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USING ADVANCED GENETIC TECHNIQUES IN STUDYING COTTON GENOTYPES APPLIED IN BREEDING

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ABSTRACT

Five Egyptian cotton varieties Giza 70,Giza 85, Giza 88, Giza89 and Giza 90 were crossed in complete diallele crosses meeting design and produced 20 F_{1s} genotypes. The genetic variability and relationships among 11 cotton genotypes (five cotton varieties and six F_{1s}) were achieved using SDS-PAGE and 10 RAPD primers. The total number of bands which produced by SDS-PAGE ranged from 17 with Giza 85 to 23 with G3 (Giza70), and the cluster analysis divided the eleven cotton genotypes into two groups. The level of polymorphism for all genotypes, as revealed by RAPD was 77.05%. In addition, RAPD analysis revealed different genetic similarity (according to Dice coefficient). Among the 11 cotton genotypes the highest similarity was between G7 and G8. However, the G8 was 61.3% similar to its female parent G90 and 78% to its male parent G89, while the lowest genetic similarity was between G6 and G10 (54.5%). Three of five Egyptian cotton varieties were characterized with eight positive and one negative unique RAPD markers.
Keywords: SDS-PAGE, RAPD, Polymorphism, Genetic similarity, Positive unique bands, Negative unique bands.

INTRODUCTION

Cotton is an economically plant allover the world. It belongs to genus Gossypium that comprises about 50 diploid and tetraploid species (Rana and Bhat, 2002). The majority of commercial cotton varieties present-day belong to *Gossypium hirsutum* L.(n=2X=26), while a few (10% of total production) belong to *Gossypium barbadense* L.(n=2X=26). Cotton is a major of oilseed crop which provide world with natural fiber.

In Egypt, the main cotton species is *Gossypium barbadense*L. which has a worldwide reputation because of its fiber length, relative staple and adaptation to different environmental conditions. However, varieties belong to this species are virtually derived from crosses between relatively few number of accessions through breeding programs. This has to lead to decrease the genetic variation among Egyptian cotton germplasm. Therefore, the improvement of cotton through breeding to diseases, insect pests, and abiotic stresses, would require an immediate practical DNA analysis to find out markers that facilitate the genetic improvement of closely related genotypes. The DNA markers could be used to increase the efficiency of breeding programs (Mohamed *et al.*, 2003).

During the last 30 years, the identification of cultivars traditionally relied on embryological and morphological characters, which cannot be assessed in differentiating between or within different species or even individuals. But nowadays, the progress of electrophoretic techniques are become useful tools for phylogenetic studies among different resources (Zhang *et al.*, 1998).

Randomly amplified polymorphic DNA (RAPD) markers have been successfully used for cultivars analysis and species identification in most plant, due to the technical simplicity and speed of RAPD methodology.(Abdel chany and Essam, 2003)

The present study aimed to make crosses between five Egyptian cotton varieties, investigate the genetic diversity among 11 cotton genotypes (five Egyptian cotton varieties and six F_{1s} genotypes) through protein banding pattern and RAPD molecular marker, and estimate the genetic relationships among the five Egyptian cotton varieties and their F_{1s} genotypes.

MATERIALS AND METHODS

Plant material

Five Egyptian cotton varieties (Giza70, Giza85, Giza88, Giza89, and Giza90) Table1, were crossed in complete diallel in season 2004 and produced 20 genotypes F_{1s} seeds. The biochemical and molecular studies was carried out on 11 cotton genotypes (five Egyptian varieties and six F_{1s} genotypes) listed in Table 2.

