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DETECTION OF GENETIC DIVERSITY IN DIFFERENT SOYBEAN (*Glycine max* L.) CULTIVARS USING SEED STORAGE PROTEIN PROFILES AND RAPD-PCR ANALYSIS

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ABSTRACT: Protein electrophoretic banding patterns of SDS-PAGE and RAPD-PCR analyses were performed to establish molecular diversity pattern for five soybean cultivars (Crawford, Giza 22, Giza 21, Giza 35 and Giza 111) and to elucidate their genetic relationships. The results of protein banding pattern showed a low level of polymorphism, so cannot be used for complete discrimination among the five cultivars under study. However, the results of protein profiles could be considered as indicators for general protein model pattern for the studied soybean cultivars. On the other hand, RAPD-PCR profiles revealed high levels of polymorphism among the five cultivars. Four 10-mer arbitrary primers successfully generated reproducible polymorphic products. Both number and size of the amplified products varied considerably with different primers and a sum of 22 polymorphic and 9 monomorphic bands were generated in all cultivars that used. A total of 8 unique bands were also identified. Two of the primers were more successful in cultivar's identification since they produced unique bands that are characteristics of each cultivar under study. The combination of all polymorphic bands generated by the four primers, were enough to discriminate between the examined soybean cultivars. Various combinations of the three RAPD decamer oligonucleotides had been used in the single-primer to increase the potential of the PCR reaction. To elucidate the genetic relationship, a dendrogram was constructed using both SDS-PAGE and RAPD profiles. The resulting dendrogram revealed three main genetic clusters; the first cluster include Giza22, Crawford and Giza35, while the second cluster, comprised the cultivar Giza21, and the third cluster comprised the cultivar Giza111. The first group has been subdivided into two subgroups; the first subgroup comprised one cultivar (Giza35) whereas the second subgroup included the two cultivars Giza22 and Crawford.

Key words: Soybean, *Glycine max* L., SDS-PAGE, RAPD-PCR.

INTRODUCTION

Soybean (*Glycine max* L.) is an important oil and protein crop in Egypt. Soybean contains about 18-24% oil and 30-50% protein with considerable amounts of essential amino acids, especially lysine as well as phosphorous, calcium and vitamins. Soybean is one of the most economic and nutritious crop as it has high content of protein and oil (Yaklich *et al*., 2002). Analysis of genetic variation in crop gene pools is a powerful tool for investigating the origin and early evolution of crop lineages and also have the potential to identify unique genetic resources for continued crop improvement (Badr, 2008). Characterization of the genetic variation in the available germplasm is important for further improvement of crop yield and to impart resistance to biotic and abiotic stresses (Kour and Singh 2004). Morphological markers were not quite enough to expose the genetic diversity between the morphological overlap of cultivars and the morphologically identical accessions (Gardiner and Forde, 1988). The electrophoretic banding patterns of total seed protein as revealed by polyacrylamide gel

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electrophoresis in the presence of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has been used for identification and differentiation of large number of crops (Abdelsalam *et al*., 1998; Aly *et al.,* 2000; Badr *et al.,* 2000; Hassan 2001 a and b). Also, SDS-PAGE was used to identify soybean cultivars and to discriminate high yielding soybean plants (Larsen, 1967; Lowry *et al.,* 1974; Mori *et al*., 1981; Gorman, 1988; Abdel-Tawab *et al*., 1993; Stejskal and Griga, 1995; Rashed *et al.,* 1997; Hsieh *et al*., 2001; Fahmy and Salama, 2002). The major limitation for using biochemical techniques is the existence of insufficient polymorphism among closely related cultivars.

