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Genetic Diversity of Walnut Revealed by AFLP and RAPD Markers

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Abstract

AFLP and RAPD methods were used to investigate the genetic diversity of walnuts in western Sichuan plateau and Qinba mountainous regions. 35 samples were collected from 8 different regions, and 32 RAPD primers and 28 AFLP primer combinations were identified with polymorphism bands among the entire. 324 and 2155 fragments were respectively produced by RAPD and AFLP makers, and 86.1 % of RAPD bands and 57.2% of AFLP bands showed polymorphic with the size of 180~2000 bp and 50~1800 bp, respectively. The average amplified were 10.1 fragments per primer by RAPD and 76.9 fragments per pair primer by AFLP. The more polymorphic for genetic resource in Western Sichuan Plateau was observed by both RAPD and AFLP. The high number of alleles and the high expected genetic diversity detected with RAPD and AFLP markers indicate that western China has an important genetic diversity pool and abundant genetic variance of walnuts.

Keywords: *Juglans regia* L, AFLP, RAPD, genetic diversity

1. Introduction

Walnut (*Juglans regia* L), rich in nutritious substances and various microelement, is one of important economic species in the world (Martínez et al., 2010). Because of its breeding characteristics, walnut has formed abundant genetic diversities through a long term evolution under complicated environment (Wu et al., 2000a; Yang, 2005). However, little has been known of the evolution connections among walnut seeds and the phylogenetic relationships among the varieties. The early research methods were main morphological and agronomical traits, biochemical markers such as cell karyotype analysis and isoenzymes (Fornari et al., 2001; Malvolti et al., 2001), these methods are sensitive to environmental factors and the number of markers is limited, thus the research of walnut diversity has limited.

The information on structure, organization, and evolution of genomes has been greatly improved by the molecular marker techniques such as RFLP, ISSR, RAPD, and AFLP (Cervera et al., 2000; Niu et al., 2007; Li et al., 2011). In comparison with other marker types, these DNA-based markers techniques can detect the genetic diversity of specie in all tissues at all stages of development without affecting by environmental condition (Di et al., 2006). Moreover, the genetic diversity within or among species can also been analysed following these molecular marker techniques (Cervera et al., 2000; Qi et al., 2011). As a result of it, the development of molecular marker technique distinctly increases the study of plant genetic diversity (Soriano et al., 2005).

China is regard as a great center for origin and genetic variation of walnut (Jia & Xu, 2006). The aim of paper was to perform variety identification of the walnuts by RAPD and AFLP makers in different environment in western China. Both of them provided a reliable and facility way to identify and characterize walnut varieties cultivated in China and to determine the genetic relationship among the populations for using, exploring and protecting the germ plasma resources of walnut in China.

2. Materials and Methods

2.1 Plant Material

Thirty-five superior walnuts were collected at the detachable time in the two different climate regions, including BaTang, XiangCheng, JiuLong, and DeRong County in Western Sichuan plateau as well as NanJiang, TongJiang, and WanYuan County in Qinba mountainous regions (Table 1). All the seeds rise in the conservatory of Sichuan

Agricultural University.

Table 1. The walnuts accessions evaluated in this study and their origin

Number	Locality	Number	Locality	Number	Locality
BT-1	Batang	JL-3	Jiulong	NJ-5	Nanjiang
BT-2	Batang	JL-4	Jiulong	TJ-1	Tongjiang
BT-3	Batang	JL-5	Jiulong	TJ-2	Tongjiang
BT-4	Batang	DR-1	Derong	TJ-3	Tongjiang
BT-5	Batang	DR-2	Derong	TJ-4	Tongjiang
XC-1	Xiangcheng	DR-3	Derong	TJ-5	Tongjiang
XC-2	Xiangcheng	DR-4	Derong	WY-1	Wanyuan
XC-3	Xiangcheng	DR-5	Derong	WY-2	Wanyuan
XC-4	Xiangcheng	NJ-1	Nanjiang	WY-3	Wanyuan
XC-5	Xiangcheng	NJ-2	Nanjiang	WY-4	Wanyuan
JL-1	Jiulong	NJ-3	Nanjiang	WY-5	Wanyuan
JL-2	Jiulong	NJ-4	Nanjiang		

2.2 DNA Extraction

DNA was extracted from fresh leaves of each accession using the method of Chen & Wang (2004) and Zhang *et al* (2003). DNA concentration and quality were detected by Ultrospec 2100 ultraviolet scanner. The extracted DNA was stored at -20℃.

