Application of molecular markers to assess genetic diversity among eight sorghum genotypes

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Youssef S. S. *, Moghaieb R. E.A. *, Rezkalla A. A. ** and Ahmed S. M. **
*Genetics Department and Genetic Engineering Research Center, Faculty of Agriculture, Cairo University, Giza, Egypt
**Genetics and Cytology Department, National Research Center, Dokki, Giza, Egypt

This study aims to assess the genetic diversity and phylogenetic relationships among eight sorghum genotypes by RAPD analysis. The total protein profiles and peroxidase polymorphism were also recorded to characterize the levels of genetic diversity in the cultivars. Thirteen random primers generated 163 total scorable bands, out of them 121 were polymorphic, indicating 74% of polymorphism, and the size of the bands ranged from 202 to 5787 bp, reflecting polymorphism. The number of bands ranged from 9 for primer OP-G14 to 20 for primer OP-A4. Only one cultivar (ICSR-93004) recorded four positive markers, followed by Giza-15 (3 bands); the remaining genotypes except ICSR-92003, showed only one unique band. However the genetic matrices based on dendrogram showed different levels of genetic similarity. Protein analysis revealed 9 bands, 5 of them were polymorphic (55.5%). The peroxidase isozyme displayed 4 bands, only the second band distinguished the cultivar Giza-15.

Key words: *Sorghum bicolor, genetic markers, RAPD analysis, SDS-PAGE, peroxidase banding pattern.*

INTRODUCTION

Sorghum is an important staple food throughout the semi-arid tropics of Asian and African regions (Ahmed *et al.*, 2000). It ranks the fifth after the four major cereal crops, wheat, maize, rice and barely, worldwide and the second most important cereal crop after maize in sub-Sahran Africa. It originated in North East Africa (Ethiopia and Eritrea), which is considered the center of diversity for the crop (Agramma and Tuinstra, 2004). In these areas there is a great demand for sorghum for human consumption, livestock feed and green manure production. The cultivated varieties are low in productivity, thus food shortage occurs frequently in the sorghum growing areas. And the extension of its cultivation to marginal lands requires extensive breeding programs followed by the introduction of new varieties fitting small scale farmer needs (Haussmann *et al.*, 2000).

Assessment of the extent and distribution of genetic variation in a crop species and its relatives is essential for understanding pattern of diversity and evolutionary relationships between accessions that helps to sample genetic resources in a more systematic fashion for conservation and plant improvement. Traditionally, the extent of diversity among sorghum genotypes was analyzed based on morphological markers (Harlan and Dewet, 1972) such as characters controlled by a small number of genes may not provide an accurate indication of the genetic divergence (Doggett, 1988).

Knowledge of genetic diversity has an important impact on the improvement of crop productivity as well as on the conservation of genetic resources (Dean *et al.*, 1999 and Simioniuc *et al.*, 2002). Several DNA-based techniques have been used successfully in DNA fingerprinting of plant genomes and for genetic diversity studies. Among them, random amplified polymorphic DNA (RAPD) analysis which is quick (Colombo *et al.*, 1998 and Fahima *et al.*, 1999) and well adapted for non radioactive DNA fingerprinting of genotypes (Cao *et al.*, 1999). However, problems with the reproducibility in amplification of RAPD markers and with data scoring have been reported (Jones *et al.*, 1998).

Molecular analysis has clarified understanding of evolution of the sorghum genome and its molecular map has reached a parallel level to most other major crops (Paterson, 1994). Sorghum is a diploid with 10 pairs of chromosomes and has a genome of about 730 Mb. Its genome has been sequenced because of its small size and its importance for food security and biofuel production in diverse environment, particularly developing countries in the tropics (Paterson *et al.*, 2009).

Isozyme markers has been used for genetic diversity analysis in many species. It is widely employed for revealing the genetic structure of plant populations and assessing the genetic relationships among different taxa (Dudnikov, 2003).