Molecular markers have been proven to be powerful tools for assessing genetic variation within and among population of plants. At present, number of PCR based molecular techniques are available for assessing genetic diversity in plants. These include identification of amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and simple sequence repeats (SSRs). Genetic analysis with RAPD is fast, less technical, less expensive and involves no radioactivity and hybridization. RAPD technique requires small amount of DNA, easy to perform and reveals dominant molecular markers of ultimate potentialities in several fields of plant science including systematics and evolution (Witkus *et al*., 1994). RAPD markers can be used in genetic diversity, cultivar identification and genetic relationships in soybean (Mienie *et al*., 1995; Thompson *et al*., 1998; Chowdhery *et al*., 2001; Baranek *et al*., 2002; Barakat, 2004) and other different plants and populations (Al-Khalifa *et al.,* 2005; Bhutta *et al*., 2006; El-Shazly and El-Metairi, 2006; Lin *et al*., 2009; Badr *et al*., 2012; Hoque and Hasan, 2012; Prasanthi *et al.,* 2012). The RAPD method also provides useful evidence for gene mapping (Barua *et al*., 1993 ; Liu *et al*., 2000). To increase the potential of the original RAPD assay to generate polymorphic DNA markers with a given set of primers was further increased by combining two primers in a single PCR (Klein-Lank *et al*., 1991) and using combining three primers (Triple RAPD-PCR) in a single PCR to generate polymorphic DNA in mango (Mansour *et al*., 2008).

In this study, SDS-PAGE and RAPD-PCR fingerprinting were used to assess the genetic variation among the studied five soybean cultivars and to determine the genetic relationship among studied cultivars.

MATERIALS AND METHODS

This study was carried out in Molecular Genetics Lab., Microbial Genetics Lab., Biochemical Genetics Lab., Genetics Dept., Biochemistry and Microbiology Departments Labs, Central Lab., Faculty of Agric. Biotechnology Lab., Faculty of Veterinary Medicine, Zagazig University.

Materials

Seed samples

Five cultivars of soybean seeds (*Glycine max* L.) were used in this investigation namely; Giza 21, Giza 22, Giza 35, Giza 111 and Crawford. Seed samples under study were obtained from the Leguminous Crops Department Research (LCDR), Field Crops Research Institute, Agricultural Research Center, Ministry of Agriculture, Giza, Egypt.

Seeds were grown in pots and DNA was extracted from leaves at age of twenty days.

Protein analysis

Characterization of proteins profiles was carried out using one dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide slab gel (12%) was prepared according to Laemmeli (1970). For each soybean cultivar, ten dry seeds were milled together to a fine powder. Then 0.2 ml of sample buffer (0.2 M Tris-HCl pH6.8, 2% SDS) was added to 0.02 g of seeds meal of each cultivar and stored overnight at 4ºC. Centrifugation was performed at 9000 rpm for 6 minutes and supernatant was collected for analysis. Protein samples were prepared by mixing clear supernatant with sample buffer (0.125 M Tris-HCl, pH 6.8; 10% SDS; 10% sucrose and 0.1% mercaptoethanol) in 1:1 ratio and denatured by heating at 90ºC for 3 minutes. Equal amount of three replicates of each sample was loaded on the gel and electrophoresis was carried out at 15 mA for about half an hour, and then at 25 mA

for 4-6 hr. Molecular weights of different bands were calibrated with Sigma wide range molecular weight marker. Protein bands were visualized by staining the gel using 0.25% Coomassie Brilliant blue (R-250).

RAPD-PCR Analysis

DNA isolation

Freshly excised leaves from ten randomly chosen plants were harvested and mixed together for each cultivar. DNA was isolated from 50 mg of leaf material using DNA extraction kit (Qiagen) (by spectrophotometeric readings at 260 and 280nm. The 260 to 280 ratios were between 1.7 to 1.8 depicting high purity of the isolated DNAs). The concentration and purity of the extracted DNA was determined. Concentration was adjusted at 6 ng for all samples using TE buffer pH 8.0.

RAPD-PCR technique

Four primers 10-bp oligonucleotide of random sequences (Amersham Pharmacia Biotch., USA) were used in the PCR reaction according to Williams *et al*. (1990). The sequences of these primers are: Primer. 1- CS-44 (5' ATTCGGCCG C 3'). Primer No. 2 CS-46 (5' GGGATCTAG C3') ; Primer No3 CS-56 (5' TGGTGGGTC C 3'); Primer No 4 OH-04 (5'GGAAGTCGC C 3').

