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Full Length Research Paper

Assessment of genetic diversity for some Iraqi date palms (*Phoenix dactylifera* L.) using amplified fragment length polymorphisms (AFLP) markers

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Amplified fragment length polymorphisms (AFLP) were used to evaluate the genetic diversity between 18 date palm (*Phoenix dactylifera* L.) varieties (11 females and 7 males) collected from the center of Iraq. Six primer pairs were applied to detect polymorphism between varieties. A total of 83 polymorphic AFLP fragments were detected with an average of 13.8 polymorphic fragments/primer combination. Genetic distance was estimated using Jaccard's genetic similarity index and was ranged from 0.07 to 0.75. Unweighted pair group method with arithmetic mean UPGMA ordered date palm varieties into two main clusters independently of their origin and sex. The first cluster consisted of three sub-clusters. The first one consisted of five female varieties and one male, while the second sub-clusters consisted of five male varieties. The third one consisted of five varieties; four were females and one male. The second main cluster consisted of the remaining two female varieties. Moreover, all primer combinations contributed to the discrimination of date palm varieties, suggesting the efficiency of AFLP method in assessing genetic diversity in date palm. A large range of genetic diversity characterized Iraqi date palm germplasm.

Key words: Genetic diversity, amplified fragment length polymorphisms (AFLP) polymorphisms, molecular characterization, *Phoenix dactylifera* L.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) ($2n = 2x = 36$) is of a great socio-economic importance in the Arabian region. It is believed to have originated in Mesopotamia (Wrigley, 1995). The numbers of known date palm cultivars distributed all over the world are approximately 5000, out of them 650 are found in Iraq (Ibrahim, 2008). During the Gulf and Iran-Iraq wars, many palm trees were destroyed and more died when the southern marshes were drained. Genetic diversity of this crop was therefore, negatively affected by these ecological stresses. The use of suitable genetic markers will allow researchers to estimate genetic diversity which will ultimately aid in the genetic conservation of date palm.

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The progress of any genetic preservation is dependent on understanding the amount and distribution of the genetic variation present in the genetic pool (Jubrael et al., 2005). Morphological traits were used to characterize the genetic variation in date palm varieties (Bashah, 1996; Askari et al., 2003). These traits are mainly related to the fruit, which are complex and are greatly influenced by the environment (Sedra et al., 1993; Askari et al., 2003). Biochemical studies, including isozyme and peroxidases activity analyses have been used to characterize date palms in Morocco and Tunisia (Bendiab et al., 1998; Salem et al., 2001; Azoqour et al., 2002). However, isozymes appear to be of a limited use due to low levels of polymorphisms (Al-Jibouri and Adham, 1990). Due to developments in the field of molecular genetics, various techniques have been emerged to analyze genetic variation. DNA fingerprinting techniques such as restriction fragment length polymorphism

Table 1. Name, gender and origin of the 18 date palm varieties of Iraq.

Variety	Gender	Origin
Barhi	Female	Basrah
Qul Husaini	Female	Diyala
Deari	Female	Basrah
Helawi	Female	Basrah
Shwethi Ahmer	Female	Basrah
Baw Adem	Female	Diyala
Leelwi	Female	Basrah
Buliani	Female	Baghdad
Qitaz	Female	Diyala
Um Al-Blaliz	Female	Karbala
Meer Haj	Female	Diyala
Risasy	Male	Baghdad
Ghanami Akhder	Male	Basrah
Ghnami Ahmer	Male	Basrah
Khekri	Male	Basrah
Smeasmi	Male	Baghdad
Ghulami	Male	Diyala
Greatli	Male	Baghdad

(RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple (short) sequence repeat (SSR) or microsatellites, representational difference analysis (RDA) and microchip technologies were widely used. Al-Khalifah and Askari (2003) analyzed the genetic diversity among 13 different cultivars of date palm using random amplified polymorphic DNA (RAPD) markers. Similar reports about date palm characterization are also known from North Africa (Sedra et al., 1998). AFLP method (Vos et al., 1995) has been used also for genetic fingerprinting and genetic mapping. AFLP fingerprinting was used to characterize 18 varieties of date palm from Iraq (Jubrael et al., 2005), 47 date accessions collected from eight locations in Egypt (El-Assar et al., 2005) and to evaluate the genetic diversity in 40 ecotypes of date palm collected from oases in Tunisia (Rhouma et al., 2007).

