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Genetic diversity and relationships among Chinese *Eucommia ulmoides* cultivars revealed by sequence-related amplified polymorphism, amplified fragment length polymorphism, and inter-simple sequence repeat markers

Y. Li, S.H. Wang, Z.Q. Li, C.F. Jin and M.H. Liu

College of Forestry, Northwest A&F University, Yangling, Shaanxi, China

Corresponding author: Z.Q. Li
E-mail: lizhouqi@nwsuaf.edu.cn

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ABSTRACT. Sequence-related amplified polymorphism (SRAP), amplified fragment length polymorphism (AFLP), and inter-simple sequence repeat (ISSR) markers were used to estimate the genetic diversity and relationships among *Eucommia ulmoides* cultivars in China. A total of 240, 192, and 150 DNA fragments were detected by 10 SRAP primer combinations, 10 AFLP primer combinations, and 10 ISSR primers, among which 89.2, 65.1, and 88.0% of the fragments were polymorphic, respectively. Cluster analysis revealed that Qinzhong No. 3, Xiaoyeci, Qinzhong No. 1, and Qinzhong No. 2 formed independent clusters. The other 15 cultivars exhibited two clusters. The results of this study will help in the selection of parents for both genome mapping and crossbreeding purposes.

Key words: *Eucommia ulmoides*; Genetic diversity; Sequence-related amplified polymorphism (SRAP); Amplified fragment length polymorphism (AFLP); Inter-simple sequence repeat (ISSR)

INTRODUCTION

Eucommia ulmoides Oliver, the single extant species of the genus *Eucommia* (Eucommiaceae) (Tippo, 1940), is strictly a dioecious perennial tree. It is an economically important plant for both herbal medicine and the organic chemical industry. Chemical constituents in the bark and leaf have high pharmacological activities and healthcare functions such as lowering blood pressure (Kwan et al., 2003) and sugar (Lee et al., 2005). The whole plant except xylem contains *Eucommia* rubber, which is an important raw material in the chemical industry (Nakazawa et al., 2009). Historically, wild *E. ulmoides* had a natural range covering much of the Yangtze River valley and central China (Tippo, 1940; Wang et al., 2003). However, wild plants rarely exist today due to the long history of utilization and overexploitation in the past three decades. Currently, *E. ulmoides* is widely cultivated in low mountains and hills of central China and other parts of the world, such as Europe, the United States (Tippo, 1940), Russia, Japan, and Korea (Zhang, 1990).

Although only a single species of *Eucommia* has survived from the Quaternary glacial period, some differentiations in physiology, morphology, and genetics have accumulated during the evolution of this plant because of influences from the natural environment, natural hybridization, and gene mutation (Zhang et al., 1999). Conventional breeding programs have mainly focused on the selection of promising plants from existing natural populations (Du et al., 1994; Zhang et al., 2004; Dong et al., 2011). These selected plants were propagated vegetatively and released as clones. Recently, these cultivars were used as parents in the crossbreeding program. Within these existing *E. ulmoides* cultivars, an accurate assessment of the genetic diversity and relationships is essential for the long-term success of breeding programs and maximizes the exploitation of the germplasm resources.

To estimate the genetic diversity, different molecular marker techniques, including sequence-related amplified polymorphism (SRAP) (Li and Quiros, 2001), amplified fragment length polymorphism (AFLP) (Vos et al., 1995), and inter-simple sequence repeat (ISSR) (Zietkiewicz et al., 1994), are available and have been applied in many crop and tree species, such as citrus (Amar et al., 2011), mulberry (Kalpana et al., 2012), curcuma (Taheri et al., 2012), and bamboo (Waikhom et al., 2012). In *E. ulmoides*, analyses of genetic diversity have been carried out using ISSRs (Wu et al., 2011) and AFLPs (Yao et al., 2012).

In this paper, we examined the use of three different marker systems to estimate the level of genetic diversity and relationships among *E. ulmoides* cultivars in China, which would help in the identification of diverse parents for both genome mapping and crossbreeding purposes.

MATERIAL AND METHODS

Plant materials

The study included 19 *E. ulmoides* cultivars selected from different areas of China. These cultivars were produced by researchers through controlled breeding of clones (Du et al., 1994; Zhang et al., 2004; Dong et al., 2011) and have been used in the crossbreeding program. Fifteen cultivars were planted in the village of Yantuo, Lingbao, Henan Province, and four cultivars (Qinzhong No. 1, Qinzhong No. 2, Qinzhong No. 3, and Qinzhong No. 4) were planted in the museum garden of Northwest A&F University. The cultivars with local names, sexes, origins, and special characteristics are mentioned in Table 1.

