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Genetic Fingerprinting and Relationships of Six Soybeans (*Glycine max* L.) Cultivars Based on Protein and DNA Polymorphism

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ABSTRACT

Electrophoretic SDS-PAGE and RAPD-PCR analyses were performed to establish fingerprints for six soybean cultivars (Clark, Crawford, Giza 83, Giza 21, Giza 35 & Giza 111) and to elucidate their genetic relationships. The resulted protein banding pattern shows low levels of polymorphism and could not be used to discriminate completely among the six cultivars under study. However, the resulted protein profiles could be considered as general biochemical fingerprints of the soybean. On the other hand, RAPD-PCR profiles revealed high levels of polymorphism among the studied cultivars. Four 10-mer arbitrary primers were successfully generated reproducible polymorphic products. Both number and size of the amplified products varied considerably with different primers and a sum of 60 polymorphic and 10 monomorphic bands were generated in all the cultivars under study. A total of 30 unique bands were also identified. Two of the primers were more successful in cultivar's identification since they produced unique bands characteristics of each cultivar under study. The combination of all polymorphic bands generated by the four primers, were enough to discriminate completely between the examined soybean cultivars. To study the genetic relationship a dendrogram was constructed using both SDS-PAGE and RAPD profiles. The resulting dendrogram revealed two main genetic clusters; the first clusters comprise the cultivar Clark, while the second cluster includes the cultivars Crawford, Giza 83, Giza 21, Giza 35 and Giza 111. The second group was further subdivided into two subgroups; the first subgroup comprise one cultivar (Crawford) whereas the second subgroup includes the four cultivars Giza 83, Giza 21, Giza 35, Giza 111.

Key Words: Soybean; SDS-PAGE; RAPD; PCR; Random primers

INTRODUCTION

Soybean represents one of the most important leguminous plants in Egypt as well as in many countries. Soybean is grown for its seed protein and oil. Utilization of soybean cultivars with potential levels of insect-resistance can increase profits by reducing the use of insecticides and lessen the risk of insecticide residues in the human food chain (Rowan *et al.,* 1991). Johnston *et al.* (1993) implicated trypsin inhibitor as a soybean component that produces antibiosis in susceptible insects. The genetically controlled resistance factors in soybean are due to one or more chemicals that are found in plants. Biochemical and molecular characterization of insect-resistance or susceptible soybean cultivars as well as amount of genetic diversity among them are considered a central task for many purposes of soybean breeding (Thomposon *et al.,* 1998; Fahmy & Salama, 2002).

The electrophoretic banding patterns of total seed protein as revealed by polyacrylamide gel electrophoresis in the presence of sodium dodecyle sulphate (SDS-PAGE) has been used for identification and differentiation of large numbers of crops (Abdelsalam *et al*., 1998; Aly *et al*., 2000; Badr *et al.*, 2000; Hassan 2001 a & b). Also, SDS-PAGE has also been 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 & Griga, 1995; Rashed *et al*., 1997; Hsieh *et al.,* 2001; Fahmy & Salama, 2002). The major limitation of the biochemical techniques is insufficient polymorphism among closely related cultivars.

Randomly amplified polymorphic DNA (RAPD) assay, on the other hand which detects nucleotide sequence polymorphisms by means of the polymerase chain reaction (PCR). A single primer of arbitrary nucleotide sequence is a useful method for generating molecular markers and provides accurate fingerprints at the molecular level (Williams *et al.,* 1990; Welsh & McClelland, 1990). DNA markers give a much higher degree of polymorphism and reproducibility. The PCR products that are not shared between some individuals act as polymorphic markers. The development of DNA markers have been recently introduced in plant discriminations and being employed for the improvement of intractable traits such as drought tolerance, resistance to foliar–feeding insects and the combination of high protein/high yield (Linc *et al.*, 1996; Lefebvre *et al*., 2001; Hassan, 2002, Lu & Myers 2002; Abdel-Tawab *et al.,* 2003). Researchers have developed

robust sets of DNA markers in soybean (*Glycine max* L.) using different techniques (Caetano-Anolles *et al.*, 1991; Prabhu & Gresshoff, 1994; Chowdhury *et al*., 2000; Brik & Sivolap, 2001a & b).

