides* populations sampled based on Nei's genetic distance, as revealed using inter-simple sequence repeat markers (**a**); sequence-related amplified polymorphism markers (**b**). The genetic similarity coefficients between populations obtained by cluster analyses are marked on branches and the x-axis. Population abbreviations are provided in Table 1.

Following the method of Evanno (2005), the ΔK values were plotted against the K numbers of the sub-groups. The maximum ΔK occurred at $K = 4$ based on ISSR markers and $K = 2$ based on SRAP markers (Figure 5). We divided the accessions into different sub-groups considering membership probabilities (≥0.25 for ISSR, ≥0.50 for SRAP). The STRUCTURE analysis (Figure 6a) indicated that the entire population could be divided into four groups based on the ISSR markers: group 1 comprised eight populations; group 2 consisted of the five populations SMX, Liu'an (LA), Guilin (GL), ZJJ, and QJ; populations Shiyan (SY) and Luoyang (LY) formed group 3, while populations HZ and NY comprised group 4. Based on the SRAP marker analysis, the 17 populations were divided into two groups (Figure 6b): group 1 included eleven populations, HZ, LY, Beijing (BJ), SY, Anguo (AG), TS, NY, LPS, JJ, XA, and AK; the other six populations [LA, GL, QJ, SMX, Nanchang (NC) and ZJJ] were clustered into group 2.

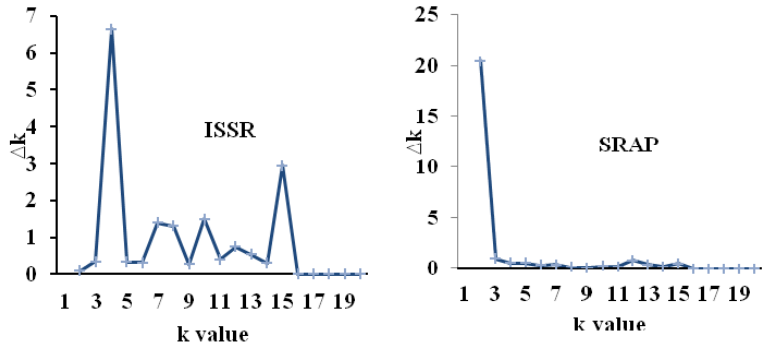

Figure 5. ΔK values for different numbers of *Eucommia ulmoides* populations assumed (K) in the STRUCTURE analysis based on inter-simple sequence repeat markers (ISSR; left panel) and sequence-related amplified polymorphism markers (SRAP; right panel). Population abbreviations are provided in Table 1.

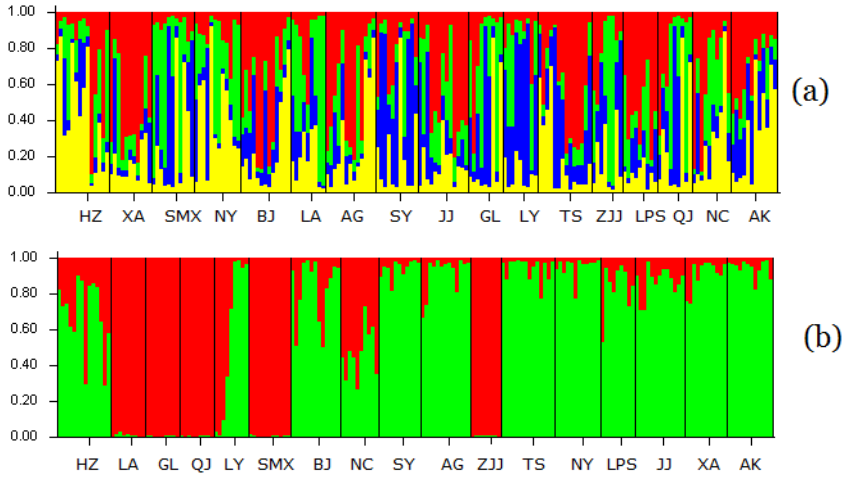

Figure 6. Population structure of *Eucommia ulmoides* prepared using the STRUCTURE program (Pritchard et al., 2000), as revealed using inter-simple sequence repeat markers (**a**); sequence-related amplified polymorphism markers (**b**). Population abbreviations are provided in Table 1.

