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Genetic Analysis of Walnut Cultivars in China Using Fluorescent Amplified Fragment Length Polymorphism

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Abstract. Informative DNA fingerprints from 50 walnut cultivars (*Juglans regia*) in China were generated using amplified fragment length polymorphism (AFLP) markers to reveal their genetic diversity and relationships. Nine primer combinations were selected from 64 *EcoR* I/*Mse* I primer combinations to amplify the accessions. An average of 132 polymorphic loci per primer set was detected from the nine primer combinations. The discrimination power of each polymorphic marker (estimated by the polymorphism information content) ranged from 0.00 to 0.37 with an average of 0.19. A moderate level of genetic diversity was observed among the 50 cultivars. Their expected heterozygosity varied from 0.38 to 0.50 (average, 0.44), and Dice's similarity coefficient ranged from 0.53 to 0.86 (average, 0.70). The cluster analysis conducted using the unweighted pair group method of arithmetic averages method showed that all of the cultivars fell into five groups at Dice's similarity coefficient of 0.68. According to the comprehensive analyses based on the specific loci, similarity coefficient, and clustering results, six cultivars (Liaoning 1, Zixin, Shanhe 4, Zha 343, Tulare, and Chandler) were considered important germplasms of walnut cultivars.

English (or persian) walnut (*Juglans regia*) is an economically important tree species throughout temperate regions of the world as a result of its nutrient-rich nut and high-quality timber. China is considered one of the most important countries for production of this species and still has wild walnut populations (Kuang and Lu, 1979; Wang et al., 2008). China contributes approximately one-fourth of the world's total walnut production (Food and Agriculture Organization of the United Nations, 2005). In the past 30 years, China has made substantial progress in walnut breeding with more than 40 cultivars developed and used for production. However, as development progressed, distinguishing walnut cultivars became problematic both for purposes of cultivar identification and for defining and protecting intellectual property given that morphological traits are greatly affected by growing season and environmental conditions. Additionally, the lack of information regarding the genetic variability and structure of the available cultivars or elite lines had the potential to limit further genetic improvement. Low genetic similarity among parents limits continued breeding success, whereas the introduction of new sources of germplasms into the breeding pool may provide the genetic variability required to enable continued progress in developing new cultivars (Birchler et al., 2003).

The AFLP technique, developed by Vos et al. (1995), is a polymerase chain reaction-based DNA diversity screening procedure. The sensitivity of the AFLP technique was increased with the introduction of fluorescent markers. It can rapidly generate hundreds of highly repeatable markers from the DNA of any organism and is superior in terms of time and cost-efficiency, repeatability, and resolution compared with many other techniques (Aggarwal et al., 2002). Therefore, AFLP markers have emerged as a major genetic marker with broad application in systematics, population genetics, DNA fingerprinting, and mapping quantitative trait loci (Drossou et al., 2004; Mullet et al., 2002; Pan et al., 2002; Vilanova et al., 2003). AFLP analysis has been used to detect DNA polymorphisms and the genetic relationships of walnut genotypes in recent years (Bayazit et al., 2007; Kafkas et al., 2005; Sutyemez, 2006). However, few applications of AFLP technology to the genetic analysis and fingerprinting of walnut cultivars have been reported.

We used the fluorescent AFLP technique to define the genetic variation and structure of 50 *J. regia* cultivars in China to identify their genetic relationships and the difference of parental genetic contribution as well as to provide molecular information for distinguishing cultivars. The results will provide insight into the genetic background of available cultivars as well as offer scientific guidance on parent selection and breeding in walnut breeding.

Materials and Methods

Plant materials. In total, 50 cultivars, of which 16 were hybrids (products of controlled pollinations) and 34 were selections (selections from open pollination), were chosen based on either extensive cultivation or predominant characteristics in growth, cropping, and stress resistance, and these cultivars were subjected to AFLP analysis. The breeding of the selected cultivars is briefly described in Table 1. Cultivars were classified according to their most commonly used names. Fresh leaves were collected from 8- to 13-year-old trees in six provinces of P.R. China [Hebei Province, Henan Province, Shanxi Province, Shandong Province, Xinjiang Province, and Yunnan Province (Table 1; Fig. 1)], then frozen quickly and stored at –80 °C until DNA extraction.