This work aims to: i) investigate the utility of biochemical and molecular approach to establish genetic fingerprint for six cultivars of soybean under study. ii) Identify commercially obtained primer sequences that generate usable polymorphism with soybean DNA and iii) to determine the genetic relationships between insect-resistance and susceptible cultivars.

MATERIALS AND METHODS

Six soybean cultivars were used in this investigation comprised of Clark, Crawford, Giza 83, Giza 21, Giza 35 and Giza 111. Seeds of these cultivars were kindly supplied by Agriculture Research Center (ARC), Giza, Egypt. The cultivars Giza 21, Giza 35 and Giza 111 are characterized for improved quality, are insect-resistance and need low amount of nitrogenous fertilizer.

Protein analysis. Characterization of proteins profiles was carried out using one dimensional sodium dodecyle sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide slab gel (12%) was prepared according to Laemmli (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 seed 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 & 0.1% mercaptoethanol) in 1:1 ratio and denatured by heating at 90ºC for 3 minutes. Equal amount of sample was loaded on the gel and electrophoresis was carried out at 15 mA four about half an hour, and then at 25 mA for 4-6 h. 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).

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 analysis. 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 No 1. -GTTTCGCTCC-3; Primer No. 2. -AAGAGCCCGT-3; Primer No 3. -AACGCGCAAC-3; Primer No 4. -CCCGTCAGCA-3

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 beads tablets (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 95 ºC for 1 min; Annealing at 36ºC for 1min; Extension at 72ºC for 2 min; Final extension at 72ºC for 5 min; Hold at 4ºC.

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) by un-weighted pair group method based on arithmetic mean (UPGMA). 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). Thirty ng from extracted DNA were used for amplification reaction. The polymerase chain reaction mixture (manufactured by Amersham Pharmacia Biotch. USA) containing all of the necessary reagents except the primer and the DNA which added to it.

The primers used were 10–bp oligonucleotide of random sequences (Amersham Pharmacia Biotch. USA). Four primers were selected as potentially useful. The sequences of these primers are: Primer No 1. GTTTCGCTCC–3; Primer No 2. AAGAGCCCGT–3; Primer No 3. AACGCGCAAC–3; Primer No 4. CCCGTCAGCA– 3

Amplification was performed in 25 µl total volume using 5 microliter of each primer. The amplification protocol was carried out as follows: 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; Hold at 4ºC.

RESULTS AND DISCUSSION

Protein analysis. Fig. 1 demonstrates the SDS-protein profiles of six soybean cultivars used in this study. A maximum of 25 bands were detected with molecular weights ranging from 149 to 6.5 kDa. Five major

Fig. 1. Seed protein banding patterns of the six soybean cultivars. Lanes 1-6 are: Clark, Crawford, Giza 83, Giza 21, Giza 35 and Giza 111 respectively. Lane M; referrers to the standard protein marker