DISCUSSION

Genetic diversity of *E. ulmoides*

E. ulmoides, the only extant species of Eucommiaceae, is one of the oldest and most effective herbal medicines, and is widely used for treatment of hypertension (Dai et al., 2013). Hence, to better conserve and utilize this endangered species, it is necessary to gather as great a diversity of material as possible. Therefore, we collected samples from 17 populations, attempting to include the main distribution range of *E. ulmoides* in China. Eight ISSR primers and eight SRAP primer combinations were used to analyze the 187 *E. ulmoides* samples representing the 17 populations. ISSR analysis revealed high genetic diversity at the species level (*PPB* = 76.9%; *H* = 0.273; *I* = 0.4034), but genetic diversity was relatively low at the population level (*PPB* = 42.63%; *H* = 0.1696; *I* = 0.2482). The same result was observed based on the SRAP data, at the species level *PPB* = 66.8%, *H* = 0.2276, and *I* = 0.3447, while at the population level *PPB* = 42.63%, *H* = 0.1589, and *I* = 0.2363. Likewise, Wang et al. (2006), Wu et al. (2011), Yao et al. (2012), and Zhang et al. (2013) all revealed relatively high genetic diversity of *E. ulmoides* at the population level.

Both Yao et al. (2012) and Zhang et al. (2013) reported that the genetic diversity of the semi-wild population was greater than the cultivated population, which suggests that ongoing cultivation has reduced the genetic diversity of *E. ulmoides*. Our study showed that the SMX population had a relatively high genetic diversity (*PPB* = 60.0% for ISSR, *PPB* = 58.6% for SRAP), and this was attributed to hybridization, which may result in high levels of genetic variation in rare species (Leimu et al., 2006). Both ISSR and SRAP analysis indicated that the genetic diversity of the AK population was lower than the other populations (*PPB* = 24.6% and 31.2%, respectively). However, only 12 individuals were found in the AK population, and the small population size may be the main factor for its low genetic diversity (Brzosko et al., 2011). Small populations are predicted to have reduced genetic diversity caused by genetic drift, founder effects, and accumulation of deleterious mutations (Young et al., 1996).

Genetic differentiation of *E. ulmoides* populations

Based on the values of G_{ST} (0.374 for ISSR, 0.294 for SRAP), we observed relatively low genetic differentiation among the populations of *E. ulmoides*. The AMOVA analysis based on the ISSR and SRAP markers further indicated that 11.21 and 7.62% of the total gene diversity, respectively, was found among populations (Table 2). Yao et al. (2012) indicated that low to moderate levels of genetic differentiation among *E. ulmoides* populations may result from the relatively high gene flow among the studied populations.

In the present study, N_m among the populations determined by ISSR and SRAP analysis was 0.836 and 1.201, respectively. The value of $N_m > 1$ indicates that no significant genetic differentiation existed among populations, and this level of migration would not restrain the continued divergence among the populations (Slatkin, 1987). Thus, the results of the SRAP analysis indicate that no significant genetic differentiation exists among populations. In addition, according to the Mantel test, no significant correlation between genetic distance and geographical distance was detected in this study. Similar results were also found in some other studies of *E. ulmoides* (Wang et al., 2006; Yao et al., 2012; Zhang et al., 2013). However, the

results of the ISSR analysis indicate just the opposite. Wu et al. (2011) also suggested that gene flow in *E. ulmoides* was low, which was based on an N_m value of 0.412 using ISSR markers.