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Table 1. Accessions chosen for the amplified fragment length polymorphism analysis of walnut cultivars in P.R. China.

Cultivar	Breeding history	Location	Year
Anbian 4	Selected from eastern European walnuts in the area of Anbian, China	Hebei Province	1987
Baofeng	Selected from walnuts from Xinjiang Province (Xinjiang walnuts)	Henan Province	1979
Chandler	Pedro 53-113 × UC 56-224	Henan Province	1978
Fenghui	'Shangsong 5' × 'Akesu 9'	Hebei Province	1978
Franquette	Selected from French walnuts	Hebei Province	Unknown
Hartley	Selected from walnuts in Napa Valley, CA	Hebei Province	1915
Hexuan	Selected from walnuts in Hetian, Xinjiang Province	Xinjiang Province	2008
Jing 861	Selected from Xinjiang walnuts	Henan Province	1982
Jinlong 1	Selected from walnuts in Fenyang, Shanxi Province	Hebei Province	1978
Jinlong 2	Selected from walnuts in Fenyang, Shanxi Province	Shandong Province	1978
Liaoning 1	Selection of 'Changlidabaopi' × selection of 'Xinjiang paper-shell walnut'	Henan Province	1980
Liaoning 2	Selection of 'Changlidabaopi' × selection of 'Xinjiang paper-shell walnut'	Henan Province	1980
Liaoning 3	Selection of 'Changlidabaopi' × selection of 'Xinjiang paper-shell walnut'	Henan Province	1989
Liaoning 4	'Damahetao' × selection of 'Xinjiangpaper-shell walnut'	Henan Province	1990
Liaoning 8	Selection of 'Xinjiangbaoke 5' × selection of 'Xinjiang paper-shell walnut'	Hebei Province	1990
Lipin 2	Selected from seedling progeny of paper-shell walnuts	Hebei Province	1977
Lübo	Selected from Xinjiang walnuts	Henan Province	1976
Luguang	'Kakazi' × 'Shangsong 6'	Hebei Province	1978
Luguo 1	Selected from Xinjiang walnuts	Shandong Province	1981
Luguo 2	Selected from Xinjiang walnuts	Shandong Province	1981
Luguo 3	Selected from Xinjiang walnuts	Shandong Province	1981
Luguo 4	Selected from Xinjiang walnuts	Shandong Province	1981
Luguo 5	Selected from Xinjiang walnuts	Shandong Province	1981
Luhe 1	Selected from Xinjiang walnuts	Shandong Province	1996
Luxiang	'Shangsong 6' × 'Xinjiangzaoshufengchan'	Shandong Province	1985
Qingxiang	Selected from walnut population in Japan	Hebei Province	1948
Shangsong 6	Selected from Xinjiang walnuts	Shandong Province	1975
Shanhe 1	Selected from 'Genianhetao' in Shanxi Province	Shanxi Province	1986
Shanhe 2	Selected from Xinjiang walnuts	Shanxi Province	1986
Shanhe 3	Selected from 'Genianhetao' in Shanxi Province	Shanxi Province	1986
Shanhe 4	Selected Xinjiang walnuts	Shanxi Province	1986
Shanhe 5	Selected Xinjiang walnuts	Shanxi Province	1986
Shanhe 6	Selected Xinjiang walnuts	Shanxi Province	1986
Tai 15	'Shangsong 5' × 'Akesu 9'	Shandong Province	1978
Tulare	'Tehama' × 'Serr'	Shandong Province	1966
Vina	'Payne' × 'Franquette'	Hebei Province	1965
Wen 185	Selected from 'Kakazi'	Henan Province	1983
Xiangling	'Shangsong 5' × 'Akesu 9'	Henan Province	1978
Xifu 1	Selected from 'Genianhetao'	Shandong Province	1981
Xifu 2	Selected from 'Genianhetao'	Shandong Province	1984
Xilin 1	Selected from Xinjiang walnuts	Henan Province	1978
Xilin 2	Selected from Xinjiang walnuts	Shandong Province	1978
Xinfeng	Selected from walnuts in Hetian, Xinjiang Province	Xinjiang Province	1976
Xinjufeng	Selected from progenies of 'Hechun 6'	Shandong Province	1983
Xinzaofeng	Selected from Xinjiang walnuts	Shandong Province	1979
Yuanfeng	Selected from Xinjiang walnuts	Shandong Province	1975
Zha 343	Selected from Xinjiang walnuts	Henan Province	1989
Zhonglin 1	Jian 9-7-3 × 'Fenyangchuanzi'	Henan Province	1989
Zhonglin 5	Jian 9-11-12 × Jian 9-11-15	Henan Province	1989
Zixin	Self-crossed progeny of 'Xinjiangmumahetao'	Yunnan Province	1998