Table 1: List of five Egyptian cotton varieties, staple length, and climatic region

Varieties	Staple length	Location
Giza70	Extra-long	Delta
Giza85	Long	Delta
Giza88	Extra-long	Delta
Giza89	Long	Delta
Giza90	Long	Upper Egypt

Table 2: List of cotton genotypes used in biochemical and genetic studies

Code no.	Genotype
G1	Giza85
G2	Giza89
G3	Giza70
G4	Giza90
G5	Giza88
G6	Giza88 X Giza70
G7	Giza85 X Giza88
G8	Giza90 X Giza89
G9	Giza88 X Giza89
G10	Giza85 X Giza90
G11	Giza90 X Giza70

Methods

I - Biochemical and genetic studies

Electrophoretic detection of protein by sodium dodecyle sulphate, polyacrylamide gel electrpophoresis was carried out to differentiate between the eleven cotton genotypes under study using the method described by Laemmili (1970) with slight modifications.

Protein isolation:

Eight cotton seeds from each samples were crushed and 5 ml of chloroform were added then shaked for 72 hours to eliminate the oil content from cotton seeds . The samples were dried and ground in liquid nitrogen. The crushed samples were transferred to 1 ml Eppendorf tube brought to 200 µl with extraction buffer (50 m M tris-HCl buffer, pH 6.8, glycerol 10 % w/v, ascorbic acid 0.1%, cycteine hydrochloride 0.1 w/v) and uncubated at 65 C for 1 hr. Centrifugation at 18,000 rpm for about 30 min, was carried out to remove debris.

The protein content in supernatant was estimated according to the method desribed by Bradford (1976) by using bovine serum albumin as a standard protein. Protein content was adjusted to 2 mg / ml per sample.

Preparation of samples:

Sodium dodecyl sulphate (SDS) was added to the sample at a rate of 4 mg SDS to each 1 mg protein, followed by adding 50 µl 2-mercaptoethanol to each 950 ml of the sample, then mixed and heated at 100° C in water bath for 3-5 min.

Gel running:

Electrophoresis was performed in a vertical slab mold (Hoefer Scientific Instruments, San Francisco, CA, USA, model LKB 2001), measuring 16 x 18 x 0.15 cm. Electrophoresis was carried out at 30 milliamper (m.A.) at 10° C for 3 hours.

Staining the gel with silver nitrate:

The silver staining method for protein described by Sammons *et al.* (1981) was used. This method of staining is sensitive and detect as little as 2 ng of protein in a single band

II- Molecular and genetic studies.

- Genomic DNA extraction and purification:

Extraction of total DNA was performed using CTAB protocol according to Probsky *et al.*, (1997). To remove RNA contamination, RNase A (Sigma Co., USA)were added to the DNA solution and incubated at 37°C for half an hour. The extracted DNA was deproteinized by adding proteinase K (Sigma Co, USA) and incubating at 37°C for 2 hours.

- Random amplified polymorphic DNA (RAPD):

Ten decamer primers were used in this work to detect the polymorphism among the 11 cotton genotypes according to Williams *et al.*,

(1990). The amplification reaction was carried out in 30 µl reaction volume containing 1X PCR buffer, 4 mM $MgCl_2$, 0.3 mM dNTPs , 60 P mol primer, 1 U/ 1µl *Taq* DNA polymerase and 30 ng template DNA. PCR amplification was performed in a T-gradient thermal cycler (Biometra; T Gradient). Programmed to fulfill 40 cycles after an initial denaturation cycle for 4 min at 94° C. Each cycle consisted of a denaturation step at 94° C for 1 min, an annealing step at 35° C for 1 min, and an extension step at 72° C for 1 min, followed by extension cycle for 7 min at 72° C in the final cycle. Amplified products were visually examined on agarose gel and the presence or absence of each size class was scored as 1 or 0, respectively.