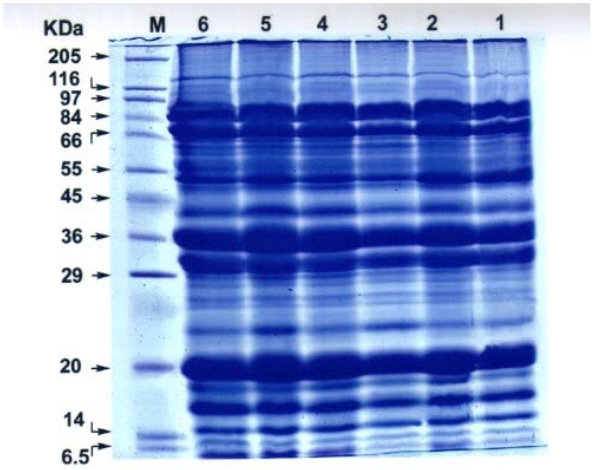
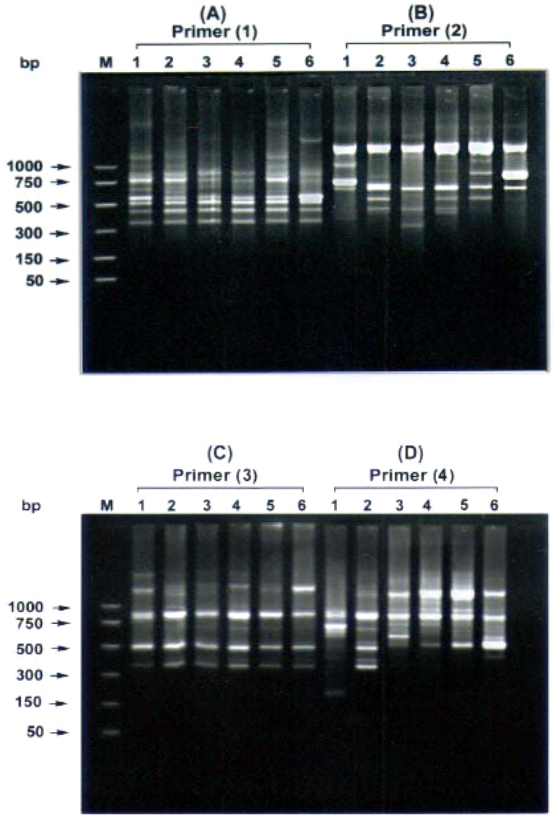


Fig. 2 (A, B, C & D). RAPD fingerprints of the six soybean cultivars using four primers. Lanes 1-6 are: Clark, Crawford, Giza 83, Giza 21, Giza 35 and Giza 111. Lane M= standard DNA marker



monomorphic bands were identified in all the cultivars at approximate molecular weights of about 16, 20, 32, 36 and 72 kDa. In addition, 20 minor monomorphic bands were also identified.

With regard to seed protein banding patterns, slight polymorphism has been identified among the six cultivars under study. The band at molecular weight of about 116 kDa is absent in the cultivars Clark and Giza 35. On the other hand, the band with molecular weight 89 kDa was found only in cultivar Giza 111. In this investigation, the obtained seed protein banding patterns could not discriminate the studied cultivars. 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 conservative nature of the seed protein (Bonfitto, *et al*., 1999). Low level of protein polymorphism was also reported in early ripening peach of Sinai (Mansour *et al.,* 1998) and 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).

Seeds protein of soybean is known to contain two kinds of proteins lipoxygenase and trypsin inhibitor which produces antibiosis in susceptible insects, (Johnston *et al.,* 1993). Trypsin inhibitor protein has approximately molecular weight of 21500 Da (Krishnan, 2001). In this investigation, the protein band at molecular weight of 20 kDa could be that of trypsin inhibitor, while the band with an approximate molecular weigh of 89 kDa (Fig. 1), found only in the cultivar Giza 111 is though to be for lipoxygenase (Stejskal & Griga, 1995). The density of protein band with molecular weigh of 20 kD was high in the more resistant cultivars (Giza 21, Giza 35 & Giza 111) as compared to the other. Researchers have estimated that as few as two (Kenty *et al.,* 1996) and as many as six genes (Rufener *et al.,* 1989) could be responsible for the partial insect resistance.

RAPD analysis. The four random primers generated strong amplification profiles with distinct bands (Fig. 2 A, B, C & D). The fingerprints generated by these primers revealed characteristic profiles for each cultivar; in terms of number and position of RAPD bands. No correlation between primer GC content and the clarity of the banding pattern was noted in the present study which is in accordance with Marillia and Scoles (1996).