Triple RAPD-PCR

To increase the potential of the PCR reaction, various combination of the three decamer oligonucleotides (Primer. 1-CS-44 (5' ATTCGG CCG C 3'). Primer No. 2 CS-46 (5' GGGATCT AG C3'); Primer No3 CS-56 (5' TGGTGGGTC C 3') had been used in the single-primer PCR as suggested by Mansour *et al*. (2008). Amplification was performed in 25 µl total volume containing thirty ng (5 µl) from extracted DNA and 5 microliter of each primer. The polymerase chain reaction mixture, PCR kits manufactured by Amersham Pharmacia Biotch. USA, containing all of the necessary reagents was used. The amplification protocol was carried out as follows using PCR unit II biometra; denaturation at 94ºC for 5 min; 45 cycle each consists of the following steps: denaturation at 95ºC for 1 min, annealing at 36ºC for 1min; extension at 72ºC for 2 min; final extension at 72ºC for 5 min; and hold at 4ºC until separation on agarose gel and statistical analysis.

Analysis of amplified PCR products

Amplified PCR products were separated on 1% agarose gel containing ethedium bromide (0.5 µg/ml) at constant voltage 75 V. After electrophoresis, the RAPD patterns were visualized with UV transilluminator. RAPD markers were scored as DNA fragments present or absent. The band size was estimated by comparison to a standard molecular DNA marker (Promega). The bands scored from SDS-protein and RAPD-PCR analysis were pooled together to construct dendrogram tree using NTsys (Numerical Taxonomy System Program) by Rohlf (1993). The concentration and purity of the extracted DNA was determined. Concentration was adjusted at 6 ng for all samples using TE buffer pH 8.0. The DNA extracted from each cultivar was used for PCR amplification using PCR unit II biometra according to Williams *et al*. (1990).

RESULTS AND DISCUSSION

SDS-PAGE of Seed Storage Proteins

In soybean, there are a lot of seed storage proteins such as glycinin (11S) and β-conglycinin (7S), and the 11S to 7S ratio (Saio and Watanabe, 1969) Protein profile using SDS-PAGE of the studied soybean cultivars detected 10 bands with molecular weights approximately ranged from 305 to 15 KDa (Fig. 2) with 40% polymorphism. Number of total protein bands ranged from 9 bands for Crawford, 8 bands for Giza 111, 8 bands for Giza 35, 7 bands for Giza 22 and 8 bands for Giza 21. The cultivar Crawford has one positive unique band at molecular weight of about 20 KDa (Table 2). Also one positive band was unique to cultivar Giza 35 with molecular weight of about 30 KDa.

The SDS-PAGE profiles of the five cultivars of soybean detected 10 bands with 40% polymorphism. Barakat (2004) reported low level of protein polymorphism among the six cultivars of soybean and five major bands were identified (72, 36, 32 20 and 16 KDa) from 25 detected bands. However Malik *et al.* (2009) detected 26 bands with 50% polymorphism

Table 1. Name, origin and pedigree of the studied cultivars soybean

Accession	Origin	Pedigree
Giza 21	Egypt	Crawford × Celest
Giza 22	Egypt	Crawford × Forrst
Giza 35	Egypt	Crawford × Celest
Giza 111	Egypt	Crawford × Celest
Crawford	USA	Williams × Colombus