RESULTS AND DISCUSSION

-SDS-PAGE analysis

The SDS-PAGE was carried out for the 11 cotton genotypes as illustrated in Figure 1 where bands with different molecular weight were detected and ranged from 14KD to 116 KD the total number of bands ranged from 17 in G1(Giza 85) to 23 in G3 (Giza70). The obtained data was used to built up a phylogenic tree as shown in Figure 2. The cluster analysis of SDS-PAGE grouped the 11 cotton genotypes into two clusters, the first cluster contains two genotypes; G10(G85XG90) and its female parent's G1(Giza85) with genetic similarity of 95.46%. The second cluster brunched into two subclusters, the first subcluster contains G8(G90XG89) and G2(G89) with genetic similarity of 98.28% with G4(Giza90), Giza89 and Giza90 represent the parents of G8.

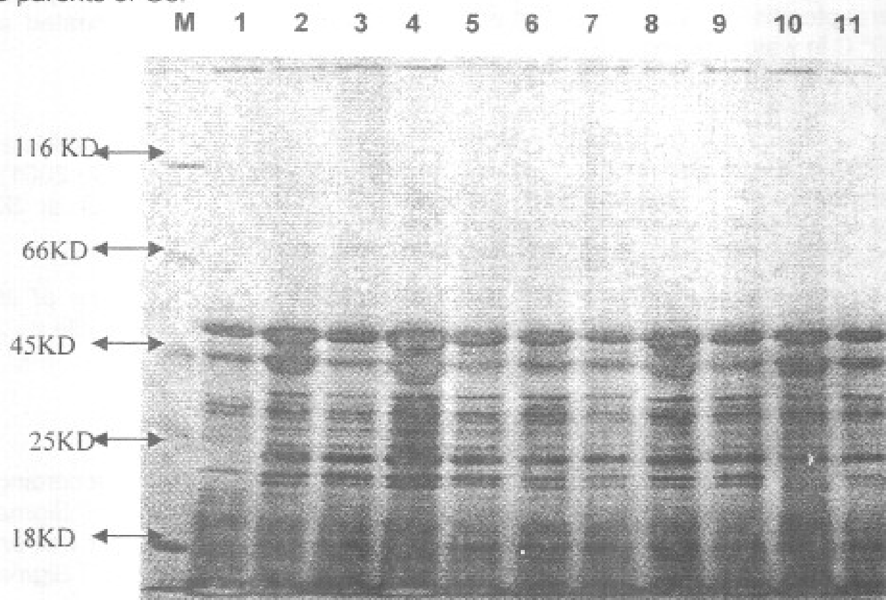

Figure1: SDS protein pattern for the 11 cotton genotypes under study. Lanes 1 through 11 refer to cotton genotypes. M : protein marker

The second subcluster also branched into three subclusters, the first one contains G3(G70) and G9(G88XG89) with highest genetic similarity of 98.78% and G7(Giza88 X Giza70) with different linkage distance. G5(Giza88) and G6(Giza88 X Giza70) formed the second subcluster with genetic similarity of 97.86%, while the third subcluster contains G11(Giza90 X Giza70) alone.