2.3 RAPD Analysis

The total volume of PCR amplification reaction system was 20 μL, which contained 40 ηg genomic DNA, 2.0 mM $MgCl_2$, 2 μL 10 × PCR Buffer, 25 μM dNTP, 1.0 U *Taq* DNA polymerase, and 0.4 μM random primer. PCR amplification were performed in programmed 5 min at 94 ºC followed by 40 cycles of 1 min at 94 ºC, 1 min at 36 ºC, 1 min 30 s at 72 ºC, and 10 min at 72 ºC. The amplified products were separated by 1.5 % agarose gel electrophoresis, and stained by EB, and taken pictures (Di et al., 2006).

2.4 AFLP Analysis

Genomic DNA was restricted with two restriction endonucleases: EcoRI and MseI, and double-stranded adaptors were ligated to the ends of DNA fragments, generating template DNA for subsequent PCR amplifications (preselectice followed by selective). Approximately 200 ng of DNA was digested with EcoRI and MseI enzymes combinations (Zhao et al., 2006). The AFLP analysis followed the method describing by Vos et al. (1995) with some modifications. Preamplification was done on PTC-100 thermal cycle using 25 cycles of 30 s at 94 ℃ followed 1 min at 56 ℃, 1 min at 72 ℃, and 10 min at 72 ℃. The selective amplification were composed of 13 cycles of 30 s at 94 ℃, 30 s at 65℃, and l min at 72℃, and the other amplification were 13 cycles of 30 s at 94 ℃, 30 s at 56 ℃, and l min at 72 ℃. PCR products were separated on 5 % w/v denaturing polyacrylamide gels and were visualized after silver staining (Zhao et al., 2006; Lu et al., 2001).

2.5 Statistical Analyses

All distinctively and unambiguously polymorphic bands were scored (1 for present and 0 for absent), and then the original data were input to computer to get the gene diversity and Genetic Distance (*GD*). The similarity coefficient was calculated to get the similarity coefficient matrix, and the cluster analysis was carried out with the sequential agglomerative hierarchical nested cluster analysis program (SAHN) and un-weighted pair group method analysis (UPGMA) in NTSYSpc 2.10e software. The cluster result of the AFLP and RAPD primer combined was used to perform Mantel test to be compared with the cluster result of AFLP or RAPD primer individually.

3. Results

3.1 RAPD and AFLP Analysis

32 pairs of RAPD primer which amplified clear bands and had abundant polymorphism were selected from 330 pairs of primer. The selected primers were used to analyze 35 walnuts and amplified 324 bands in total. The amplified bands ranged from 180 to 2000 bp. In these bands, there were 279 polymorphic bands and the

percentage of polymorphic loci arrived at 86.1 %.On an average, 10.1 bands were amplified per primer. Figure 1 shows an example of RAPD banding profile with primer O-6.

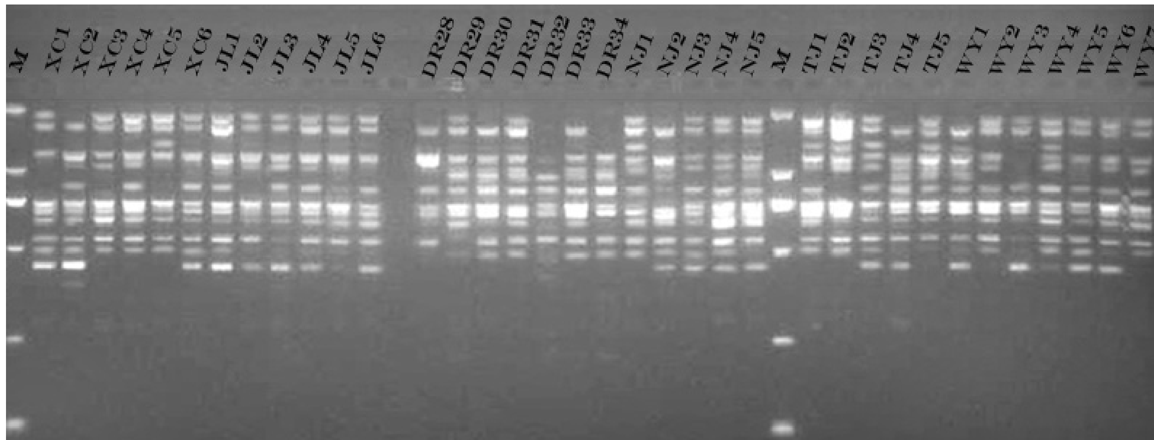

Figure 1. RAPD patterns amplified with the primer O-6

48 AFLP primer combinations (Eco Ⅰ +3 and Mse Ⅰ +3 primers) were used to detect the polymorphism among walnuts samples, and 28 primer combinations were polymorphic. 2155 polymorphic AFLP loci were to screen the same 35 genotypes of walnuts, and the polymorphic bands ranged from 50 to 1800 bp. The polymorphism rate was about 57.2 %, and the bands amplified by a pair of primer combination were 76.9 in average. Genetic polymorphism was rich among the 35 domestic walnut genotypes by AFLP. An example of banding profiles with E-AAG/M-CCC primer combination is given in Figure 2.