DNA isolation. DNA was extracted from the leaves previously ground in liquid nitrogen. The extraction protocol followed Doyle and Doyle (1990) with minor modifications (Wang et al., 2008). The concentration and intactness of the extracted DNA were determined using a spectrometer at 260 and 280 nm and by electrophoresis in 0.8% (w/v) agarose gels against standard solutions of lambda DNA.

Amplified fragment length polymorphism analysis. AFLP analysis was carried out according to the manual provided with the AFLP Kit (Dingguo Bio-technology Co., Beijing, China). The primer combinations were fluorescently labeled, and then fragments were detected by laser and accurately sized with an internal standard and with an automated DNA sequencer (ABI PRISM 377; Applied Biosystems, Foster City, CA). Digitally

converted raw data were saved as samples migrated past the fluorescence detector. Multilocus profiles were visualized using ABI GENESCAN software (Applied Biosystems).

A total of 64 *EcoR* I/*Mse* I primer combinations were applied to the genomic DNAs, and nine primer combinations with more polymorphisms were selected to analyze the different accessions of walnut samples (Table 2).

Statistical analysis. Only bands with strong intensity were scored. The AFLP band patterns obtained were classified as present (1) or absent (0) across all 50 walnut cultivars for each primer combination, and the values were used to a compile binary data matrix. Markers with a molecular weight lower than 70 bp were excluded from the data matrix.

The discrimination power of each AFLP marker was evaluated by polymorphism information content [PIC (Anderson et al., 1993)] as follows:

$$PIC = 1 - \sum_{i=1}^{n} P_i^2 - 2\sum_{i=1}^{n}\sum_{j=i+1}^{n} P_i P_j = 2\sum_{i=1}^{n}\sum_{j=n+1}^{n} P_i P_j \left(1 - P_i P_j\right),$$

where n is the number of alleles and P_i and P_j are the frequencies of each allele.

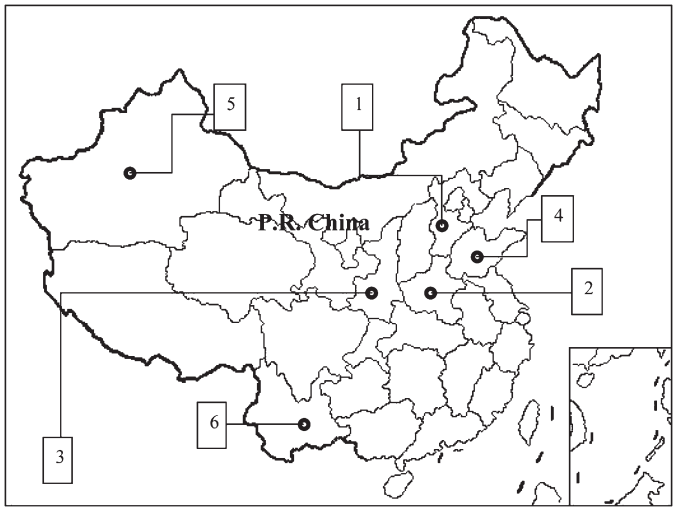

Fig. 1. Names and locations of 50 walnut cultivars sampled in P.R. China; 1 = Hebei Province, 2 = Henan Province, 3 = Shanxi Province, 4 = Shandong Province, 5 = Xinjiang Province, 6 = Yunnan Province.

Table 2. Estimates of genetic diversity among 50 walnut cultivars from P.R. China.