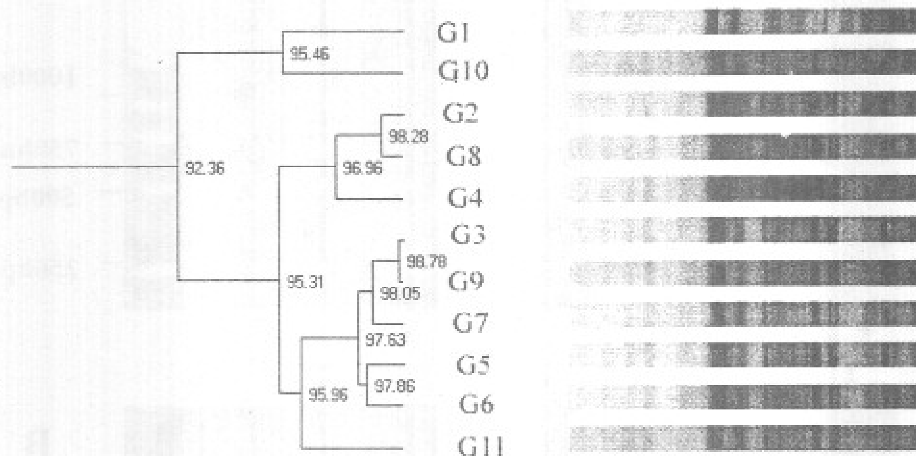

Figure2: Cluster analysis as revealed by protein data

-Polymorphism detected by RAPD markers

The RAPD profile were generated using 10 different decamer primers. The 10 primers amplified 61 DNA fragments, 14 of them were monomorphic and 47 fragments were polymorphic (77.05%), in one or other of 11 cotton genotypes. Donger and Kharbikar (2004) employed 86 random primers to study 25 cotton (*G.hirsutum*) accessions, sixty three primers detected polymorphism. A total of 296 DNA fragments were generated by the 63 primers, 225 of them were polymorphic (76.01%).

The different primers revealed different level of polymorphism among the 11 cotton genotypes as illustrated in Table 3. The number of amplified DNA fragments were scored for each primers, the highest number of amplified DNA fragments was 9 with primers opB05 and opC02, while the lowest number was 4 with primers opB06, opB08 and opB17, with an average of 6.1 per each primer across the 11 cotton genotypes. The number of polymorphic amplicons per primer ranged from 2 (primer opB17) to 9 (primer opC02) with an average of 4.7 per each primer. Kumar *et al.*, (2003) studied the genetic diversity in a set of 30 elite cotton germplasm lines using RAPD markers, 25 primers were used to amplified a total of 108 bands. Total bands amplified for each primer ranged from 1 to 8, with an average of 4.4 fragment per primer .