Table I summarizes the banding pattern obtained with these primers. RAPD bands were between 4027 to about 269 bp size produced. Soybean cultivars Crawford, Giza 83, and Giza 35 produced the greatest number of amplified products (average of 8.2 per primer), followed by the cultivars Giza 21, Clark, and Giza 111 (average of 7.7, 6.2 & 6.0 per primer, respectively). RAPD profiles produced by all four primers were used to evaluate the similarity between the studied cultivars. A total of 70 reproducible marker bands (Table II; Fig. 2 A, B, C & D) were scored with 15,

21, 14 and 20 DNA bands generated by the primers No. 1, 2, 3 and 4 respectively. The four primers generated a total of 60 polymorphic (85.71% polymorphism) and 10 monomorphic (14.29%) bands in all the cultivars under study. The number of polymorphic bands ranged from 10 to 19 bands per primer.

A total of 30 polymorphic bands were scored as unique one (Table II) and these unique bands could be used to discriminate among the cultivars on the basis of one or more unique bands (Table III).

Primer No 1 gives amplified a maximum of 15 bands ranging from 389 to 3526 bp in size. A total of 10 polymorphic and 5 monomorphic bands were scored in the profiles generated by this primer. The most observable polymorphic bands were recorded at approximately molecular size of about 1518, 1216, 714 and 625 base pairs (Table I & II). This primer generated two unique bands in the cultivars Clark and Giza 83 at approximate sizes of 673 and 1003 bp respectively (Table III & Fig. 2A). These bands therefore are considered as specific markers for these cultivars.

Primer No. 2 was the most informative, compared to other, and therefore as this primer revealed clear variations in RAPD profiles of cultivars (Fig. 2 B). A total of 19

Table I. Summary of data obtained by RAPD analysis using four 10-bases long primers for identification of six cultivars of soybean (*Glycine max* L.)

Primer No.	Primer sequence 5`→ 3`	% GC	No. of bands						Approx. band size bp
			Lane 1 Clark	Lane 2 Crawford	Lane 3 Giza 83	Lane 4 Giza 21	Lane 5 Giza 35	Lane 6 Giza 111	
1	-GTTTCGCTCC -	60 %	10	10	8	6	11	7	3526-389
2	-AAGAGCCCGT-	60 %	5	8	8	9	7	6	4027-269
3	-AACGCGCAAC-	60 %	5	8	8	8	5	5	2325-300
4	-CCCGTCAGCA-	70 %	5	7	9	8	10	6	2325-293
Total			25	33	33	31	33	24	

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

Primer No.	Polymorphic bands		Total polymorphic bands (a)	Monomorphic bands	Amplification products (b)	Polymorphic bands Size with bp	% of poly-morphism (a/b x 100)
	Unique Bands	Non-unique bands					
1	2	8	10	5	15	3474, 2296, 1518, 1216, 989, 950, 714, 625.	66.66%
2	14	5	19	2	21	4027, 3226, 1496, 516, 460	90.48%
3	4	8	12	2	14	2325, 1841, 1818, 1682, 1516, 1201, 706, 300	85.71%
4	10	9	19	1	20	2325, 1990, 1497, 1265, 1069, 763, 680, 589, 479	95%
Total	30	30	60	10	70		85.71%

The most prominent bands are underline

Table III. Molecular size in bp of the amplified polymorphic (unique) DNA bands generated by four DNA random primers used for identifying the six soybean cultivars