The aim of this study was the use of AFLP markers to identify the genotypes and genetic characterization of 18 date palm varieties of Iraq.

MATERIALS AND METHODS

Plant materials

A total of 18 well-defined reference Iraqi date palm varieties were collected from two date palm stations belonging to the Ministry of Agriculture. Nine female varieties were collected from Al-Mahaweel date palm station at Hilla Governorate south of Baghdad. The remaining nine varieties (2 females and 7 males) were collected from Al-Zaafarania Station in Baghdad. These varieties originated from four different regions of Iraq; Baghdad, Diyala, Karbala and Basrah (Table 1). AFLP analysis was carried out in the biotechnology laboratories of the international center for agricultural researches in the dry areas (ICARDA), Aleppo, Syria.

DNA extraction and AFLP analysis

Total DNA was extracted from young and healthy leaves according to Benito et al. (1993) with some modifications. AFLP analysis was carried out by following the method of Vos et al. (1995), with few modifications. 250 ng of DNA from each sample was digested for 3 h at 37°C with 10 U each of two restriction enzymes, *Tru* 9I (recognition site 5' T↓TAA 3') and *Pst*I (recognition site: 5' CTGCA↓G 3'), in 20 µl final volume of the reaction mixture containing 1x one-phor-all buffer (Pharmacia Biotech, Uppsala, Sweden). After digestion, specific adapters were ligated with the DNA fragments by adding 3 µl of a solution containing 50 pmol of *Tru* 9I-adaptor and 5 pmol of *Pst*I-adaptor, 0.3 U of T_4 DNA-ligase, and 10 mM rATP in 1x one-for-all buffer. The incubation was continued for 3 h at 37°C. After ligation, the reaction mixture was diluted to 1:4 using sterile distilled water. Pre-selective amplification was performed in a reaction volume of 20 µl containing 50 ng of each of the two oligonucleotide primers (*P00* and *M00*) corresponding to the *Tru* 9I and *Pst*I adaptors, 2 µl of ligated-DNA, 1 U *Taq* polymerase and 1x PCR buffer (Roche, Mannheim, Germany 1999). Amplification was performed in a thermocycler (PE Applied Biosystems PCR 9600, Applied Biosystems, Foster City, CA, USA) programmed for 30 cycles, each cycle comprising 30 s at 94°C, 30 s at 60°C and 1 min at 72°C. The preamplified product was diluted to 1:4 then 2 µl was used as a template for selective amplification. Selective amplification was conducted using *Tru* 9I and *Pst*I primer combination listed in Table 2. Amplification was performed in a thermocycler (PE Applied Biosystems PCR 9600) programmed for 36 cycles with the following cycle profile: a 30 s DNA denaturation step at 94°C, a 30 s annealing step and a 1 min extension step at 72°C. The annealing temperature was varied: in the first cycle it was 65°C; in each subsequent cycle for the next 12 cycles it was reduced by 0.7°C (touchdown PCR) and for the

Table 2. Number of fragments amplified, polymorphic bands, primer efficiency and discrimination power of the six primer combinations used for AFLP analysis.

Primer combination	Total fragment (number)	% Primer efficiency	Polymorphic fragment (number)	% Polymorphsim	%Discrimination power
P11-aa/M88-tgc	63	25	12	19	14
P104-aagc/M95-aaaa	33	13	7	21	8
P74-ggt/M95-aaaa	46	18	26	57	31
P11-aa/M95-aaaa	31	12	5	16	6
P293-taca/M62-ctt	45	18	21	47	25
P101-aacg/M95-aaaa	34	13	12	35	14
Total	252		83(33%)		

Table 3. Major allele frequency, gene diversity and PIC estimated by AFLP markers in the 18 date palm varieties of Iraq.