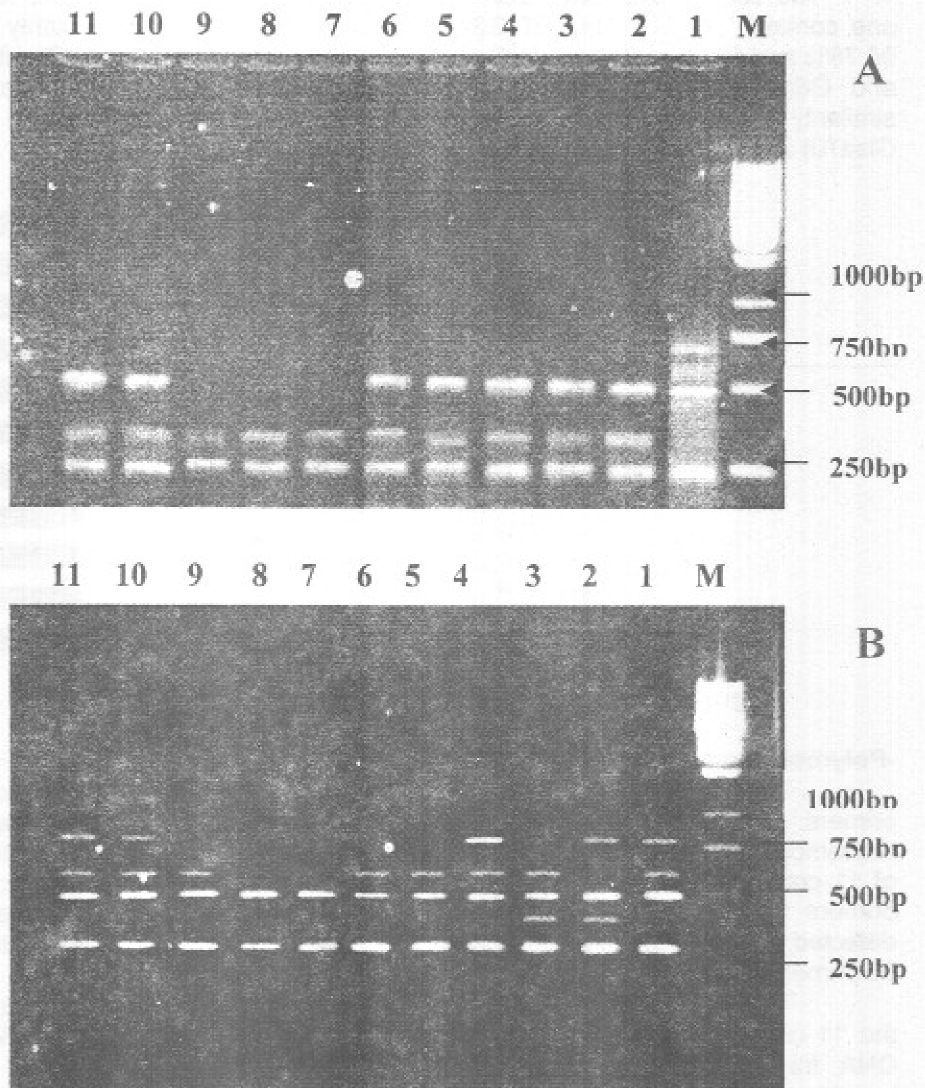

Figure 3: RAPD profile of the 11 cotton genotypes amplified using (A)Primer opA03, (B) primer opC15. M: 1Kbp lader DNA Molecular marker. G1 through G11 refer to cotton genotypes.

Table 3: Number of amplicons and polymorphic bands as revealed by each RAPD primer, total number, and level of polymorphism.

RAPD primer	Total number of amplicons	Polymorphic amplicons	Percentage of polymorphism
opA03	7	5	71.43
opA10	7	4	57.14
opB05	9	7	77.78
opB06	4	4	100
opB08	4	3	75
opB09	6	4	66.67
opB10	6	6	100
opB17	4	2	50
opC02	9	9	100
opC15	5	3	60
Total	61	47	77.05

The total number of amplicons revealed for each cotton genotypes are illustrated in Table 4, results cleared that the highest number of amplicons was 43 which produced with G1(Giza85), while the G10 (G85XG90) produced the lowest number of amplicons (28).

Table 4: The number of amplicons DNA fragments for each RAPD primer with the 11 cotton genotypes, and the total number of amplicons revealed with each genotype.

RAPD primer	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11
opA03	7	3	3	3	3	3	2	2	2	3	3
opA10	5	6	4	5	4	5	4	5	4	4	4
opB05	5	4	5	6	6	4	6	7	6	3	7
opB06	3	3	3	1	2	3	3	3	2	2	3
opB08	4	4	4	2	1	4	4	4	1	2	2
opB09	4	5	4	3	3	4	3	3	3	3	2
opB10	2	2	3	4	2	2	2	2	2	2	2
opB17	4	4	4	2	2	4	4	3	3	2	2
opC02	5	5	5	3	5	6	7	5	5	3	4
opC15	4	4	4	4	3	3	2	2	3	4	4
Total	**43**	**40**	**39**	**33**	**31**	**38**	**37**	**36**	**31**	**28**	**33**

The number of amplicons for each F_1 genotype, the number of bands presented in parental genotypes and the total number of ands for F_1 parents obtained with the ten random primers are illustrated in table 5. Among the F_{1s} genotypes the G6 (G88 X G70) produced the highest number of amplicons(38), 20 fragments of them were present in Giza88 and Giza70, while 3 fragments were present in Giza88 and absent in Giza70, and the rest