Fig 1: Seeds and seedlings of soybean

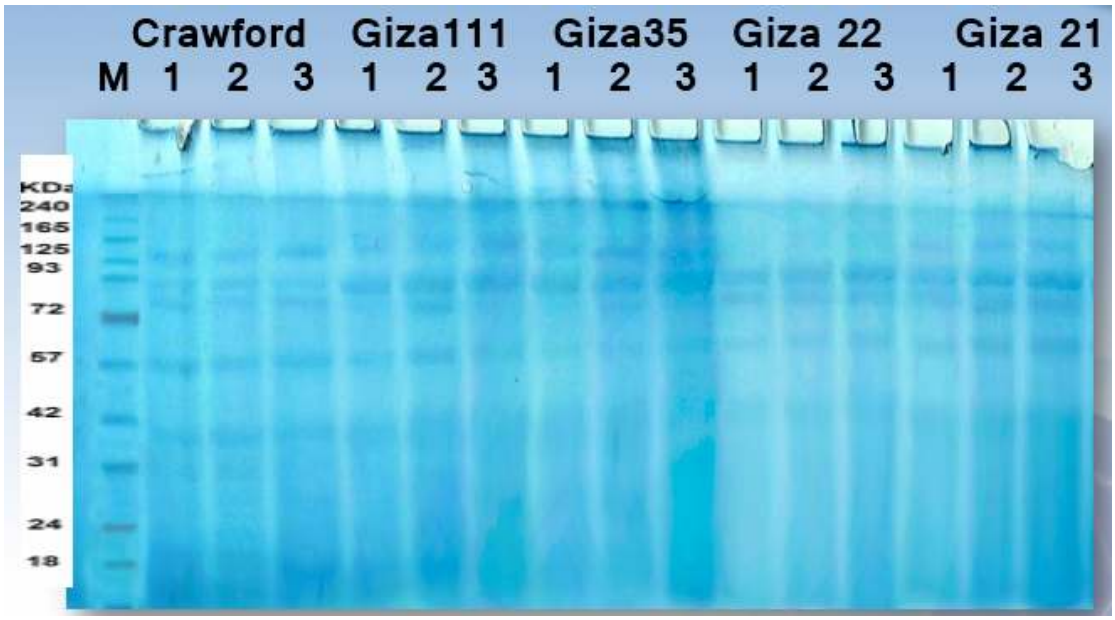

Fig. 2. SDS-PAGE of five cultivars of Soybean

Table 2. Presence and absence of SDS-PAGE protein bands of the studied five soybean cultivars

		Crawford			Giza 111			Giza 35			Giza 22			Giza 21		
N	**MW**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
1	305	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	144	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1
3	90	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4	87	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5	78	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1
6	56	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
7	40	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
8	30	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0
9	20	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
10	15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

1=band present 0=band absence

among ninety-two accessions of *Glycine max*. From these results, the number of bands of soybean seed protein patterns are ranged from 22 to 26 bands and protein profile can be used as a general biochemical fingerprint for the soybean (Barakat, 2004). Three of the studied cultivars (Giza22, Giza 35 and Giza 83) are distinguished by cultivar specific bands. The unique markers may help in identifying these cultivars. Also, these three cultivars are different in the weights of 100 seeds and having different pedigrees at least in one parent (Table 1). Some soybean cultivars were identified by biochemical genetic markers (Abdel-Tawab *et al*. 1993; Fahmy and Salama, 2002). Many investigators used seed storage protein to study genetic diversity; Ahmad *et al.* (1997), Badr *et al*. (2000) in the genous Lathyrus, Mustafa and El-Kholy 2008, among accessions of *Vicia faba* and Radwan *et al.* (2013) among accessions of Lathyrus inconspicuous. Many studies used SDS-PAGE for distinguishing cultivars and at infra specific levels (Signor *et al*., 2005; Sammour *et al*., 2007 a, b; Mustafa and El-Kholy, 2008).

However, these protein profiles could be used as a general biochemical fingerprint for the soybean. The low level of protein polymorphism could be attributed to the conservative nature of seed proteins. (Bonfitto *et al*., 1999). Low level of protein polymorphism was also reported in mung bean cultivars (Hassan, 2001a). However many investigators have used seed storage protein variability for the identification and characterization of species and cultivars (Abdelsalam *et al*., 1998; Badr *et al*., 2000; Ibrahim, 2003).