Table 1. List of *Eucommia ulmoides* cultivars.

No.	Cultivar	Sex	Origin	Special characteristics
1	Yecongzhi	Male	Songxian, Henan	Leaf: oval, oblong, and dense; bark: smooth; branch: stubby; ornamental tree
2	Xiaoyexiong	Male	Luoyang, Henan	Leaf: oval, oblong, small, and dense; bark: smooth
3	Longguai	Male	Luoyang, Henan	Leaf: ovate; bark: smooth; branch: dragon-shaped; ornamental tree
4	Yanci	Female	Lingbao, Henan	Leaf: oval and oblong; bark: smooth; high yield of fruit
5	Ziye	Male	Cili, Hunan	Leaf: ovate and purple; bark: smooth; ornamental tree
6	Nanyang No. 1	Male	Nanyang, Henan	Leaf: oval and oblong; bark: smooth
7	Luochao No. 3	Male	Luoyang, Henan	Leaf: oval and oblong; bark: smooth
8	Qinzhong No. 1	Male	Lueyang, Shaanxi	Leaf: oval and oblong; bark: rough; high content of bioactive constituents and *Eucommia* rubber; high resistance to drought
9	Qinzhong No. 2	Male	Cili, Hunan	Leaf: oval and oblong; bark: smooth; high content of bioactive constituents and *Eucommia* rubber; high resistance to cold
10	Qinzhong No. 3	Female	Dujiangyan, Sichuan	Leaf: ovate; bark: smooth; high content of bioactive constituents; high resistance to drought
11	Changbing	Female	Lingbao, Henan	Leaf: oval and oblong; petiole: long; bark: smooth
12	Huazhong No. 5	Male	Wuling Mountain	Leaf: ovate; bark: rough; high yield
13	Huazhong No. 2	Female	Luoyang, Henan	Leaf: ovate; bark: rough; high yield; high resistance to diseases and insect pests
14	Daguo	Female	Lingbao, Henan	Leaf: oval and oblong; bark: smooth; fruit: large
15	Daye	Female	Lingbao, Henan	Leaf: ovate and large; bark: smooth
16	Xiaoyeci	Female	Lingbao, Henan	Leaf: oval, oblong, and small; bark: smooth
17	Qinzhong No. 4	Female	Lueyang, Shaanxi	Leaf: ovate; bark: smooth; high content of bioactive constituents; high resistance to cold
18	Juye	Female	Lingbao, Henan	Leaf: ovate and large; bark: rough
19	Huazhong No. 4	Female	Yunyang, Hubei	Leaf: ovate; bark: smooth; high yield

DNA isolation

Genomic DNA was isolated from young leaves of *E. ulmoides* using a modified cetyltrimethyl ammonium bromide (CTAB) method (Porebski et al., 1997). Samples of young leaves (0.2 g) were ground to a fine powder in liquid nitrogen and incubated at 65°C for 1 h in 2 mL CTAB isolation buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 2% CTAB, 2% β-mercaptoethanol, and 2% polyvinyl pyrrolidone). After incubation, an equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed well with inversions, and centrifuged at 12,000 rpm for 10 min. The upper aqueous phase was transferred to new tubes and extracted again with an equal volume of chloroform:isoamyl alcohol (24:1). The supernatant was mixed with about two volumes chilled absolute alcohol and 1/10 volume 3 M sodium acetate to precipitate the DNA. The tubes were centrifuged at 10,000 rpm for 5 min. DNA pellets were washed twice in 70% ethanol, air-dried, and dissolved in 0.5 mL Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA). Samples were treated with 2 µL RNase (10 mg/mL) at 37°C for 20 min. For further purification, the sample was extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0) followed by two extractions with chloroform:isoamyl alcohol (24:1). The supernatant was precipitated by adding two volumes of chilled absolute alcohol, air-dried, and dissolved in TE buffer. DNA quality was ascertained through gel electrophoresis on a 0.8% agarose gel while the DNA quantity was estimated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). DNA was diluted to a concentration of 30 ng/µL for polymerase chain reaction (PCR).