Primer combination	Major allele frequency	Range of gene diversity	Average of gene Diversity	Range of PIC	Average of PIC
P11-aa/M88-tgc	0.75	0.10-0.48	0.29	0.10-0.36	0.23
P104-aagc/M95-aaaa	0.78	0.10-0.48	0.29	0.10-0.36	0.23
P74-ggt/M95-aaaa	0.72	0.10-0.50	0.30	0.10-0.38	0.24
P11-aa/M95-aaaa	0.75	0.10-0.49	0.30	0.10-0.37	0.24
P293-taca/M62-ctt	0.72	0.10-0.50	0.30	0.10-0.38	0.24
P101-aacg/M95-aaaa	0.70	0.20-0.50	0.35	0.18-0.38	0.28
Mean			0.31		0.25

remaining 23 cycles, it was 56°C. The amplified products were separated on 6% denaturing polyacrylamide gels. DNA fragments were visualized by silver staining using silver staining kit (Promega, 1993) as described by the supplier. Silver-stained gels were scanned to capture digital images for the gels after air drying.

Data analysis

Total bands numbers were scored visually. Polymorphic bands were also scored. Polymorphism percentage was calculated by dividing number of polymorphic bands amplified by the total number of bands amplified by the same primer combination. Discrimination power for each primer combination was calculated by dividing the number of polymorphic bands amplified by primer combination by the total number of polymorphic bands obtained. AFLP bands were visually scored as present (1) or absent (0) to create the binary data set. Major allele frequency, gene diversity and polymorphism information content (PIC) and genetic distance between the 18 varieties were estimated using software PowerMarker V3.25 (Liu and Muse, 2005). For the genetic similarity analysis, Jaccard's coefficient of similarity (Jaccard, 1908) was used. A dendrogram was generated by cluster analysis using the unweighted pair group method of the arithmetic averages (UPGMA). Principal coordinated analysis (PCA) was also carried out to show multiple dimension of the distribution of the accessions in a scatter-pot by PAST software version 1.62 (Hammer et al., 2001)

RESULTS

A total of 252 easily scorable bands were generated from six selective AFLP primer combinations (Table 2). The number of amplified fragments per variety varied from 31 to 63 with an average of 42 fragments per primer combination. Among the 252 fragments scored across all the varieties, 83 bands (33%) were polymorphic for at least one of the varieties. The primer combination P74/M95 amplified 26 polymorphic bands (57% polymorphism) showing the highest discrimination power (31%) and the primer combination PI1/M95 amplified only five (16% polymorphism) with only 6% discrimination power (Table 2). Major allele frequencies of the selective primer combinations are shown in Table 3. The primer combination P104/M95 had 0.78 allele frequency, while it was 0.70 in P101/M95. The average Nei's gene diversity (Nei, 1973) detected by different primer combinations also varied (Table 3). The range of genetic diversity value was ranged from 0.29 (P11/M88 and P104/M95) to 0.35 (P101/M95) with a total average of 0.31. Polymorphism information content (PIC) also ranged from 0.23 (P11/M88 and P104/M95) to 0.28 (P101/M95) with total average of 0.25 PIC. The pair-wise Jaccard's genetic similarity index (Jaccard, 1908) was calculated for the 83 polymorphic fragments of the 18 varieties (Table 4). It varied among varieties between 0.075 to 0.755. The variety 'Ghulami' was highly divergent from 'Deari' and closely related to 'Ghnami Ahmer' which was very closely related to 'Ghnami Akhder'. UPGMA ordered the date palm varieties into two main clusters irrespective of their origin and gender at similarity levels of 0.42 (Figure 1). The first cluster consisted of three sub-clusters at

Table 4. Genetic distance's values among the 18 date palm varieties of Iraq as revealed by AFLP analysis.