RAPD-PCR Analysis

The RAPD fingerprinting by the four primers generated 31 bands (Fig. 3) with 70.96% polymorphism. Both primers 3 and 4 generated more than 70 % polymorphism (Table 3). From a total of 31 bands, 7 bands are unique to 3 cultivars from the 5 studied cultivars and 9 bands are monomorphic. Primer 3 produced a total of 11 bands comprised 3 bands common among the five soybean cultivars. Besides the high polymorphic bands detected by both primers 3 and 4, there are positive and negative unique bands to be specific for some cultivars. Three positive bands are unique to cultivar Giza 21with molecular sizes of about 1651 bp, 1560 and 1272 bp, one positive unique band specific to Giza 111 at approximately molecular size 1163 bp and 3 postive bands are unique to cultivar Giza 35 by all primers (Fig. 3 and Table 3). However, for the negative unique bands, one negative unique band specific to Giza 111 and Crawford was observed at approximately molecular size of 691 and 448, bp respectively. The maximum number of total RAPD bands (11 bands) was produced by primers 3 with five positive bands (1560, 1163, 727, 561 and 505 bp) in cultivars Giza 21, Giza 35 and Giza 111 and one negative band with a molecular size of about 448 bp unique to cultivar Crawford.

RAPD amplification profile revealed 70.96% polymorphism among the studied soybean cultivars using the four primers and more than 70% polymorphism using 3 and 4 primers. Various combination of the three decamer oligonucleotides (primer 1 ,primer 2 and primer 3) had been used in PCR run to increase the potential of the PCR reaction. The combination of the three primers gave high polymorphism percentage (93.75%) than the single three primers (65.21%) (Fig. 4 and Table 4). Mansour *et al*. (2008), found that various combination of three decamer oligonucleotides tended to increase the potential of the PCR reaction and the polymorphism in some Mango cultivars.

This relatively high polymorphism has been observed in many studies of genetic diversity on soybean cultivars (Abdel Noor *et al*., 1995; Mienie *et al*., 1995; Thompson and Nelson, 1998; Thompson *et al*., 1998; Chowdhery *et al*., 2001; Baranek *et al*., 2002; Tu *et al*., 2003; Barakat, 2004) reported that there was a low degree of RAPD polymorphism (46%) among 19 soybean accessions included in the Czech National Collection of Soybean Genotypes.

Amplified RAPD fragments were differentiated among the studied cultivars and characterized by presence or absence of unique cultivar specific bands. Cultivar Crawford is distinguished by one negative band. Giza 111 one positive band and one negative band, Giza 35 (three positive bands) and Giza 21 (three positive bands) are also distinguished by cultivar specific bands. The RAPD markers are being successful to distinguish