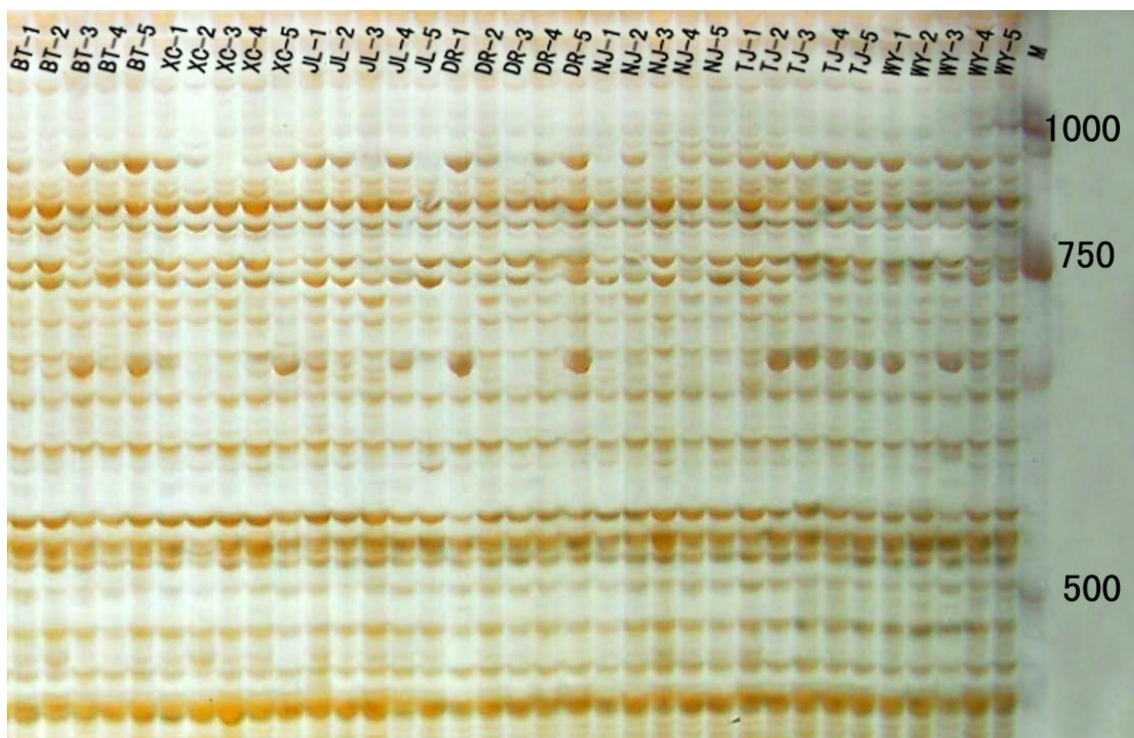

Figure 2. AFLP fingerprint of 35 walnuts genotypes using primer comparison of E-AAG/M-CCC.

The comparison of RAPD and AFLP methods revealed intensity differences in level of polymorphism rate and measuring efficiency (Table 2). And two independent species genetic similarity matrices were produced for the RAPD and AFLP data to calculate genetic distance. The RAPD *GD* of Western Sichuan plateau and Qinba mountainous regions is 0.289 and 0.175, respectively. And for the AFLP, it is 0.266 and 0.206, respectively. The *GD* of all walnut samples are similar (Table 2).

Table 2. Comparison of bands generated by RAPD and AFLP markers among 35 walnuts

Parameter	RAPD maker	AFLP maker
Number of assay units	32	28
Number of polymorphic bands	279	1233
Number of loci	324	2155
Polymorphic rate	86.1%	57.2%
Genetic Distance (*GD*)	0.273	0.271

3.2 Cluster Analysis

A dendrogram was established for the 35 walnuts genotypes with UPGMA cluster analysis based on the *GD*s from either RAPD data or AFLP data presented in Figure 3 and Figure 4. Both have a high co-phenetic coefficient and therefore show a good fit with *GD* values.