Lower gene flow is commonly found in rare or endangered species because the distribution and population size of these species are often dramatically reduced. Nevertheless, our SRAP analysis in *E. ulmoides* indicated that this endemic plant has a high level of gene flow. The mode of pollen and seed dispersal, which determine gene flow among populations, may partly account for the status of gene flow. *E. ulmoides* is a wind pollinated and outcrossing species and the distances among populations are too large for pollen dispersal. Thus, seeds appear to be the main genetic material exchanged from one geographic region to another. Local farmers collect seeds of *E. ulmoides* randomly and mix them together before planting. Occasionally, the germplasm may be dispersed to other places by farmers. This frequent exchange of seeds further improves the maintenance of genetic variation (Guo et al., 2007).

Cluster analysis

Similar results were obtained from the STRUCTURE analysis using ISSR and SRAP markers. According to the SRAP analysis, the populations were clustered into two main groups, but the HZ, LY, and NC populations displayed some degree of mixed ancestry. The NC population was especially admixed and exhibited inheritance from two different ancestors; accessions in the NC population were similar to both main groups. This result was further confirmed by ISSR analysis, with the LY and NC populations clustered into group 3 and group 4, respectively. There was no significant correlation between the ISSR and SRAP dendrograms, except for the AK population, which formed a genetically distinct group. A Mantel test showed no significant relationship between genetic and geographic distances among the sampled populations (r = 0.022 for ISSR, r = -0.046 for SRAP). This corroborates previous reports showing there was no statistically significant correlation between the pairwise genetic distance and the corresponding geographic distance among the populations (Wu et al., 2011; Yao et al., 2012).

Implications for conservation

A long cultivation history of *E. ulmoides* has played an important role in shaping the present genetic distribution pattern. In traditional forestry breeding, conventional breeding of *E. ulmoides* has mainly focused on the selection of promising plants from existing natural populations. These selected plants were propagated vegetatively and released as clones (Li et al., 2014). However, clonal reproduction for desirable characteristics narrows genetic variation and is not appropriate for conservation because this material includes only a portion of the gene pool. To protect the TCM germplasm resource, the "medicinal plant core collection" conception was proposed recently (Liu et al., 2012). Seed collection may be a better consideration at present and in the future for the germplasm conservation of *E. ulmoides*.

The main objective of genetic resource conservation is to maintain high levels of genetic variability. According to our present study, the SMX, BJ, LY, SY, LA, AG, and NY populations possess higher genetic variation than the mean, suggesting that these seven populations should be given high priority in conservation. These seven populations were closely clustered together in the ISSR and SRAP dendrograms, while the dendrograms all showed that the AK population formed a genetically distinct group. Thus, the AK population should be given pri-

ority in germplasm collection, and a proper conservation measure should be instituted for its low genetic diversity at the inter-population level, which is mainly due to its small population size. A decreased population size and increased isolation strengthen the effects of genetic drift, which is capable of reducing the variation within populations and increasing the differentiation among populations (Li et al., 2005). Based on the STRUCTURE analysis, the HZ, LY, and NC populations are also important for conservation programs. Furthermore, most work by conservationists on medicinal plants could be achieved by cooperating with the people who own, manage, or make use of these species (Hamilton, 2004). Most *E. ulmoides* populations are cultivated, and the cultivators should be encouraged to protect this highly rare tree species, not only for economic benefits but also for the responsibility of protecting endangered plants.

In conclusion, our results based on ISSR and SRAP analysis indicate that these 17 cultivated populations of *E. ulmoides* exhibit high genetic diversity at the species level, with a relatively lower genetic diversity at the population level. Additionally, a low level of inter-population genetic differentiation and a high level of intra-population genetic differentiation were found in our present study, which is supported by a moderate level of gene flow. Exchange of seeds among local farmers has played an important role in shaping the present genetic distribution pattern. Based on our research, several suggestions were proposed for genetic diversity conservation of *E. ulmoides* in China.

Conflicts of interest

The authors declare no conflict of interest.

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