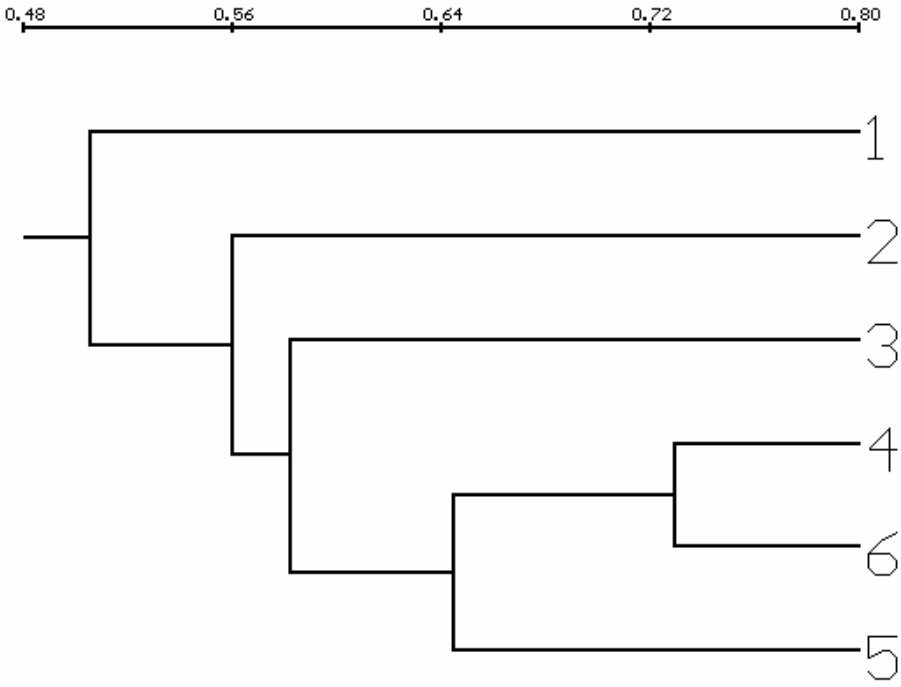
Primers	Unique bands in different soybean cultivars					
	Clark	Crawford	Giza 83	Giza 21	Giza 35	Giza 111
1	673	----	1003	----	----	----
2	1234 - 769	918 - 725	2509 – 1540 – 878 – 564- 269	1064 – 427	1339 - 989	905
3	1639 - 890	-----	-----	346	-----	1704
4	989 – 724 –645	504 – 449- 342 -293	1265	1200	1704	----

The most prominent bands are underlined.

Table IV. Similarity indices among the six soybean cultivars estimated by seed protein and RAPD analyses

	Clark	Crawford	Giza 83	Giza 21	Giza 35	Giza 111
Clark	1.000					
Crawford	0.525	1.000				
Giza 83	0.424	0.559	1.000			
Giza 21	0.458	0.525	0.559	1.000		
Giza 35	0.559	0.627	0.559	0.627	1.000	
Giza 111	0.559	0.525	0.627	0.729	0.661	1.000

Fig. 3. Dendrogram of the six cultivars based on the pooled results derived from both seed protein and RAPD. 1= Clark, 2= Crawford, 3= Giza 83, 4= Giza 21, 5= Giza 35 and 6= Giza 111



polymorphic bands were scored the most prominent at approximate molecular sizes of about 516 and 460 bp (Table II). Out of nineteen polymorphic bands, fourteen unique bands were identified which clearly discriminate between the six cultivars under study. The noticeable unique bands generated by primer No. 2 were identified at about 769,725, 269, 427, 1339 and 905 bp in the cultivars Clark, Crawford, Giza 83, Giza 21, Giza 35 and Giza 111, respectively (Table III).

A maximum of 8 polymorphic and four unique bands were scored in the RAPD profile generated by primers No. 3. The most clear unique band was identified at about 890 bp in the soybean cultivar Clark (Table III). This cultivar is distinguished from the other cultivars by the presence of this unique band and the absence of the band with approximately molecular size of 308 bp (Fig. 2C).