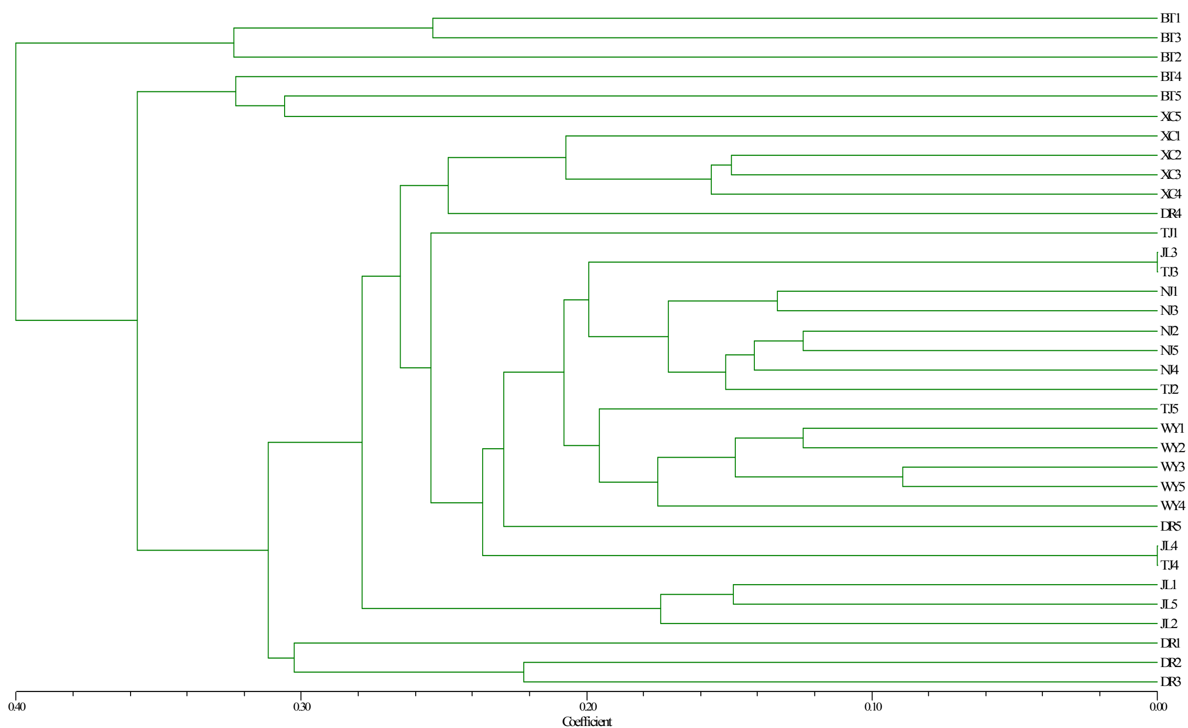

Figure 3. Dendrogram of the 35 walnut cultivars included in this study generated by un-weighted pair group method analysis (UPGMA) cluster analysis from the similarity matrix obtained using RAPD genetic distance

Clustering based on RAPD data divided the 35 walnuts genotypes into three clusters at *GD*=0.34 level (Figure 3), designated Ⅲ. Cluster Ⅰ included three genotypes: BT-1, BT-3, BT-2; cluster Ⅱ was composed of three genotypes: BT-4, BT-5, XC-5; and of the others were grouped in cluster Ⅲ.

The dendrogram derived from AFLP data also clustered samples into four groups using 28 combination primers, which was in agreement with the results based on RAPDs except for minor difference (Figure 4). Figure 3 and Figure 4 reveals the relationships among the 35 genotypes of walnuts. The lowest *GD* (0.06 or 0.09) was found in the different genotype, such as 0.06 (TJ-3 VS JL-3) based on RAPDs and 0.09 (JL-1 VS JL-2) based on AFLPs. The greatest *GD*, also in the different genotype, 0.53 (BT-2 VS XC-5) based on RAPDs and 0.53 (BT-3 VS DR-2) based on AFLPs. It is possible that the different between clusters was resulted from different molecular markers. The cophenetic correlation analysis on cluster results from AFLP or RAPD data indicated the correlation of co-phenotype was remarkable (P<0.001), the *Rcs* of AFLP was higher than that RAPD indicating the results of AFLP were more reliable than that of RAPD. Results revealed there was abundant genetic variance among walnuts varieties in China.