Stability of protein profile is one of the main features of the seed protein; for this reason it has been suggested as an additional tool for species identification beside the other traditional biosystematics approaches. The uniformity of the profile and its additive nature make seed protein electrophoresis as a powerful tool in studying the origin and evolution of cultivated plants (Ladizinsky and Hymowitz, 1979). Electrophoretic patterns of total seed protein, as revealed by polyacrylamide gel electrophoresis (PAGE) provide valid evidence for addressing taxonomic and evolutionary problems in plants (Crawford, 1990).

The main objectives of this study were: 1) Determination of genetic relationships among studied sorghum genotypes. 2) Comparing the application and utility of RAPD technique to analyze genetic diversity among sorghum genotypes. 3) Assessing genetic similarity using molecular markers polymorphism and comparing it with cultivar regional information. 4) Characterization the levels of cultivars genetic diversity using isozyme and protein analysis.

MATERIALS AND METHODS

Plant materials

Eight genotypes of *Sorghum bicolor* of different origins cultivated in Egypt were evaluated using RAPD technique. Cultivar names, their origins and line grouping are listed in Table (1). The seeds of the eight cultivars were kindly provided by the Field Crops Institute, Agricultural Research Center (ARC), and Giza, Egypt. This study was carried out during the period from 2008 to 2011 at the Genetic Engineering Research Center, Faculty of Agriculture, and Cairo University.

Table (1): The Source and main agronomic characteristics of the eight sorghum genotypes.

No	Cultivar	Source	Agronomic characterization
1	Giza-15	Egypt	White seed, long stem and resistant to Acremonium strictum, Fusauium moniliforme and downy mildew.
2	NEB-Dorado-V9	Nebrasca (U.S.A)	White seed, short stem, stays green at maturity and resistant to Acremonium strictum, Fusauium moniliforme and downy mildew.
3	ICSR-93004	ICRISAT	Creamy seed, short stem and stays green at maturity.
4	ICSV-273	ICRISAT	White seed, dwarf stem and stays green at maturity.
5	ICSB-88006	ICRISAT	Growth duration 60-62 days, stays green, white seed and length 125 cm.
6	ICSB-88005	ICRISAT	Grain, growth duration 60-62 days, stays green, white seed and long stem.
7	ICSR-92003	ICRISAT	Grain, stays green, creamy seed and short stem.
8	ICSR-93001	ICRISAT	Grain, growth duration 62 days, white seed and short stem.

⇒ ICRISAT = International Crop Research Institute for the Semi-Arid Tropics.

Genetic differences between eight sorghum genotypes by RAPD technique

DNA preparation

Seeds were grown in a growth chamber at 27°C with 12 hr d /light. Genomic DNA was isolated from the leaves collected from 10- 15-day old seedlings according to the protocol described by Dellaporta *et al.* (1983).

RAPD primers

Total genomic DNA was isolated using the method descried in Rogers and Bendich (1985). PCR reactions were conducted using arbitrary 10-mer primers (Operon Technology, Inc., Alameda, CA, USA). The names and sequences of the primers that gave clear bands are presented in Table (2).

Table (2): Types and sequences of the RAPD primers used in this study.

Primer	Sequence
OPA-01	5`-CAGGCCCTTC -.3`
OPA-04	5`-AATCGGGCTG -.3`
OPB-05	5`-TGCGCCCTTC -.3`
OPB-10	5`-CTGCTGGGAC -.3`
OPC-08	5`-TGGACCGGTG -.3`
OPC-11	5`-AAAGCTGCGG -.3`
OPD-10	5`-GGTCTACACC -.3`
OPF-15	5`-CCAGTACTCC -.3`
OPG-14	5`-GGATGAGACC -.3`
OPK-02	5`-GTCTCCGCAA -.3`
OPK-04	5`-CCGCCCAAAC -.3`
OPM-10	5`-TCTGGCGCAC -.3`
OPN-11	5`-TCGCCGCAAA -.3`

PCR amplification and electrophoresis

PCR amplification was performed in a volume of 20 µl containing 30 ng of template DNA, 1µM of primer, 200 µM of each dNTP, 3 mM MgCl2 and 1 U Red Hot Taq polymerase (AB gene Housse, UK) and 10-X Taq polymerase buffer (AB gene Housse, UK). Reactions were conducted in Biometra T1 Thermocycler (Germany) with initial denaturation step to 94°C for 5 min and then subjected to 35 cycles of 1 min at 94°C; 1 min at 35°C and 1 min at 72°C. The RAPD reaction products were evaluated for polymorphisms on 1% agarose gel. After staining with 1 µg mL-1 ethidium bromide for 30 to 60 min, the gels were photographed by gel documentation system.