Primers/indices	Ne (mean ± SD)[z]	I (mean ± SD)[z]	He (mean ± SD)[z]
E-AAC/M-CAA	1.40 ± 0.33	0.39 ± 0.23	0.50 ± 0.37
E-AAC/M-CTT	1.34 ± 0.32	0.34 ± 0.23	0.39 ± 0.35
E-AAG/M-CAA	1.41 ± 0.36	0.38 ± 0.25	0.41 ± 0.32
E-AAG/M-CAG	1.36 ± 0.35	0.34 ± 0.25	0.46 ± 0.37
E-ACA/M-CAC	1.39 ± 0.33	0.37 ± 0.23	0.45 ± 0.38
E-ACT/M-CTA	1.45 ± 0.35	0.41 ± 0.23	0.38 ± 0.31
E-AGC/M-CAG	1.45 ± 0.35	0.41 ± 0.23	0.46 ± 0.34
E-AGC/M-CAT	1.41 ± 0.35	0.37 ± 0.26	0.50 ± 0.37
E-AGC/M-CTT	1.37 ± 0.32	0.37 ± 0.23	0.40 ± 0.33
Average	1.39 ± 0.03	0.37 ± 0.03	0.44 ± 0.05

[z]Ne = effective number of alleles; I = Shannon' s information index; He = expected heterozygosity.

A similarity matrix was calculated with the Dice's similarity coefficient (Dice, 1945), and cluster analysis was carried out using unweighted pair group method of arithmetic averages [UPGMA (Gil-Vega et al., 2006)] with NTSYSpc 2.11F software package (Rohlf, 1998).

Factorial correspondence analysis (FCA) was performed with GENETIX 4.05 software (Belkhir et al., 2000; Breton et al., 2008).

The percentage of polymorphic bands was calculated using POPGENE32 Version 1.32 (Yeh et al., 1997), which was also used to carry out the effective number of alleles (Ne) and Shannon's information index (I) on the premise of the Hardy-Weinberg equilibrium. The expected heterozygosity (He) index was estimated according to Nei (1987) as

$$He = 1 - \sum_{i=1}^{n} P_i^2,$$

where Pi is the frequency of the ith allele scored as 0 (band absent).

Results

Amplified fragment length polymorphisms. Nine primer combinations produced clear-cut AFLP profiles for the 50 walnut cultivars, and a total of 1263 AFLP fragments were detected. The fragment size scored in all accessions ranged from 70 to 500 bp with the majority distributed between 80 and 300 bp. A total of 1189 fragments among the total 1263 tested exhibited polymorphisms. The number of fragments per primer combination ranged from 116 (*E*-ACA/*M*-CAC) to 171 (*E*-AAG/*M*-CAA) with an average of 140, whereas polymorphic fragments varied from 110 (E-ACA/M-CAC) to 160 (*E*-AAG/*M*-CAA) with an average of 132, and the percentage of polymorphism ranged from 88.7% to 99.3% with an average of 94.1%. Eight of the nine primer combinations generated greater than 90% of the polymorphisms detected.

Genetic diversity. Three indexes (Ne, I, and He) were calculated based on the allele frequencies in nine primer combinations (Table 2). Ne varied from 1.00 to 2.00 with an average of 1.39. I changed from 0.00 to 0.69 with an average of 0.37. He of each allele ranged from 0.00 to 1.00 with an average of 0.44.

Cultivar discrimination. The frequency of polymorphic bands observed among the 50 cultivars ranged from 0.14 to 0.99 with an average of 0.67. The discrimination power of each marker was estimated by the PIC (results not shown) and ranged between 0.00 and 0.37 (the expected maximum value for a biallelic locus was 0.50) with an average of 0.19. *E*-ACT/*M*-CTA showed the highest PIC value of 0.37. A large proportion of markers had a high discrimination power above 0.30.

The discrimination power of the nine primer combinations was estimated by computing the number of possible groups into which the 50 cultivars could be separated. Examination of all loci revealed that the 50 cultivars could be distinguished by each of the nine primer combinations, and the identification percentage per primer combination was 100%.

The Dice's similarity coefficient among cultivars ranged from 0.53 to 0.86 with an average of 0.70.