Primer CS- 44

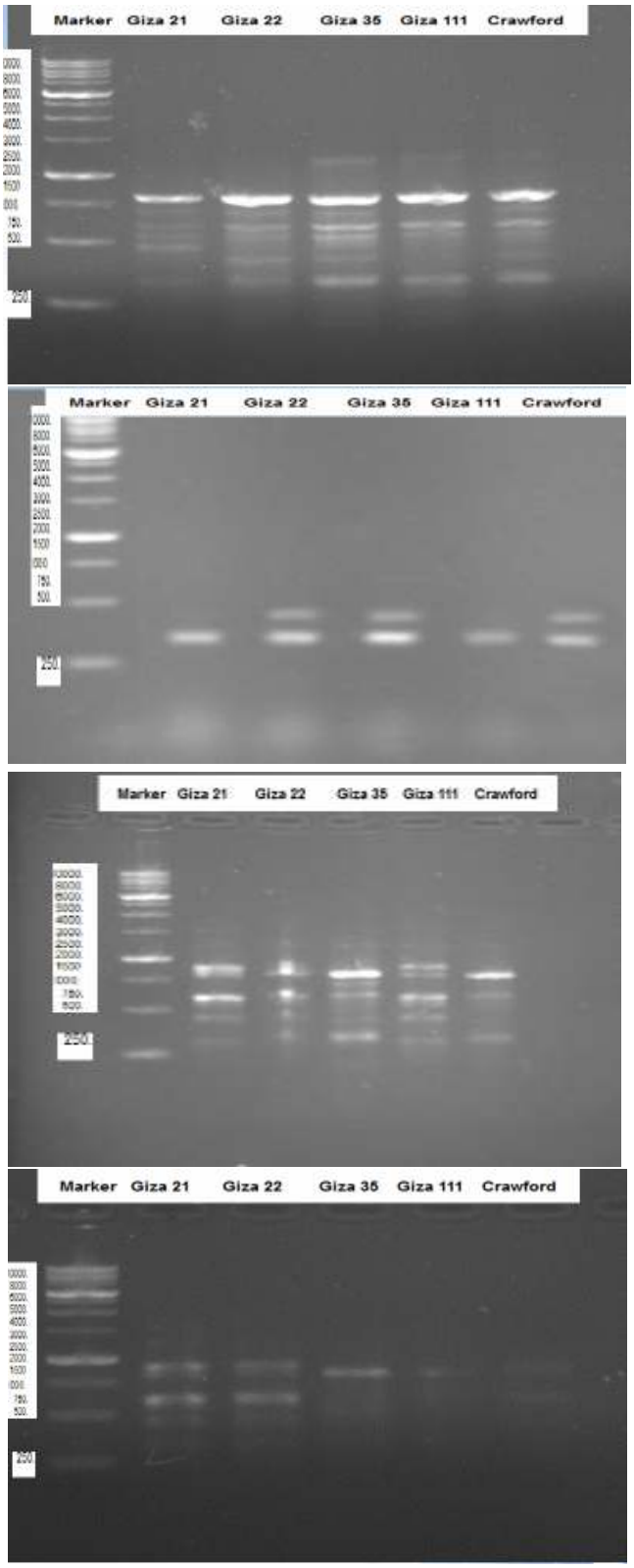

Fig. 3. DNA polymorphism of the five soybean genotypes using four random primers

Table 3. Number of amplification, polymorphic, monomorphic products and percentage of polymorphism generated by four random primers used for identifying five cultivars of soybean (*Glycine max* L.)

Primer	Range of fragment size	Giza 21	Giza 22	Giza 35	Giza 111	Crawford	Total No. of fragments	Monomorphic Fragments	Polymorphic Fragments	Polymorphism (%)
1	**333-426 bp**	1	2	2	1	2	2	1	1	50%
2	**250-1211 bp**	6	6	10	7	8	10	4	6	60%
3	**324-1560 bp**	6	5	8	6	4	11	3	8	72.72%
4	**309-1651 bp**	7	5	3	2	3	8	1	7	87.5%
Total	**250-1651 bp**	20	18	23	16	17	31	9	22	70.96%
Average		5	4.5	5.75	4	4.25	7.75	2.25	5.5	

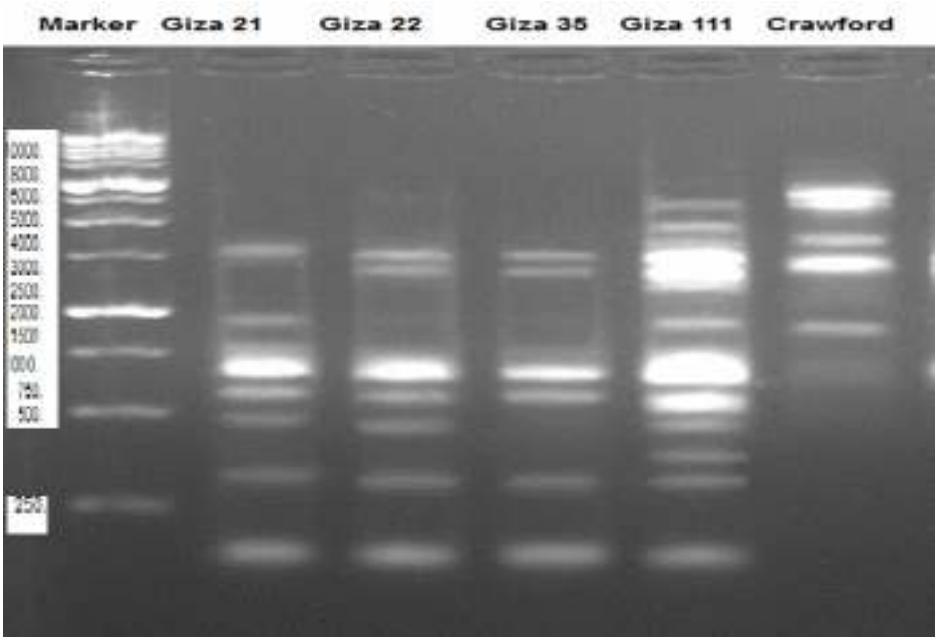

Fig. 4. DNA polymorphism of five soybean genotypes using triple RAPD-PCR

Table 4. Comparison between RAPD results using three separately primers (1, 2, 3) and Triple RAPD PCR (1, 2, 3) products using the same primers