Band scoring and cluster analysis

The RAPD gel images were scanned using the Gel Doc 2000 Bio-Rad system and analyzed with Quantity One Software v. 4.0.1 (Bio-Rad Laboratories, Hercules, CA USA). The bands were sized and then binary coded by 1 or 0 for their presence or absence in each genotype. The systat ver. 7 computer program was used to calculate the pairwise differences matrix and plot the dendrogram among sorghum cultivars (Yang and Quiros, 1993). Cluster analysis was based on similarity matrices obtained with the unweighed pairgroup method (UPGMA) using the arithmetic average to estimate the phenogram.

Total proteins and isozyme electrophoretic analysis

Five seeds were planted in plastic pots (3L) each containing a mixture of sandy soil and peat moss (1:1 / V: V). Seedlings were irrigated daily with 400 ml of Hoagland solution (Hoagland and Arnon, 1950). Isozyme and total proteins were extracted from plant leaves. Peroxides and protein analysis were performed using SDS-PAGE according to Stegemann *et al.* (1983) and Laemmli (1970), respectively.

RESULTS AND DISCUSSION

Reproducibility in amplification of RAPD technique is a major concern (Jones *et al.*, 1998). The RAPD products of the eight sorghum genotypes with respect of the 13 random primers are presented in Table (2). The RAPD profiles of the used genotypes were compared to find out the differences among them by occurrence of polymorphic bands. Through the tested primers, the total scorable bands were 163, their size ranged between 202- 5787 bp (Table 3). The number of produced bands per primer varied from 9 for (OP-G14) to as many as 20 for (OP-A 4). Out of 163 total bands, 121 were polymorphic, which revealed 74% polymorphism that reflected genetic diversity among the genotypes (Fig.1).

The genetic diversity assessed among eight cultivars of sorghum in this study using RAPD markers showed 74% polymorphism which was the same as previously found in sorghum by Menkir *et al.* (1997), and lower than (93.2%) detected by Thimmaraju *et al.* (2000), and much higher than the level of 55% reported by Tao *et al.* (1993). The variations in the genetic diversity observed in this study and in those referred to above, could be due to the use of different random primers as well as the variations that naturally occur in the genotypes (Nagaral *et al.*, 2009). High level of polymorphism based on RAPD technique has been reported among the genotypes of sorghum (Veirling *et al.*, 1994) and also in other crop species (David, 1995). The part of genetic variation observed in cultivated sorghum could be attributed to geographical diversity and domestication of sorghum from wild relatives (Doggett, 1988). From Table (4), it could be noticed that cultivar ICSR-93004 gave the highest number (4 bands) of

positive RAPD markers (unique bands), followed by Giza- 15 (3 bands); the other five cultivars showed just one unique marker. On the contrary, the cultivar ICSR-92003 was not detected by any specific marker using these 13 RAPD primers. These data also support the previous conclusion, on the attributes that affect genetic diversity.