SRAP, AFLP, and ISSR markers

A total of 256 SRAP primer combinations, 64 AFLP primer combinations, and 100 ISSR primers were pre-selected for PCR amplifications. Initially, PCR amplifications were carried out with Qinzhong No.1 and Yecongzhi as representative cultivars using these primers individually. The primers that gave clear and polymorphic amplification patterns were selected for further analysis. As a result, 10 SRAP primer combinations, 10 AFLP primer combinations, and 10 ISSR primers (Table 2) were used for the PCR amplifications of the 19 *E. ulmoides* cultivars. The SRAP primers consist of the core sequences and three selective nucleotides at the 3'-end. The core sequence of the forward primers is TGAGTCCAAACCGG. The core sequence of the reverse primers is GACTGCGTACGAATT. Only the three selective nucleotides are presented in Table 2. For AFLP analysis, the adaptor sequences were as follows: 5'-CTCGTAGACTGCGTACC-3' and 3'-CTGACGCATGGTTAA-5' (*Eco*RI adaptors), and 5'-GACGATGAGTCCTGAG-3' and 3'-TACTCAGGACTCAT-5' (*Mse*I adaptors). The *Eco*RI pre-amplification primer sequence was 5'-GACTGCGTACCAATTC-3'. The *Mse*I pre-amplification primer sequence was 5'-GATGAGTCCTGAGTAA-3'. The three selective nucleotides were presented in Table 2.

SRAP analysis was performed according to Li and Quiros (2001), with some modifications. The reaction was carried out in a 25-µL reaction volume containing 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM forward primer, 0.4 µM reverse primer, 1.5 U Taq DNA polymerase, and 30 ng genomic DNA. PCR conditions were as follows: 5 min at 94°C; 5 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 1.5 min; 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min; and a final extension of 10 min at 72°C.

AFLP analysis consisting of genomic DNA digestion with *Eco*RI and *Mse*I restriction enzymes, adaptor ligation, pre-amplification, and selective amplification using *Eco*RI plus three and *Mse*I plus three selective nucleotide primers were similar to those from Vos et al. (1995), with modifications that were described by Wang et al. (2011). The following cycling parameters were used for pre-amplification: 94°C for 2 min; 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 80 s; and a final extension of 5 min at 72°C. The PCR conditions for selective amplification were as follows: 94°C for 2 min; 14 cycles of 94°C for 30 s, 65°C for 30 s (reduced by 0.7°C/cycle), and 72°C for 80 s; 23 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 80 s; and 5 min at 72°C.

The ISSR reaction was performed according to the protocols of Zietkiewicz et al. (1994) and Wu et al. (2011). The reaction mixture was as described above for SRAP, except a single primer was used. The thermal cycling conditions were as follows: 94°C for 4 min; 38 cycles of 94°C for 30 s, 41.4°-56°C for 45 s, and 72°C for 1.5 min; and a final extension step of 72°C for 5 min.

PCR was carried out in an S1000 Thermal Cycler (BioRad Laboratories, Inc., USA). Amplification products were separated on 6% polyacrylamide gels and visualized by silver nitrate staining. To test the reproducibility of the profile, the reactions were repeated at least twice.

Data analyses

Amplified and separated DNA fragments were scored as present (1) or absent (0). Only reproducible fragments were considered, and faint or ambiguous fragments that appeared unstable on gels were ignored. Data were analyzed using NTSYS-pc Version 2.10e (Rohlf, 1998) to generate Jaccard's similarity coefficient (Jaccard, 1908). These similarity coefficients were used to produce a dendrogram using the unweighted pair-group method using arithmetic average algorithm to depict the genetic relationships.

RESULTS

Nineteen *E. ulmoides* cultivars selected from different parts of China were amplified using 10 SRAP primer combinations (Figure 1 and Table 2). All of the chosen primers amplified clear and reproducible fragments across the 19 cultivars. The 10 primers produced 240 fragments, including 214 that were polymorphic and accounted for 89.2% of total fragments. Three of the 10 primer combinations (Me4Em7, Me5Em7, and Me8Em10) exhibited 100% polymorphism, while the least polymorphism (50.0%) was shown by Me12Em5. Average numbers of loci and polymorphic loci per primer were 24.0 and 21.4, respectively.

The 10 AFLP primer combinations produced a total 192 fragments across 19 cultivars; of these, 125 were polymorphic, accounting for 65.1% of total fragments (Figure 1 and Table 2). The polymorphism rate ranged from 50.0% (E2M5) to 76.9% (E1M5). Average numbers of loci and polymorphic loci per primer were 19.2 and 12.5, respectively.