Genetic structure and relationships. FCA calculated on cultivar polymorphism AFLP data revealed that the first axis (axis 1) explained 8.40% of the percentage variation when the third axis (Axis 3) explained 5.87% and it also revealed two

main groups of cultivars comprising 39 individuals, whereas the remaining 11 cultivars were dispersed (Fig. 2). Group 1 included 21 walnut cultivars collected from Shanxi Province, Shandong Province, and Xinjiang Province, whereas Group 2 comprised 18 cultivars collected from Hebei Province and Henan Province. Interestingly, 'Shanhe 2' and 'Shanhe 4' were clustered together but isolated from all others as were 'Baofeng' and 'Xinzaofeng'. 'Liaoning 1', 'Franquette', 'Zha343', 'Tai 15', and 'Zixin' were dispersed. 'Chandler' and 'Tulare' were also separated in the FCA.

A dendrogram was generated from the data with the Dice index, UPGMA, and the program NTSYSpc 2.10t (Fig. 3). No ties were encountered during the UPGMA analysis.

The dendrogram indicated that the 50 cultivars were divided into five groups (I to V) at a level of 0.68 (the mean genetic similarity coefficient investigated) (Fig. 3). Group I contained the cultivar Liaoning 1; Group III consisted of 'Zha 343'; Group V had the cultivars Tulare and Chandler; Group II contained 24 cultivars; and Group IV was comprised of 22 cultivars (Fig. 3).

Regardless of the estimator applied, 'Luguo 1' and 'Luguo 3' were the most closely related cultivars with a Dice similarity of 0.8587. 'Liaoning 1' and 'Chandler' had the lowest genetic similarity with a Dice similarity of 0.5318.

The lowest genetic distances were observed among closely related cultivars (Fig. 3). 'Liaoning 2' and 'Liaoning 8' were highly similar to the 'Xinjiang paper-shell walnut' (with a Dice similarity of 0.8145), whereas 'Zhonglin 5', 'Xiangling', and 'Luguang' shared origins with walnuts from Xinjiang Province (hereafter referred to as Xinjiang walnuts). 'Vina' was generated from 'Franquette' (with a Dice similarity of 0.8310), and 'Baofeng' and 'Zhonglin 1' were generated from Xinjiang walnuts (with a Dice similarity of 0.8090). 'Luguo 1', 'Luguo 3', and 'Luguo 4' were derived from a seedling progeny of Xinjiang walnuts in Shandong, and 'Xinjufeng', 'Luguo 2', and 'Luxiang' shared close relatedness with Xinjiang walnuts such that these six cultivars were clustered. 'Hexuan' and 'Xinfeng' were both selected from Xinjiang walnuts in Hetian County (with a Dice similarity of 0.8321), whereas 'Shanhe 1' and 'Shanhe 3' were derived from 'Genianhetao' (with a Dice similarity of 0.8118). 'Shanhe 2', 'Shanhe 4', 'Shanhe 5', and 'Shanhe 6' were selected from seedling progenies of Xinjiang walnuts in Shanxi Province.

The genetic similarity coefficient and dendrogram among 'Shangsong 6' and its progenies 'Luxiang' (with a Dice similarity of 0.8301) and 'Luguang' (with a Dice similarity of 0.6413) indicated a higher genetic similarity with the female parent. The dendrogram showed a degree of variation among full-sib families ('Liaoning 1' and 'Liaoning 3'; 'Fenghui', 'Xiangling', and 'Tai 15').

Discussion

A variety of phenotypic, biochemical, and molecular markers have previously been used for the genetic characterization of walnut genotypes, including phenotypic markers (Zeneli et al., 2005), isozymes (Fornari et al., 2001; Vyas et al., 2003), randomly