15 fragments were present in Giza70 and absent in Giza88, so the total of female parent's fragments was (23 + 8), the eight fragments were present in Giza88 and absent in Giza70 and G6, and the total of male parent (Giza70) were (35+6), the six fragments were present in Giza70 and absent in Giza88 and G6. In G10(G85 X G90) which produced the lowest number of amplicons (28), 21 of them were present in both Giza85 and Giza90, 2 in Giza85 (23+20), and 5 in Giza90 (26+7). While the G7(G85 X G88) produced 37 DNA fragments 21 of them were present in both parents and the total of parents was 33+10 and 25+6, respectively. While the total fragments which produced by G8(G90 X G89) was 36 fragments, 18 of them were present in both Giza90 and Giza89, 3 in Giza90 (21+12), 15 in Giza89(33+7). The hybrid between Giza88 and Giza89 (G9) produced 31 DNA fragments, 19 present in both parents, 9 in the female parent (Giza88), and 3 in male parent (Giza89), the total was 28+3 and 22+18, respectively. While G11(G90 X G70) produced 33 fragments, 21 of them were present in both parents, 5 in Giza90 (26+7) and 7 in male parent Giza70 (28+11).

Table 5: The number of amplicons for each F_1 genotype across the 10 RAPD primer, number of bands which present in the parents genotypes and the total number of amplicons for F_1 and parents

F1 / Primers	opA03	opA10	opB05	opB06	opBo8	opB09	opB10	opB17	opC02	opC15	Total	
G6(G88XG70)	3	5	4	3	4	4	2	4	6	3	38	
G88&G70	3	3	4	0	1	2	1	2	1	3	20	
G5(G88)	0	1	0+2	1+1	0	0+1	0+1	0	1+3	0	3	23+8
G3(G70)	0	1	0+1	2+1	3	2	1+2	2+2	4	0+1	15	35+6
G7(G85XG88)	2	4	6	3	4	3	2	4	7	2	37	
G85&G88	2	4	4	1	1	3	1	2	1	2	21	
G1(G85)	0+5	0+1	0+1	2	3	0+1	1	2	4	0+2	12	33+10
G5(G88)	0+1	0	2	0+1	0	0	0+1	0	2+2	0+1	4	25+6
G8(G90XG89)	2	5	7	3	4	3	2	3	5	2	36	
G90&G89	2	4	2	0	2	3	0	2	1	2	18	
G4(G90)	0+1	0+1	3+1	0+1	0	0	0+4	0	0+2	0+2	3	21+12
G2(G89)	0+1	1+1	2	3	2	0+2	2	1+1	4	0+2	15	33+7
G9(G88XG89)	2	4	6	2	1	3	2	3	5	3	31	
G88&G89	2	3	4	0	1	3	1	2	1	2	19	
G5(G88)	0+1	1	2	1+1	0	0	1	0	3+1	1	9	28+3
G2(G89)	0+1	0+3	0	1+2	0+3	0+2	0+1	1+1	1+3	0+2	3	22+18
G10(G85XG90)	3	4	3	2	2	3	2	2	3	4	28	
G85&G90	3	4	2	0	2	3	0	2	1	4	21	
G1(G85)	0+4	0+1	0+3	1+2	0+2	0+1	1+1	0+2	0+4	0	2	23+20
G4(G90)	0	0+1	1+3	1	0	0	1+3	0	2	0	5	26+7
G11(G90XG70)	3	4	7	3	2	2	2	2	4	4	33	
G90&G70	3	4	3	1	2	2	0	2	1	3	21	
G4(G90)	0	0+1	3	0	0	0+1	1+3	0	0+2	1	5	26+7
G3(G70)	0	0	1+1	2	0+2	0+2	1+2	0+2	3+1	0+1	7	28+11

Dongre and Parkhi (2005) studied the hybrid cotton H '6' and its parent G.Cot.10 (male) and G.Cot.100 (female) using RAPD marker with 20 primers. Primer opA11 was found to be useful in differentiating parents and hybrid,

tow bands were generated by opA11 the first on was present in all genotypes and the other about 700bp was present in male parent and hybrid and absent in female

Genetic Relationships Revealed by RAPD Data:

Information about genetic relationships among and between species is very important in plant breeding, in which estimation of genetic relationship might have a great impact by assessing in the selection of parents for hybridization (Al-Said, 2001).