	Ghnami Ahmer	Ghnami Akhder	UmAl-Blaliz	Baw Adem	Buliani	Barhi	Deari	Ghulami	Helawi	Qul Husaini	Khekri	Qitaz	Greatli	Leelwi	Meer Haj	Risasy	Shwethi Ahmer	Smeasmi
Ghnami Ahmer	0.000																	
Ghnami Akhder	0.075	0.000																
Um Al-Blaliz	0.324	0.393	0.000															
Baw Adem	0.548	0.635	0.487	0.000														
Buliani	0.412	0.487	0.324	0.324	0.000													
Barhi	0.393	0.507	0.487	0.569	0.468	0.000												
Deari	0.590	0.681	0.527	0.612	0.635	0.376	0.000											
Ghulami	0.260	0.292	0.412	0.487	0.358	0.569	0.755	0.000										
Helawi	0.376	0.449	0.393	0.507	0.308	0.507	0.468	0.358	0.000									
Qul Husaini	0.376	0.487	0.430	0.507	0.412	0.507	0.507	0.393	0.341	0.000								
Khekri	0.156	0.156	0.358	0.468	0.376	0.468	0.635	0.260	0.412	0.412	0.000							
Qitaz	0.468	0.507	0.487	0.341	0.260	0.449	0.658	0.449	0.358	0.393	0.507	0.000						
Greatli	0.308	0.341	0.358	0.507	0.412	0.468	0.548	0.292	0.308	0.412	0.341	0.430	0.000					
Leelwi	0.412	0.487	0.393	0.430	0.341	0.468	0.590	0.430	0.341	0.487	0.412	0.393	0.412	0.000				
Meer Haj	0.244	0.276	0.358	0.681	0.376	0.393	0.590	0.358	0.276	0.341	0.376	0.430	0.276	0.449	0.000			
Risasy	0.156	0.156	0.358	0.507	0.449	0.430	0.635	0.292	0.412	0.487	0.244	0.468	0.308	0.449	0.276	0.000		
Shwethi Ahmer	0.393	0.468	0.376	0.612	0.324	0.412	0.569	0.412	0.292	0.430	0.468	0.449	0.292	0.324	0.292	0.468	0.000	
Smeasmi	0.358	0.358	0.376	0.487	0.358	0.487	0.612	0.341	0.358	0.590	0.324	0.412	0.358	0.292	0.393	0.393	0.376	0.000

similarity levels of 0.50. The first one consisted of five female varieties (Helawi, Meer Haj, Shwethi Ahmer, Qul Husaini, Um Al-Blaliz) and one male 'Greatli'. The second sub-clusters consisted of five male varieties (Risasy, Ghnami Ahmer, Ghanami Akhder, Khekri and Ghulami). The third consisted of five varieties, four were females: Baw Adem, Buliani, Qitaz, Leelwi and one male 'Smeasmi'. The second main cluster consisted of the remaining two female varieties Deari and Barhi. In order to confirm these results, the data were analyzed by multivariate PCA. The scatter diagram of the first two (PC1 and PC2) based on 252 AFLP bands is shown in Figure 2. The results exhibited the same four clusters of varieties as in the dendrogram.

DISCUSSION

Genetic identification of date palm cultivars using morphological markers is usually not possible until fruits are produced and frequently requires a large set of phenotypic data that is often difficult to assess and some-times variable due to environmental influences (Sedra, 2001; Sedra et al., 1998). DNA fingerprinting techniques have an advantage in that the DNA content of a cell is independent of environmental conditions, organ specificity and growth stage (Ainsworth et al., 1996).

None of the diagnostic marker techniques so far applied to organisms has fulfilled all of the requirements in terms of cost, ease of use and cultivar identification. However, AFLPs satisfy more conditions than any other technique and are becoming the tool of choice for many applications and organisms. The advantage of AFLP is that, its utility can be assessed with a small number of primer pairs that can be extended for more studies. AFLP is a powerful DNA fingerprinting technique that uses PCR to amplify a limited set