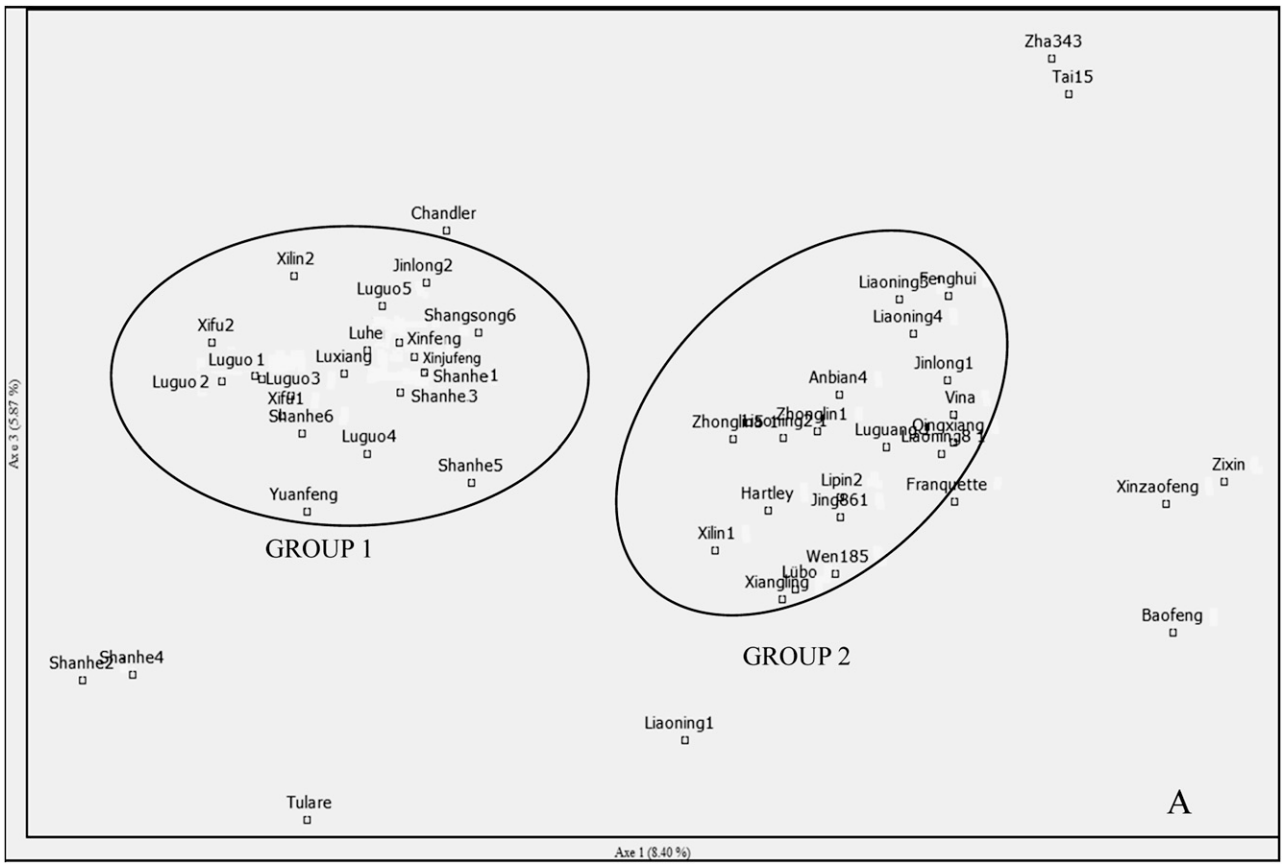

Fig. 2. Factorial correspondence analysis (FCA) based on polymorphisms at amplified fragment length polymorphism loci for 50 cultivars from P.R. China chosen to represent the molecular diversity of walnuts.