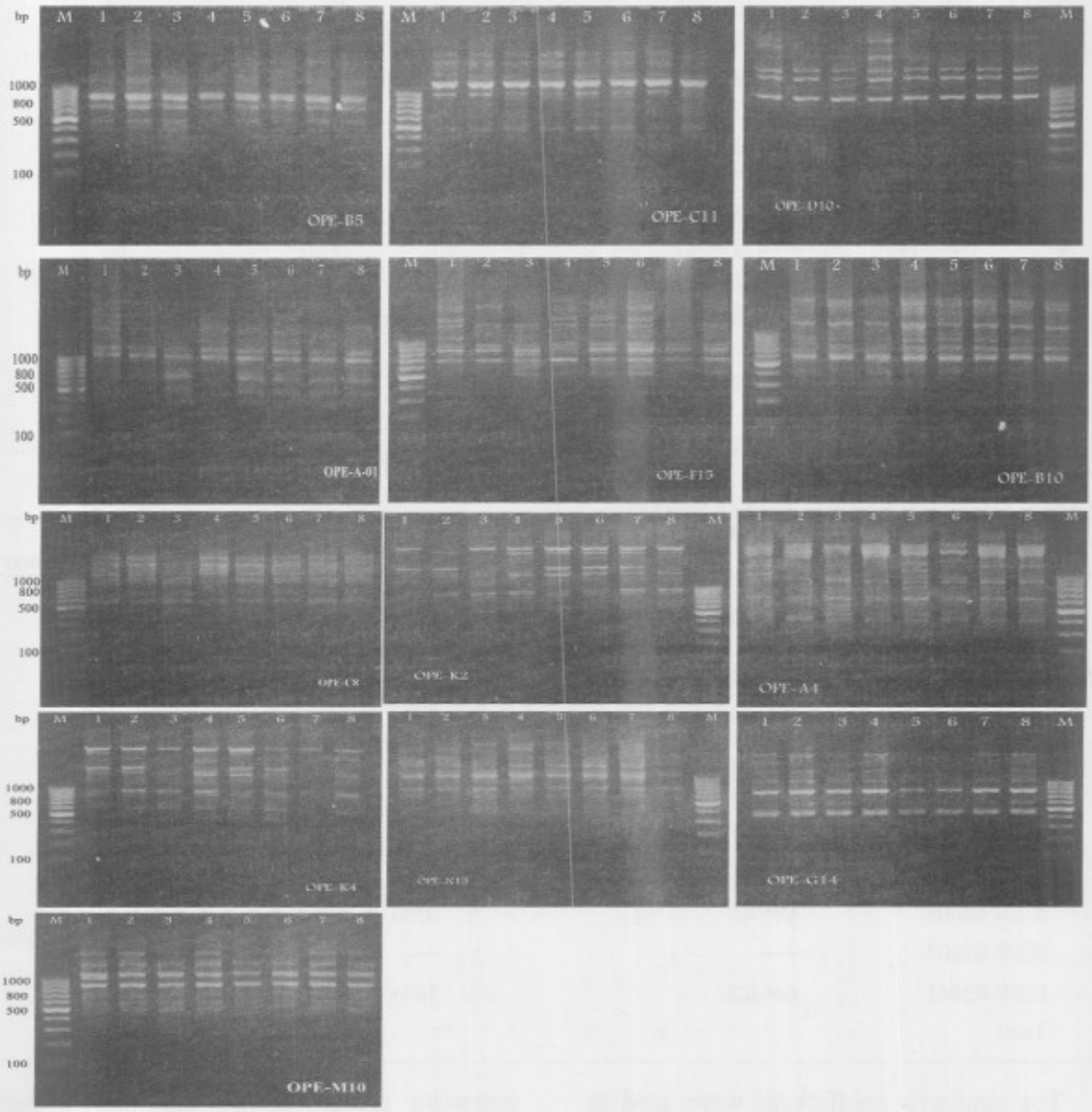

Fig. (1): RAPD banding patterns among eight sorghum genotypes using thirteen selected random primers, M: 100 bp plus DNA ladder (fermentas), 1-8: the sorghum cultivars Giza-15, NEB-Dorado-V9, ICSR-93004, ICSV-273, ICSB-88006, ICSB-88005, ICSR-92003 and ICSR-93001, respectively.

Table (3): The number of generated bands per primer, number of polymorphic bands and the percentages of polymorphism among eight sorghum genotypes revealed by RAPD markers.

No	Primers	Band size range (bp)	Generated bands	Polymorphic bands	% polymorphism
1	OP-A1	330-3703	11	8	72
2	OP-A4	240-2891	20	17	85
3	OP-B5	202-3387	10	6	60
4	OP-B10	304-2459	10	5	50
5	OP-C8	352-3217	13	8	61
6	OP-C11	423-2949	10	6	60
7	OP-D10	953-4078	10	7	70
8	OP-F15	618-4728	15	11	73
9	OP-G14	411-5787	9	6	66
10	OP-K2	411-4656	15	14	93
11	OP-K4	463-4499	16	15	93
12	OP-M10	324-2942	12	8	66
13	OP-N13	457-4030	12	10	83
	Total		163	121	74

Table (4): Genotype specific RAPD markers.