The 10 ISSR primers produced 150 fragments (Figure 1 and Table 2). Of the 150 amplification products, 132 fragments (88.0%) were polymorphic. Six of the 10 primers exhibited 100% polymorphism, while the least polymorphism (45.5%) was shown by UBC834. The average number of fragments per primer was 15.0, and the average number of polymorphic fragments was 13.2.

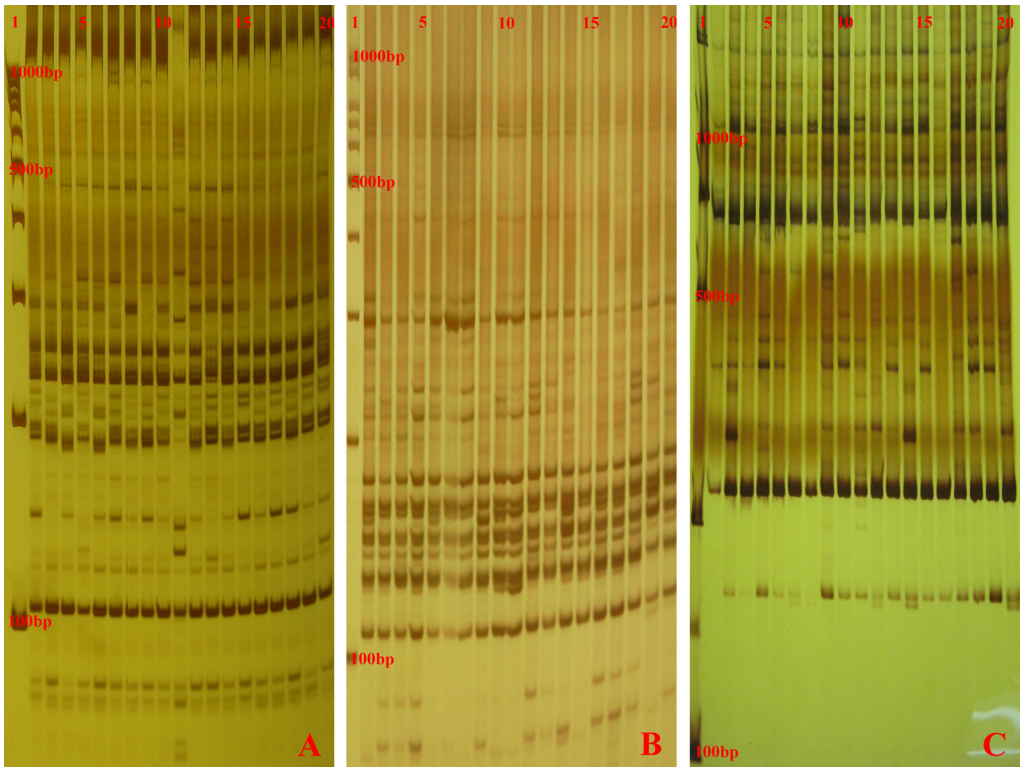

Figure 1. Amplification products of sequence-related amplified polymorphism (SRAP), amplified fragment length polymorphism (AFLP), and inter-simple sequence repeat (ISSR) primers in Chinese *Eucommia ulmoides* cultivars. **A.** SRAP primer combination Me4Em11. **B.** AFLP primer combinations E8M5. **C.** ISSR primer UBC808. *Lane 1* = molecular weight markers. *Lanes 2-20* = DNA fragments amplified from *E. ulmoides* cultivars 1-19 (see Table 1).

The genetic similarity of the *E. ulmoides* cultivars was calculated from the Jaccard's similarity coefficient considering the SRAP, AFLP, and ISSR approaches individually and together. The SRAP-based similarity coefficients ranged from 0.37 to 0.86. The AFLP-based similarity coefficients ranged from 0.40 to 0.94. The ISSR-based similarity coefficients ranged from 0.45 to 0.85. Considering the three marker systems together, the similarity coefficients varied from 0.42 to 0.81. The dendrograms based on SRAPs, AFLPs, ISSRs, and pooled data showed partially similar clustering patterns. A dendrogram based on pooled data is shown in Figure 2. Four cultivars, Qinzhong No. 3, Xiaoyeci, Qinzhong No. 1, and Qinzhong No. 2 formed independent clusters. The other 15 cultivars exhibited two clusters. The first cluster had seven cultivars including Daye, Juye, Huazhong No. 2, Daguo, Huazhong No. 5, Qinzhong No. 4, and Changbing. The second cluster was further divided in two subclusters. Huazhong No. 4 and Yanci occupied the first subcluster, while Luochao No. 3, Nanyang No. 1, Ziye, Xiaoyexiong, Longguai, and Yecongzhi occupied the second subcluster.