Primer	Range of fragment size	Giza 21	Giza 22	Giza 35	Giza 111	Crawford	Total No. of Fragments	Monomorphic Fragments	Polymorphic Fragments	Polymorphism (%)
1	**333-426 bp**	1	2	2	1	2	2	1	1	50%
2	**250-1211 bp**	6	6	10	7	8	10	4	6	60%
3	**324-1560 bp**	6	5	8	6	4	11	3	8	72.72%
Total	**250-1560 bp**	13	13	20	14	14	23	8	15	65.21
1,2,3	**169-5578 bp**	8	8	8	13	6	16	1	15	93.75

these four cultivars from other studied cultivars. These results are in agreement with those obtained by Barakat (2004) who reported that RAPD markers can successfully be used to produce variety specific fingerprints in soybean. Mienie *et al*. (1995) suggested that RAPD markers can be used for identification in South African soybean cultivars. RAPD marker is a valuable tool for assessing genetic diversity levels in 47-East Asian vegetable soybean varieties and showed high level when compared to other morphological markers (Chowdhery *et al*., 2001). However, Weian *et al*. (2009) suggested that AFLP and SSR are more suitable than RAPD for genetic diversity studies in soy bean. SSR markers are efficient to differentiate between wild soybeans, cultivars and varieties (Tantasawat *et al*., 2011).

RAPD-PCR markers have been used in different plants to assess variations. Badr *et al*. (2012) reported the analysis of morphological variations and RAPD polymorphism distinguished among populations of *Artenesia* in Central and North Saudi Arabia.

Cluster Analysis

The cluster analysis classified the studied cultivars into three groups (Table 5 and Fig. 5). The first group included Giza 22, Crawford and Giza35, while the second and third groups included Giza21 and Giza 111, respectively, where each cultivar was in separate group. The two cultivars; Giza22 and Giza35 are clustered in one group with Crawford as the pedigree information showed that Crawford is a common parent for them. The highest similarity coefficient between Giza22 and Crawford (0.792) and the dendrograms indicate that these two cultivars are closely related. The lowest similarity coefficients are recorded between cultivar Giza35 and Giza111 (0.636) although their parents are the same. As well Crawford and Giza 111 present lowest similarity coefficients (0.636) although Crawford is a parent of Giza 111.

The genetic relationship among barley cultivars (El-Shazly and El-Metairi, 2006) and turf grass (Al-Khalifa *et al*., 2005) were determined by RAPD markers. RAPD analysis was also used for the characterization and grouping of wheat genotypes (Bhutta *et al*., 2006).

The identification of cultivars with maximum genetic divergence should optimize the choice of progenitors for crosses having specific objectives such as plant breeding or the construction of genetic maps. The genetic relationship between the studied cultivars based on combination of protein and RAPD have been estimated. The seed storage proteins revealed variations among the five soybean cultivars as well RAPD-PCR showed high polymorphism and discriminated between them. Two primers (Primer CS- 56, Primer OH- 04) from the four primers revealed high degree of polymorphism among studied cultivars. The genetic relationships among some soybean cultivars in this study agree with pedigree information and others disagree. This is consistent with Abdelnoor *et al.* (1995) who reported that RAPD data of soybean cultivars were coherent for most pedigree data while Doldi *et al.* (2006) reported that the dendrogram derived from RAPD data showed some divergence from the pedigree information available for soybean lines.