Genotype	RAPD primer	RAPD specific or unique marker size (bp)	Total bands/primer
Giza-15	OP-F15	1436	3
	OP-M10	1250	
	OP-C11	1863	
NEB-Dorado-V9	OP-A4	2392	1
ICSR-93004	OP-A4	273	4
	OP-B5	202	
	OP-M10	324	
	OP-K4	3730	
ICSV-273	OP-B10	304	1
ICSB-88006	OP-B5	2281	1
ICSB-88005	OP-A1	3703	1
ICSR-92003	-----	----	---
ICSR-93001	OP-K2	2874	1
Total			12

The similarity coefficients were used as an input data for cluster analysis and the resulting dendrogram is shown in (Fig. 2). Based on the dendrogram, the eight used genotypes are grouped into two major clusters A and B. Cluster B contained only one genotype (ICSB-88006) that shows highly divergent nature. The major cluster A comprised all the other genotypes which were further divided into two sub clusters (A1, contained only genotype ICSB-88005, and A2 contained six genotypes which were further

separated into two sub-clusters A3 and A4. Sub-cluster A3 contained ICSR-93001 only, while A4 was divided into ICSR-92003 (A5) and Giza-15 (A6) separately. The last sub-cluster A7 was finally divided to comprise NEB–Dorado-V9 (A8) and to the branch A9 which contained ICSR- 93004 (A10) and ICSV- 273 (A11) separately.

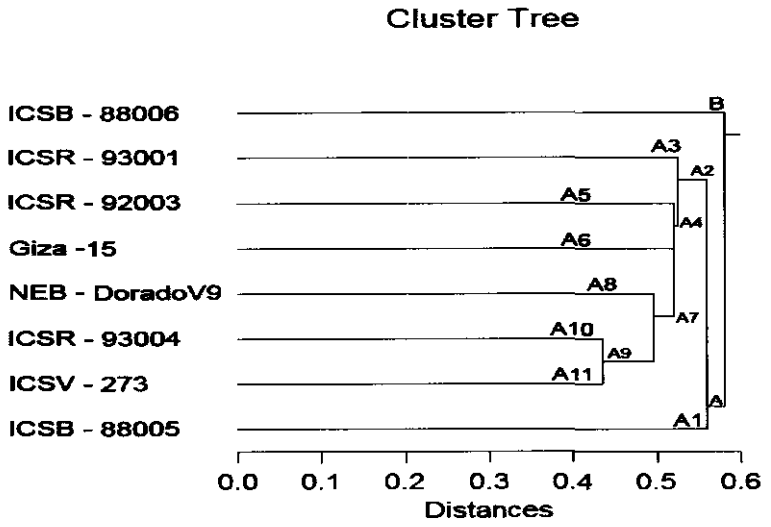

Fig. (2): Dendrogram for the eight sorghum genotypes by RAPD analysis.

Table (5): Genetic similarity matrices computed according to Dice Coefficient from RAPD of the eight sorghum genotypes of different origins.

	Giza-15	NEB-Dorado-V9	ICSR-93004	ICSV-273	ICSB-88006	ICSB-88005	ICSR-92003	ICSR-93001
Giza-15	1.000							
NEB-Dorado-V9	0.730	1.000						
ICSR-93004	0.760	0.631	1.000					
ICSV-273	0.650	0.760	0.601	1.000				
ICSB-88006	0.699	0.650	0.687	0.644	1.000			
ICSB-88005	0.809	0.668	0.668	0.656	0.803	1.000		
ICSR-92003	0.791	0.730	0.680	0.625	0.680	0.754	1.000	
ICSR-93001	0.638	0.680	0.662	0.638	0.674	0.656	0.717	1.000