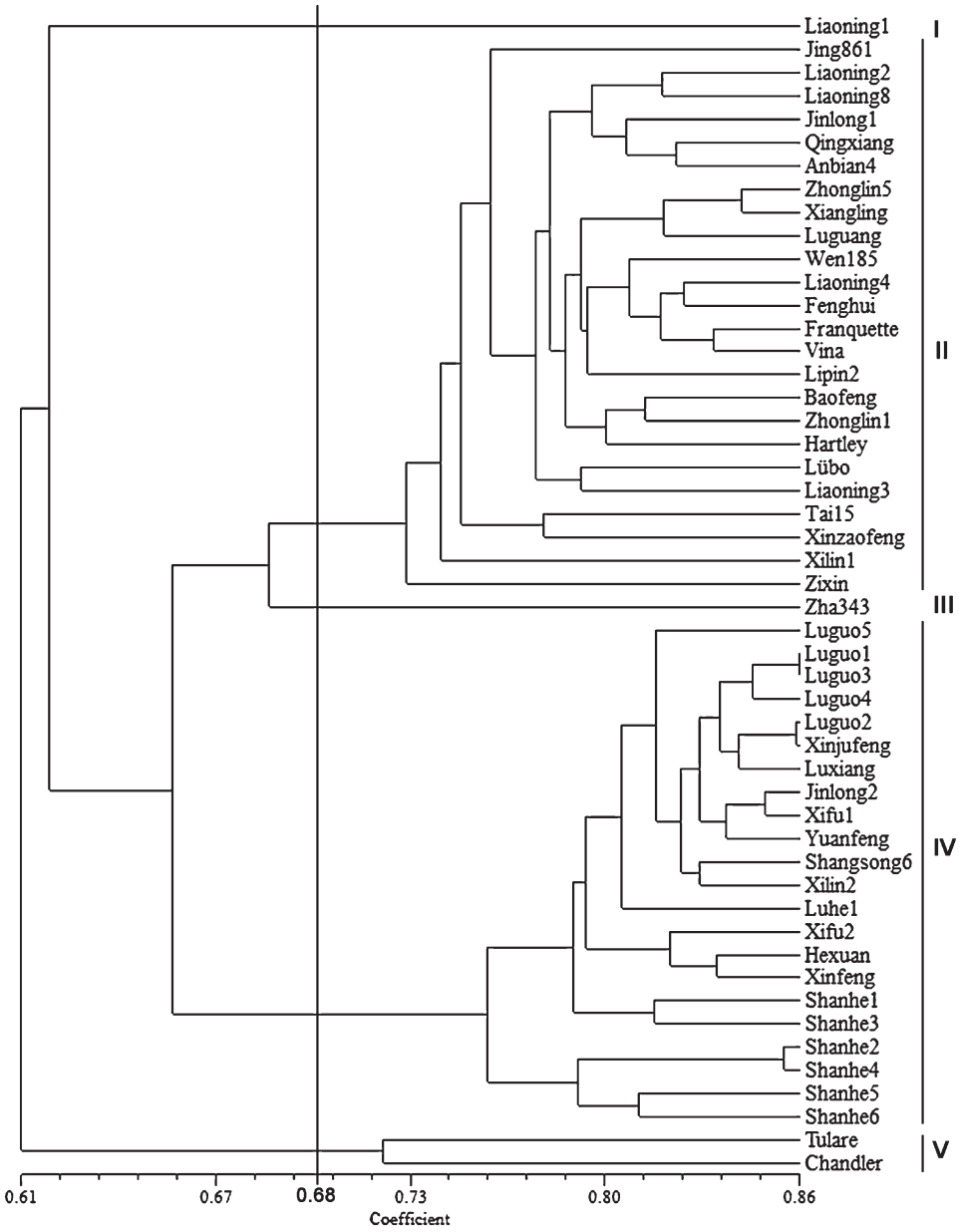

Fig. 3. Dendrogram of 50 walnut cultivars from P.R. China based on amplified fragment length polymorphism analysis conducted using nine primer combinations.

amplified polymorphic repeats (RAPD) (Nicese et al., 1998), restriction fragment length polymorphisms (RFLPs) (Fjellstrom and Parfitt, 1994), intersimple sequence repeats (ISSRs) (Potter et al., 2002), simple sequence repeats (SSRs) (Dangl et al., 2005; Foroni et al., 2005; Victory et al., 2006; Wang et al., 2008), and AFLP (Bayazit et al., 2007). Here, AFLP markers were successfully used for the identification and genetic analysis of walnut cultivars. The results were repeatable, precise, and robust, and the data revealed a high level of polymorphisms among walnut cultivars with an average polymorphism rate of 94.1% (88.7% to 99.3%). This was relatively high compared with that estimated using other marker systems such as RAPD [25% in walnut (Nicese et al., 1998)], ISSR [51.52% in Arecaceae (Roncal et al., 2007)], SSR [83.33% in *Psathyrostachys huashanica* (Wang et al., 2006a)]. It was comparable to those in *Boesenbergia* [98.7% to 100% (Techaprasan et al., 2008)], *Erythroxylum* [94.4% (Johnson et al., 2005)], *Amaranthus* [94% to 99% (Xu and Sun, 2001)], *Soldanella* [98.38% to 99.29% (Zhang et al., 2001)] but higher than those in walnut [50% (Bayazit et al., 2007), 28.4% (Sutyemez, 2006)]. The technique was demonstrated to be effective for discriminating genotypes, defining genetic structure, and assessing genetic relationships among walnut cultivars. The discrimination power of each polymorphic marker (estimated by the PIC) ranged between 0.00 and 0.38 with an average of 0.19. The most polymorphic primer combination revealed 160 polymorphic markers with an average of 132 per primer combination, suggesting that the AFLP technique was very efficient for cultivar identification. It was previously reported that AFLP technology could distinguish among cultivars in which no difference was apparent on the basis of RFLP data (Lombard et al., 2000). Here, all of the 50 cultivars could be effectively distinguished using only one primer combination.