(SRAP), (AFLP), and markers

Table 2. Primers selected for amplification and polymorphism detection.

Primer	Sequence		Amplified fragments	Polymorphic fragments	Polymorphism rate (%)
SRAP[a]	Forward[a]	Reverse[a]			
Me1Em2	ATA	AAT	23	19	82.6
Me4Em7	ACC	CAA	45	45	100.0
Me4Em11	ACC	CTA	19	15	78.9
Me5Em7	AAG	CAA	18	18	100.0
Me8Em10	ACT	CAT	23	23	100.0
Me9Em1	AGG	AAT	26	25	96.2
Me10Em14	AAA	CTT	26	24	92.3
Me12Em5	AGA	AAC	14	7	50.0
Me12Em10	AGA	CAT	21	16	76.2
Me16Em6	AGT	GCA	25	22	88.0
		Total	240	214	89.2
AFLP[b]	*Eco*RI[b]	*Mse*I[b]			
E1M5	AAC	CTA	26	20	76.9
E1M8	AAC	CTT	28	21	75.0
E2M5	AAG	CTA	24	12	50.0
E2M6	AAG	CTC	18	10	55.6
E3M4	ACA	CAT	20	13	65.0
E4M2	ACT	CAC	11	8	72.7
E4M8	ACT	CTT	15	8	53.3
E7M4	AGC	CAT	15	11	73.3
E7M8	AGC	CTT	18	11	61.1
E8M5	AGG	CTA	17	11	64.7
		Total	192	125	65.1
ISSR[c]		T (°C)[c]			
UBC808	$C(AG)_8C$	56.0	27	23	85.2
UBC824	$(TC)_8G$	50.0	8	8	100.0
UBC834	$(AG)_8YT$	56.0	11	5	45.5
UBC835	$(AG)_8YC$	43.6	11	7	63.6
UBC848	$(CA)_8RG$	56.0	19	19	100.0
UBC860	$(TG)_8RA$	56.0	11	11	100.0
UBC866	$CT(CCT)_5C$	52.6	12	12	100.0
UBC867	$(GGC)_6$	41.4	11	7	63.6
UBC868	$(GAA)_6$	46.3	18	18	100.0
UBC873	$(GACA)_4$	50.0	22	22	100.0
		Total	150	132	88.0

[a]SRAP = sequence-related amplified polymorphism; Forward = the three selective nucleotides of SRAP forward primers; Reverse = the three selective nucleotides of SRAP reverse primers. [b]AFLP = amplified fragment length polymorphism; *Eco*RI = the three selective nucleotides of *Eco*RI primers; *Mse*I = the three selective nucleotides of *Mse*I primers. [c]ISSR = inter-simple sequence repeat; T = annealing temperature.

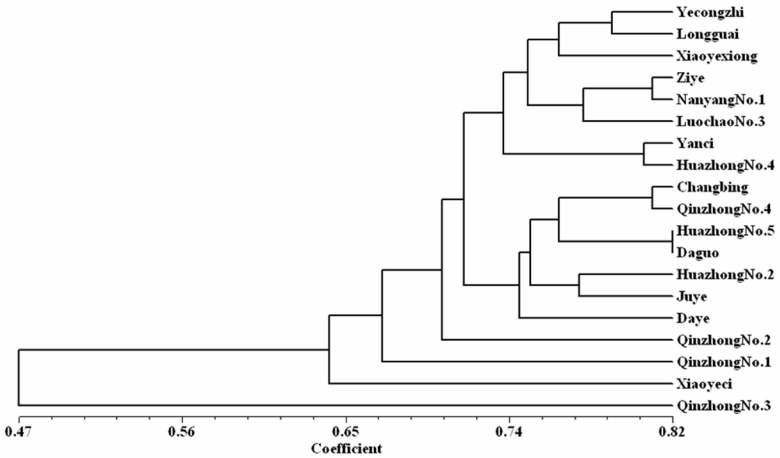

Figure 2. Dendrogram generated using UPGMA analysis showing genetic relationships among 19 *Eucommia ulmoides* cultivars based on pooled SRAP + AFLP + ISSR data.