The genetic relationships among the eight sorghum genotypes, based on the RAPD results from the 13 primers, were analyzed it using the Dice similarity coefficient to get the

genetic similarity matrices shown in Table 5. From the estimation of the relationships between the Egyptian sorghum cultivar Giza 15 and the other genotypes of different origins cultivated in Egypt as shown in Table (5) and the dendrogram (Fig. 2), it could be suggested that (Giza-15) local cultivar is closely related to ICSB-88005 (80.9%), ICSR-92003 by 79.1%, ICSR-93004 by 76% respectively, and to NEB-Dorado-V9 by 73%. While it was distantly related to ICSB-88006, ICSV-273 and ICSR- 93001 by 69.9, 65 and 63.8%, respectively. On the other hand, the examined genotypes represented different levels of genetic similarity percentages i.e. 80.3% between ICSB 88005 and 88006, 76% between NEB-DoradoV9 and ICSV-273 and 75.4% between ICSR-92003 and ICSB-88005. The least genetic distance (60.1 %) was found between ICSR-93004 and ICSV-273.

It can be concluded that RAPD markers are suitable to assess genetic diversity between the eight sorghum genotypes. Their genetic distance determined by RAPD markers may help to identify suitable germplasm for introgression into breeding stocks. This is in agreement with Nagaral *et al.* (2009) and Tao *et al.* (1993).

Peroxidase analysis in the sorghum plants

Isozymes are useful markers for studying genetic variation and evolution at the species level (Crawford, 1990). Peroxidase are enzymes related to polymer synthesis in cell wall (Bowles, 1990), as well as it plays an important role in the prevention of oxidative damage caused by environmental stress to the membrane lipids (Kalir *et al.*, 1984). In the present study, isozyme pattern are presented in Fig. (3) and Table (6) show the peroxidase profiles and the RF values of the eight sorghum genotypes were very similar where, all of them showed 4 bands at RF 0.20, 0.33, 0.52, and 0.61. While the band at RF 0.33 was not found in the Egyptian cultivar Giza-15. It could be concluded that isozyme profiles of peroxidase of the eight genotypes of *Sorghum bicolor* showed relatively relationship among them. This was reflected in some differences such as the absence of band (RF.0.33) in Giza -15. This agrees with Morden *et al.* (1990) who reported that the wild sorghums exceed the cultivars in all measures of diversity that they assessed. And Aldrich *et al.* (1992) reported that the levels of genetic diversity are greater in wild sorghum than in cultivated sorghum.

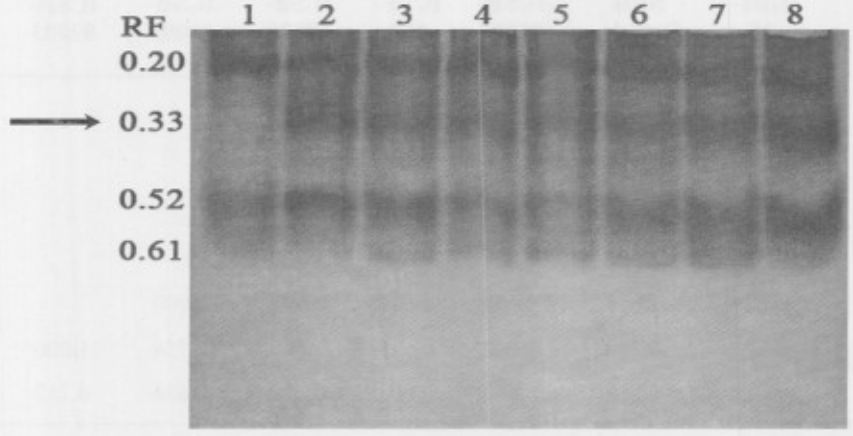

Fig.(3): Peroxidase isozyme profile among the eight sorghum genotypes, 1-8: the sorghum cultivars Giza 15, NEB-Dorado-V9, ICSR-93004, ICSV-273, ICSB-88006, ICSB-88005, ICSR-92003 and ICSR-93001, respectively.

Table (6): RF values for peroxidase isozyme in eight sorghum genotypes.