A total of 19 polymorphic bands and only one monomorphic band were detected in the RAPD profile generated by primer No. 4. The best observed polymorphic bands are 1497, 1069, 763 and 479 bp (Table II & Fig. 2d). Soybean cultivar Clark was characterized by the presence of three unique bands at approximate molecular sizes of 989, 724 and 645 bp and the absence of the band at about 1497 bp which is present in the other cultivars, where as the cultivar Crawford was characterized by the presence of four unique bands at about 504, 449, 342 and 293 bp and the absence of the bands of about 1060, 763 and 479 bp. Only one unique band at about 1265, 1200 and 1704 bp was detected in cultivars Giza 83, Giza, 21 and Giza 35 respectively. No unique band was produced in soybean cultivar Giza 111 by this primer (Table II; Fig. 2d).

From the previous results, it can be concluded that, the four utilized primers generate relatively extensive polymorphism within the studied soybean cultivars (85.71%). The primer No. 2 and 4 were more successful in cultivar identification. The former primer generated 14 unique bands while the latter primer produced 10 unique bands. The primer No. 2 produced clear unique banding patterns for all soybean cultivars under study and can be used to distinguish between them. These results are not in agreement with that obtained with AiMin *et al*. (1998) who reported that no single primer out of seven could distinguish sixteen *Brassica juncea* cultivars via single analysis. Many investigators recommended the use and applications of RAPD analysis as rapid and more powerful method to identify and characterize different plant species and cultivars (Linc *et al.*, 1996; Hassan, 2002, Weder 2002; Abdel-Tawab *et al*., 2003). RAPD markers were used to detect genetic differences between soybean cultivars and to map major genes and quantitative traits loci (Jhy-Jhu Lin *et al*., 1995; Keim *et al.,* 1995; Boerma & Mian, 1998; Ferreira & Keim, 1998; Chung *et al.,* 1998; Liu *et al*., 2000; Csanadi *et al.,* 2001; Fahmy & Shadia, 2002). Miller (1995) used RAPD to apply molecular marker technology in the development of new soybean varieties with increased protein content. More than 700 molecular markers were used by Caglar-Karakaya *et al.* (2002) in mapping of the mutation in soybean while 62 RAPD markers were used by

Liu *et al.* (2000) to construct the molecular map of soybean genome. RAPD profiles were also, used to differentiate between 27 feed legume species including soybean (Weder, 2002).

The results generated from seed protein and DNA-RAPD profiles were pooled for drawing the genetic relationships among the six soybean cultivars under study. The similarity indices among these cultivars were estimated for ea3h pair-wise group (Table IV). The similarity coefficients were then used to construct dendrograms using the un-weighted pair group method with arithmetic averages (UPGMA) from the NTsys-pc program (Rohlf, 1993). The constructed dendrogram tree revealed two main genetic clusters (Fig. 3). The first cluster comprise the cultivar Clark only while the second cluster includes the other cultivars, Crawford, Giza 83, Giza 21, Giza 35, Giza 111. The second group was further divided into two subgroups; the first subgroup comprise one cultivar (Crawford) whereas the second subgroup includes the four cultivars Giza 83, Giza 21, Giza 35, Giza 111.

The highest similarity values (0.729 & 0.661) were recorded between the two cultivars Giza 21 and Giza 111 and between Giza 35 and Giza 111 respectively; indicating that these cultivars were closely related to each other. The cultivars Giza 21, Giza35 and Giza 111 are characterized by improved quality. They are insect-resistance cultivars and need low amount of nitrogenous fertilizer. On the other hand, the lowest value (0.424) was recorded between the two cultivars Giza 83 and Clark indicating that these cultivars were genetically distant cultivars (Table IV).

CONCLUSION

Considering all data of the present study, it can be concluded that the biochemical markers like seed protein analysis are not powerful enough to discriminate between closely related varieties. RAPD markers on the other hand are much more sensitive. RAPD analysis was more effective in discriminating between them and revealed complete identification than detecting changes at the DNA level and can successfully be used to produce variety specific finger prints.

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