DISCUSSION

The 19 *E. ulmoides* cultivars from different areas of China are rich in phenotypic diversity in terms of leaves, flowers, fruits, barks, branches, and content of bioactive constituents and rubber (Table 1). It was not surprising to find a high level of polymorphism among these cultivars using SRAP (89.2%), AFLP (65.1%), and ISSR (88.0%) markers. The genetic diversity within and among the wild and cultivated populations of *E. ulmoides* has been assessed using ISSR (Wu et al., 2011) and AFLP (Yao et al., 2012) markers. These analyses detected a high level of genetic diversity using ISSRs, which yielded 85.5% polymorphism, and a moderate genetic variability using AFLP markers (78.4%). The genetic variability of plants results from the interaction of mutation, selection, random genetic drift, and migration. Geographical, ecological, and reproductive isolation have a material effect on the level of genetic diversity. *Eucommia*, being relic plants, had a wide natural range covering much of the world (Wang et al., 2003). However, most members of the *Eucommia* genus died out during the Quaternary glacial period. Hence, the rich phenotypic diversity and high level of polymorphism in *E. ulmoides* cultivars might be due to a high inherent variability of the ancestral species. Moreover, the living trees of *E. ulmoides* existed in a wide range of climatic and geographic regions in China, and rich variability and high adaptability accumulated during the cultivation history of more than 2000 years (Hu, 1979). In addition, the outcrossing and long-lived woody perennial species, such as *E. ulmoides*, commonly have higher levels of genetic diversity (Hamrick and Godt, 1996).

SRAP was more efficient than AFLP and ISSR with respect to polymorphism detection, as the polymorphism level yielded by SRAP markers in this study was higher than in the other cases. In addition, the average number of polymorphic loci per primer was higher for SRAP than for AFLP and ISSR. Similarly, SRAP was reported to be highly effective in revealing polymorphism and variance among 24 germplasm accessions of citrus (Amar et al., 2011). SRAP has several advantages over other systems: simplicity, reasonable throughput rate, numerous co-dominant markers, easy isolation of DNA fragments for sequencing, and, most importantly, it targets open reading frame regions (Li and Quiros, 2001). Hence, the SRAP marker system is a simple and efficient marker system that can be adapted for a variety of purposes, including genomic and cDNA fingerprinting, map construction, gene tagging, and map-based cloning.

There were some differences among the genetic similarities and dendrograms that were generated by SRAPs, AFLPs, and ISSRs. For example, in the SRAP analysis, the highest similarity was found between Ziye and Nanyang No. 1 (0.86), while the highest similarity based on the three marker systems together was between Huazhong No. 5 and Daguo (0.82). A possible explanation for the difference was that the three marker techniques target different portions of the genome. Another explanation could be the different number of PCR products that was used for analysis (214 for SRAP analysis, 125 for AFLP analysis, and 132 for ISSR analysis). Combined data from different marker systems proved to be especially reliable and effective for estimating the level of genetic diversity and relationships among cultivars or germplasms of trees (Amar et al., 2011; Kalpana et al., 2012).

The dendrogram revealed clear genetic relationships among *E. ulmoides* cultivars (Figure 2). According to the dendrogram, Qinzhong No. 3, Xiaoyeci, Qinzhong No. 1, and Qinzhong No. 2 were distinctly grouped and showed genetic dissimilarity when compared with the rest of the cultivars. The other 15 cultivars exhibited two clusters, and there was a high similarity among *E. ulmoides* cultivars in the respective cluster. A possible explanation

for this observation might be that frequent gene flow from one cultivar to another occurred because of natural hybridization. Between the two genetically distinct clusters, there were few chances of crossing, and thus, gene exchange was limited. It is possible to gain new cultivars or to create novel germplasms of *E. ulmoides* through hybridization between the genetically distinct cultivars in this study. Furthermore, our results could be useful to identify diverse parents to generate a segregating population for map construction and quantitative trait locus analysis of important traits such as high content of bioactive constituents and rubber.

From this study, we can conclude that the combined SRAP, AFLP, and ISSR analyses were useful for evaluating the genetic diversity and relationships among *E. ulmoides* cultivars. Furthermore, we can conclude that SRAP was a powerful tool for the generation of potential polymorphic markers. Genetic diversity and relationships among cultivars of *E. ulmoides* could contribute to the selection of parents to generate appropriate populations for both genome mapping and crossbreeding purposes.

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