The genetic similarity based on Dice coefficient (Sneath and Sokal, 1973) for the 11 cotton genotypes was calculated and presented in table 6. The highest degree of similarity was between G7(G85 X G88) and G8(G90 X G89) (88.9%), while the lowest genetic similarity was between G6(G88 X G70) and G10(G85 X G90) (54.5%). Linos *et al.*(2002) used RAPD analysis to study 28 upland cotton (*Gossypium hirsutum* L.) and they found out that the genetic similarity ranged from 0.614 to 0.922.

Among the five Egyptian cotton varieties, the genetic similarity ranged from 80% between Giza 85 and Giza 89 to 57.6% between Giza 89 and Giza 90, while the hybrid between Giza90 and Giza 89 (G8) was 61.3% similar to Giza 90 and 78% to Giza 89. The G7 which showed the highest genetic similarity with G8 had the same genetic similarity with its parents 76.9%, 76.3%, respectively.

The G6 was 58.8% similar to Giza 88 and 75% to Giza 70, while G10 had 55.1%, 54.5% genetic similarity with its parents (Giza85, Giza90), respectively. G9 (G88 X G90) showed high similarity with its female parent 85.2% comparing with male parent 60%, and G11(G90 X G70) showed (67.7%, 72.5%) genetic similarity with its parents, respectively. Mehetre *et al.* (2004) used RAPD analysis to determine the hybrid nature of crosses between *Gossypium hirsutum* 761H and *G.raimondii*. G. hirsutum 761H x G. raimondii, the hybrid offspring was 65% similar to the male parent, *G.raimondii* and 64% similar to female parent *G. hirsutum* 761H.

Table 6: Genetic similarity matrices computed according to Dice coefficient from RAPD data

	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11
G1	**100**										
G2	**80**	**100**									
G3	**77.3**	**76.7**	**100**								
G4	**67.6**	**57.6**	**68.9**	**100**							
G5	**64.8**	**60.9**	**62.5**	**73.7**	**100**						
G6	81	80.5	75	58.5	58.8	100					
G7	76.9	76.3	64.8	56.3	65.7	85.3	100				
G8	78.9	78.4	69.6	61.3	61.5	82.2	**88.9**	100			
G9	63.9	60	58.5	65.5	85.2	63.8	76.5	69.7	100		
G10	55.1	62.7	64.5	69.1	79.1	**54.5**	55.4	57.1	74.4	100	
G11	65.8	70.3	72.5	57.7	67.7	65.8	61.1	71.4	63.6	73	100

Cluster Analysis as Revealed by RAPD Data:

The dendrogram developed based on Dice similarity matrix reveled the genetic relationships among 11 cotton genotypes with different linkage

distances is shown in Figure 3. The dendrogram grouped the 11 cotton genotypes into two clusters, the first cluster brunched into three subclusters; the first subcluster contains two genotypes (G7 and G8) (which have the highest genetic similarity) with G6, Giza85 and Giza89 formed the second subcluster , while G3(Giza70) was alone in the third subcluster.

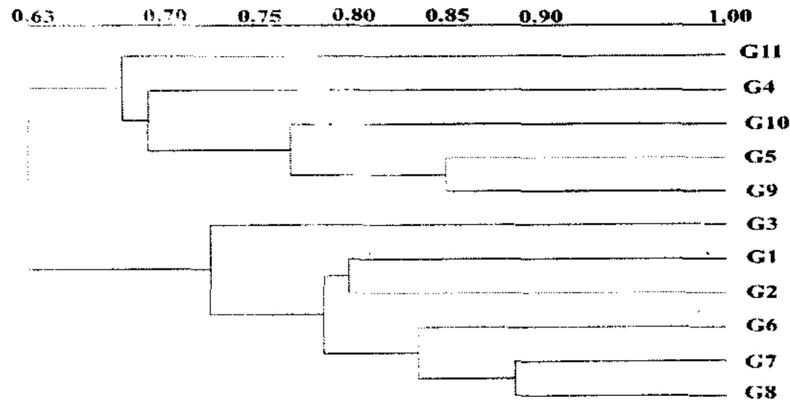

Figure4: Cluster analysis as revealed by RAPD data

The second cluster brunched also into three subclusters, the first subcluster contained G9(G88 X G90) and its female parent Giza88 and G10 (G85 X G 90) alone with different linkage distance, while the second and third subclusters contain only one genotype, G11(G90 X G70) in the third subcluster and its female parent Giza90 was in the second subcluster.