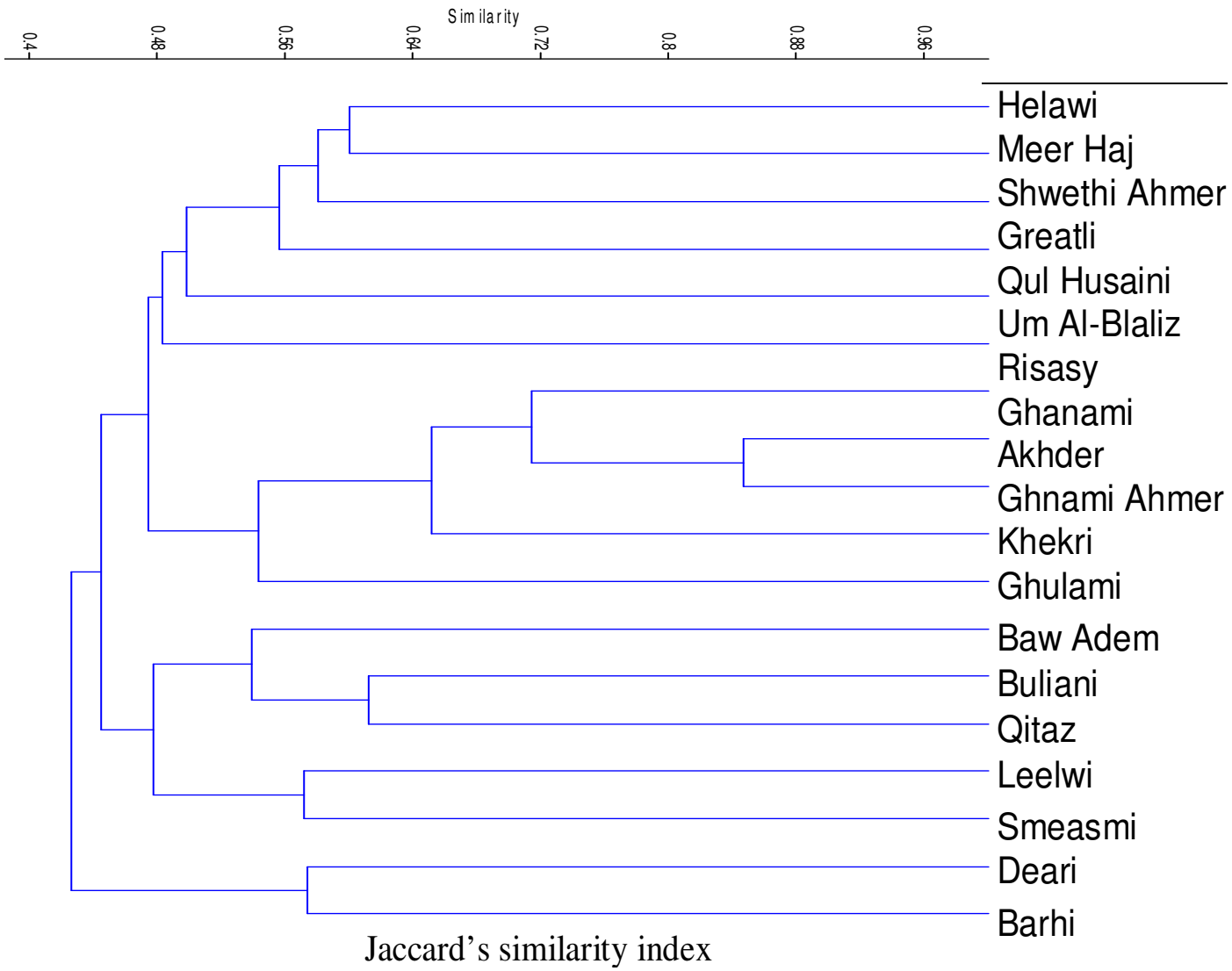

Figure 1. Genetic relationships among the 18 date palm varieties estimated by AFLP analysis.

of DNA fragments from a specific DNA sample (Blears et al., 1998; Vos et al., 1995). The reliability of the RFLP technique is combined with the power of PCR. Our AFLP marker study showed that all primer combinations used in this study were effective in distinguishing date palm varieties when used individually, revealing high level of polymorphism. Jaccard's genetic similarity index showed clusters that consisted of five male varieties. Two of these varieties 'Ghnami Ahmer and Ghanami Akhder' were very closely related with 85% similarity reflecting high similarity in their morphological traits (Ibrahim, 2008). Results also showed that there is large genetic diversity among the studied date germplasm. Jubrael et al. (2005) reported that high level of intervarietal polymorphism among another 18 Iraqi date palm varieties could be partly due to the strong out-crossing mechanism in this species, which is likely to increase the degree of polymorphism. Iraqi date palm varieties have been grown in Iraq for many years. However, Jaccard's similarity index and PCA revealed diverse relationships among them. In addition, the topology of the dendrogram and the distribution of varieties by PCA analysis showed that a typically continuous genetic diversity characterizes Iraqi date palm germplasm. In fact, the varieties were clustered independently of their geographic origin in spite of their phenotypic distinctiveness.