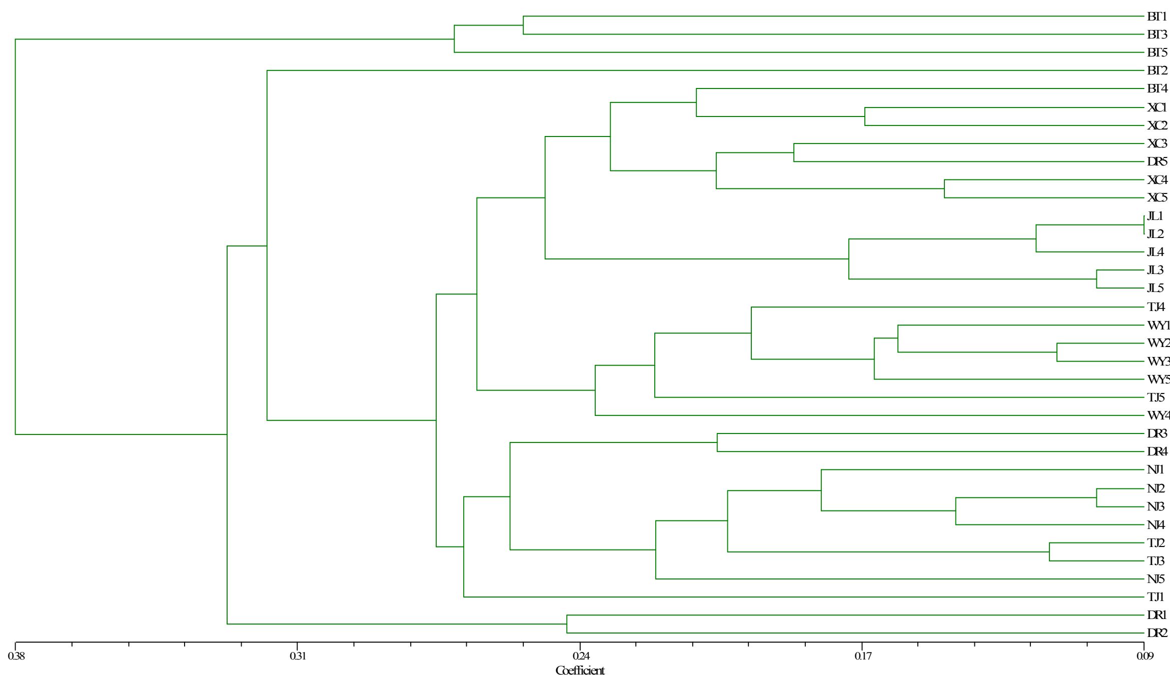

Figure 4. Dendrogram of the 35 walnut cultivars included in this study generated by un-weighted pair group method analysis (UPGMA) cluster analysis from the similarity matrix obtained using AFLP genetic distance

4. Discussion and Conclusion

Although there are abundant walnut germplasm resources in China, very little progress has been made in the AFLP research, especially on the genetic background information. There is no comparative study on RAPD and AFLP for walnut at the same times, only several papers on RAPD or AFLP analysis documented up to now (Cervera et al., 2000; Wu et al., 2000b; Niu et al., 2007). In the study, in comparison with AFPL markers, RAPD markers detected higher genetic loci per primer and level of polymorphism. However, AFLP revealed more polymorphic bands in a single lane than that in RAPD. Moreover, false positive products can were easily recognizable in AFLP, and subsequence gel had high resolution, making a more precise identification of similar and dissimilar AFLP allele. This indicated that AFLP marker provided a more detailed coverage throughout the genome than RAPD for walnut.

In this study, the researchers detected the genetic divergence among 35 varieties of walnuts in West of China by RAPD and AFLP markers. The degree of polymorphism (86.1%) revealed by RAPD was higher than previous studies on walnuts (Wu et al., 2000a). The percentage of polymorphic AFLP fragments in the study (57.2%) was also higher than the results reported by Kafkas et al (2005). Wu et al (2000b) studied that genotypes from different cluster are genetically more dissimilar than those originating from the same clusters and it is same to this study, which indicated the genetic diversities of two different regions were not in agreement with the climate. The walnuts in Qinba mountainous regions, there are similar climate and frequent genetic communication. But the walnuts in Plateau of western Sichuan plateau was opposition, geographic separation, inconvenience in traffic, lack of genetic communication, and the local diversity resources were preserved. The polymorphism of walnuts in Qinba mountainous regions was lower than that of walnuts in Western Sichuan Plateau.

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