The dendrogram assigned five groups (I to V) among the cultivars with the SC level set at 0.68 (Fig. 3). Groups I and III contained only one cultivar each, Liaoning 1 and Zha 343, respectively. Group V included two cultivars (Tulare and Chandler), and Groups II and IV comprised 24 and 22 cultivars, respectively. Cultivars in Group IV showed a common original background; they were developed from hybrids or selections of Xinjiang walnuts with the exception of 'Jinlong 2', which was selected from progenies of 'Fenyanghetao', and 'Xifu 1', 'Xifu 2', 'Shanhe 1', and 'Shanhe 3', which were selected from progenies of 'Genianhetao'. Additionally, all of the Group IV cultivars except 'Jinlong 2' were precocious types. Group II showed more diversity in terms of cultivar origin, including both hybrids and natural selections, both precocious and non-precocious maturing types and both Chinese local cultivars and introduced non-Chinese cultivars. The FCA showed a similar clustering result to the dendrogram with two main groups. Specifically, cultivars collected from Shandong Province, Shanxi Province, and Xinjiang Province were clustered [Group 1 (Fig. 2)] as were those from Henan Province and Hebei Province [Group 2 (Fig. 2)]. However, several cultivars from these six provinces were also dispersed. This could reflect their complex selection history.

The results of this study suggest moderate genetic diversity among walnut cultivars in China compared with other tree species

(Wang et al., 2008) with their D varying between 0.64 and 0.83. Among the 50 cultivars examined, some important germplasms were identified according to their similarity coefficients or their specific loci. 'Liaoning 1', 'Zixin', 'Shanhe 4', 'Zha 343', 'Tulare', and 'Chandler' varied substantially from the other cultivars with similarity coefficients less than 0.68. Furthermore, the specific loci on which only one single cultivar generated a band among 'Liaoning 1', 'Zixin', 'Tulare', and 'Chandler' ranged from 10 to 28. 'Zha 343' was a selection of Xinjiang walnuts, whereas 'Zixin' was derived from the self-crossed progeny of 'Xingjiang Muma' in Yunan Province. 'Chandler' and 'Tulare' are introduced elite cultivars from the United States. 'Liaoning 1' has the same parent as 'Liaoning 2' and 'Liaoning 3', but they were grouped apart, which remains unexplained but may suggest that relatively dramatic genetic events took place during the breeding process of 'Liaoning 1' compared with 'Liaoning 2' and 'Liaoning 3'.

Notably, cultivars with the same genetic origin were not assigned to the same groups. For example, 'Fenghui', 'Xiangling', and 'Tai 15' were hybrids that shared the same parents as 'Shangsong 5' and 'Akesu 9'; however, these cultivars did not cluster together and were instead dispersed among several subgroups. These results may be indicated that dominant AFLP markers cannot make pure clusters or that the level of diversity selected for separating groups fell within the range observed within a segregating family generated by hybridizing two highly heterozygous parents. Similar results were obtained for *Ginkgo biloba* (Wang et al., 2006b).

'Luguo 1', 'Luguo 3', 'Luguo 2', and 'Xinjufeng' were identified as the most closely related cultivars. These were selected from Xinjiang walnuts, although they differ morphologically (Xi and Zhang, 1996; Zhang et al., 2008), suggesting that they exhibit relatively less genetic variation or that they might be derived from a mutant or that they may be segregating for the morphological trait or traits.

The AFLP profiles and the clusters established here can be used for germplasm identification. Additional work will be required to determine if the fingerprint, genetic diversity, and similarity information can provide molecular solutions for parent selection in cross-breeding and effective marker-assisted genetic improvement.

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