In conclusion, AFLP is a powerful method to discriminate date palm genotypes and to assess genetic diversity in this fruit crop. Obviously, this would be enhanced by using more primer sets and/or larger number of male varieties. Work is currently in progress to discover AFLP markers linked to agronomic traits, as well as those involved in sex determination in date palm. Such markers would be important to assist in the selection or to

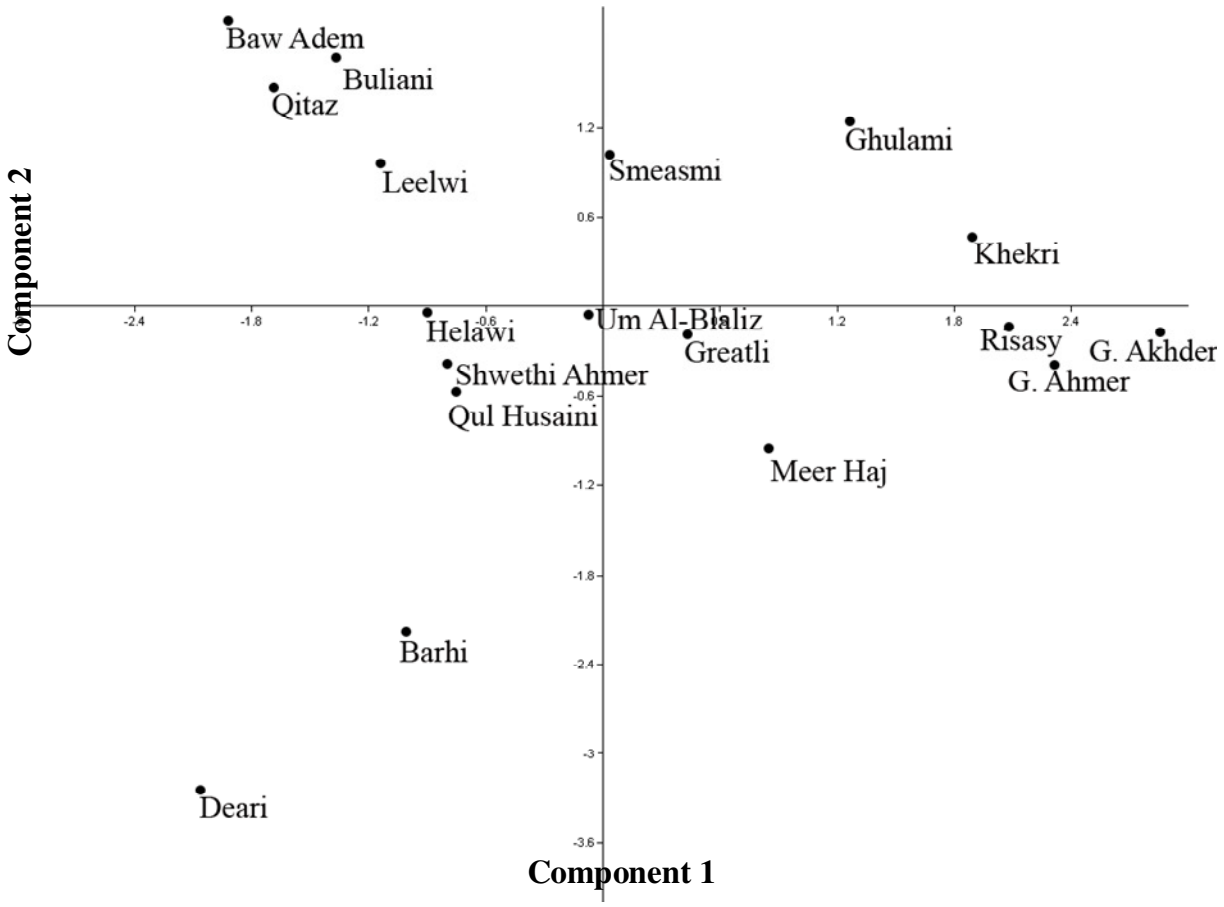

Figure 2. Principle component analysis of the 18 date palm varieties estimated by AFLP markers.

improve cultivation of this fruit crop.

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