II. 4. Genotype identification by unique DNA markers:

Genotype identification depending on morphological and physiological characters has several disadvantages, in addition to time consuming; these characters have been influenced by environmental conditions and growth stage of plant (Tanksley *et al.,* 1989). Nowadays DNA-based markers such as RAPD are used for genotypes identification due to their sensitivity and accuracy. Two types of unique markers should be observed; the amplified bands were present in one genotype and absent in all other investigated genotypes; these bands are called positive unique marker; while the negative unique marker when the bands were present in all investigated genotypes except one .

In this work three of The five Egyptian cotton varieties were characterized by 8 positive and 1 negative unique RAPD markers, G1(Giza85) was characterized with 4 positive unique markers, and G2(Giza89) was characterized with two positive unique markers, while G4(Giza90) was characterized with two positive and one negative unique markers (Table 7). Al-Said (2001) estimated the genetic relationships among 12 Egyptian cotton varieties by using 49 RAPD primers, 15 primer of them characterized only 6 genotypes with 31 positive and 28 negative unique bands.

Table7: Genotype identification by unique RAPD marker, the size of marker bands(positive and negative), primers revealed marker bands,and the number of marker bands among three cotton varieties .

Genotype	Unique positive marker			Unique negative marker			Grand total
	Size of the marker band(bp)	Primer	Total no. of marker variety	Size of the marker band(bp)	Primer	Total no.of marker variety	
G1	350,450,575,700	opA03	4	——	——	——	4
G2	750 800	opA10 opB09	2	——	——	——	2
G4	450,500	opB10	2	475	opB10	1	3

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استخدام الطرق الو راثية الحديثة في دراسة التراكيب الو راثية للقطن المستخدمة في التربية

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1-قسم الوراثة -كلية الزراعة – جامعة القاهرة

2- قسم المحاصيل -كلية الزراعة – جامعة القاهرة

أجريت هذه الدراسة على خمسة أصناف من القطن المصري هي (جيزة 70، جيزة 85، جيزة 88، جيزة 89 و جيزة 90) حيث تم تهجين هذه الأصناف تهجينا دائريا وأنتجت 20 تركيب وراثي للجيل الأول F_1.تم دراسة التباين الو راثي بين إحدى عشر تركيب وراثي (خمسة أصناف قطن مصري و ستة تراكيب وراثية للجيل الأول) باستخدام تفريد البروتينات الكلية و عشرة بادئات عشوائية RAPD .تراوح عدد الواسمات الذي أنتج بواسطة تفريد البروتينات بين 17 واسما في الصنف جيزة 85 و 23 واسما في جيزة 70. وضع الدوندروجام الناتج عن تفريد البروتين الإحدى عشر تركيب وراثي في مجموعتين منفصلتين، أما مستوى التباين الوراثي الناتج بواسطة البادئات العشوائية كان 77.05%، وقد اظهرت هذه التحليلات درجات قرابة وراثية مختلفة حيث كانت أعلى نسبة قرابة بين G7و G8 (88.9%) وأقل درجة قرابة بين G6و G10 (54.5%) . أظهر التركيب الوراثي G8(G90XG89) 61.3% درجة قرابة مع الأم جيزة 90 و 78% مع الأب جيزة 89. لقد ميزت تقنية البادئات العشوائية ثلاثة من الأصناف المصرية الخمسة بواسطة ثمانية واسمات فريدة موجبة وواحد سالب .