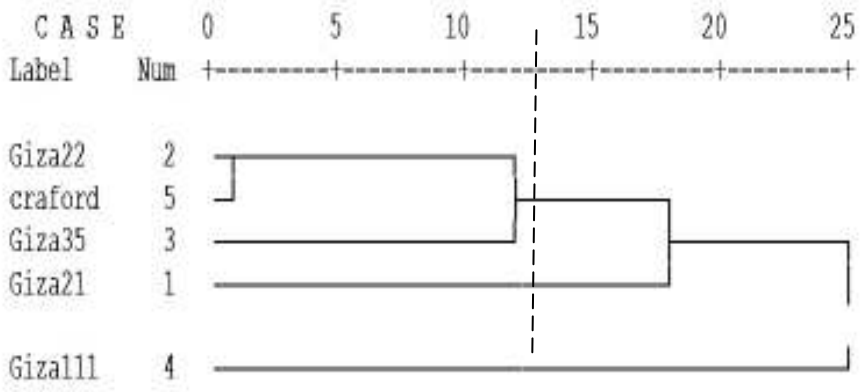

Fig. 5. Dendrogram using average linkage distance between soybean cultivars based on the combined analysis of storage protein and RAPD Profile

Table 5. Similarity coefficients among the five soybean cultivars estimated by seed protein and RAPD analysis

Cultivar	Giza 21	Giza 22	Giza 35	Giza 111	Crawford
Giza 21	1.000				
Giza 22	0.753	1.000			
Giza 35	0.649	0.740	1.000		
Giza 111	0.649	0.688	0.636	1.000	
Craw ford	0.675	0.792	0.714	0.636	1.000

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تعيين التنوع الوراثي لبعض أصناف فول الصويا باستخدام طرز بروتينات البذور المختزنة وتحليل الواسمات الجزيئية RAPD-PCR

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تم استخدام التفريد الكهربى للبروتين والواسمات الجزيئية RAPD لدراسة التنوع والاختلافات بين خمس أصناف من نبات فول الصويا (جيزة ٢١- جيزة ٢٢ - جيزة ٣٥- جيزة ١١١- كرافورد)، وتوضيح العلاقات الوراثية بينهم، وأوضحت نتائج حزم البروتين مستويات منخفضة من التنوع الوراثى ولا يمكن استخدامها للتمييز الكامل بين الخمس أصناف تحت الدراسة وعلاوة على ذلك يمكن اعتبار صورة البروتين هذه بصمة وراثية كيميائية لفول الصويا، وعلى جانب آخر أوضحت نتائج المعلومات الوراثية RAPD مستوى عالى من التنوع الوراثى بين الأصناف تحت الدراسة، وقد وجد أن أربع بادئات عشوائيه أوضحت فروق بين هذه الأصناف من حيث عدد وحجم الحزم تنوع معنويا باختلاف البادئات ووجد ٢٢ حزمة مختلفة بين الأصناف تحت الدراسة و٩ حزم فقط كانت متشابهة، ووجد أن هناك اثنين من البادئات كانوا أكثر نجاحا فى تحديد الأصناف حيث أنتجوا حزم فريدة خاصة لكل صنف على حدى تحت الدراسة وكان مجموع هذه الحزم الفريدة ٨ حزم، وكان تعدد المظاهر فى الحزم المختلفة الخاصة بهذه الأربع بادئات كافية للتمييز بين أصناف فول الصويا، ولتوضيح العلاقة الوراثية بين الأصناف تحت الدراسة تم تصميم الشكل العنقودى باستخدام بيانات التفريد الكهربى والواسمات الجزيئية (RAPD) وتم تقسيم الأصناف تحت الدراسة إلى ٣ مجاميع، المجموعة الأولى احتوت على الأصناف جيزة ٢٢ وصنف كرافورد وجيزة ٣٥ بينما المجموعة الثانية احتوت على جيزة ٢١ وأخيراً المجموعة الثالثة احتوت على جيزة ١١١والمجموعة الأولى انقسمت إلى تحت مجموعتين إحداهما تحتوى على الصنفان جيزة ٢٢ وكرافورد والأخرى تحتوى على الصنف جيزة ٣٥.

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