RF	Giza-15	NEB-Dorado-V9	ICSR-93004	ICSV-273	ICSB-88006	ICSB-88005	ICSR-92003	ICSR-93001
0.20	+	+	+	+	+	+	+	+
0.33	-	+	+	+	+	+	+	+
0.52	+	+	+	+	+	+	+	+
0.61	+	+	+	+	+	+	+	+

(+) presence and (-) absence.

Leaf protein profiles for sorghum cultivars (SDS-PAGE)

Comparative electrophoresis of proteins is a valuable method in plant biosystematics. The theoretical significance of using proteins for evaluating and taxonomic relationships among taxa has been amply discussed by Boulter *et al.* (1967). In the present study, protein banding patterns of the eight sorghum genotypes are presented in Fig. (4) and Table (7). SDS-protein patterns exhibited a maximum number of 9 bands, which are not necessarily present in all samples; five of them were polymorphic (55.5%). The protein band with the molecular size of 65.05 KDa is considered as a genotype specific band for the cultivar ICSB-88006 (Table 5). These data agree with Youssef *et al.* (2007) who reported that the low level of polymorphism was 21.4% in other crop (*Brassica napus* L.).

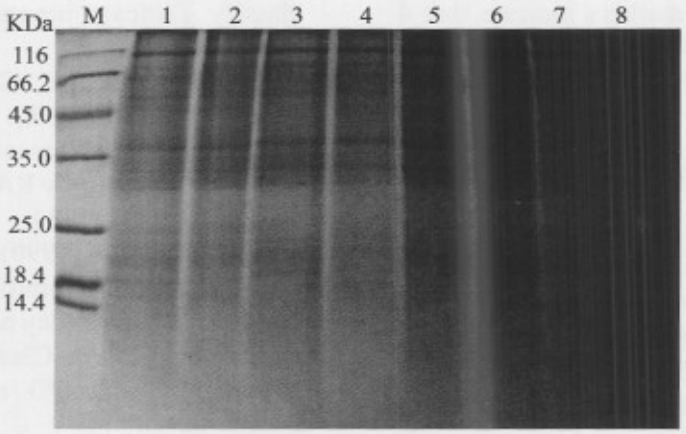

Fig. (4): The SDS-PAGE of total protein extracted from the leaves of eight sorghum cultivars the lanes M: protein marker, lanes 1-8 are the sorghum cultivars Giza-15, NEB-Dorado-V9, ICSR-93004, ICSV-273, ICSB-88006, ICSB-88005, ICSR-92003 and ICSR-93001, respectively.

Table (7): SDS-protein patterns in eight sorghum genotypes where (+) means presence of the band and (-) means absence. Broad-range protein marker was used to detect M.W. of the protein bands.

M.W (KDa)	Giza- 15	NEB-Dorado-V9	ICSR-93004	ICSV-273	ICSB-88006	ICSB-88005	ICSR-92003	ICSR-93001
80.57	+	+	+	+	+	+	+	+
74.36	+	+	+	+	+	+	+	+
65.05	-	-	-	-	+	-	-	-
56.00	-	-	-	-	-	+	+	-
40.19	+	+	+	+	+	+	+	+
39.76	-	+	+	+	+	+	+	+
34.79	+	+	+	+	+	+	+	+
32.10	+	+	+	-	+	+	+	+
20.93	+	+	+	-	+	+	+	+

As PCR techniques have been developed over the last 15 years, a wealth of new DNA marker technologies has arisen enabling the generation of high-density molecular maps for all the major crop species. Molecular markers have also been extensively used in analysis of genetic diversity in sorghum crop. Based on the data obtained by RAPD analysis, it was possible to discriminate between the eight sorghum genotypes used in the present study. The genotype-specific markers indicate that 4 markers distinguish the cultivar ICSR-93004 and three markers distinguish the cultivar Giza-15.

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استخدام الواسمات الجزيئية لتحديد التباعد الوراثي بين ثمانية تراكيب وراثية من السورجم

سوسن سامي يوسف* . رضا علواني مغيب* . عايدة ألفونس رزق الله** و سوزان مسعد أحمد**
*قسم الوراثة – كلية الزراعة – جامعة القاهرة *
**قسم الوراثة والسيتولوجي – المركز القومي للبحوث - الدقي - الجيزة

تهدف هذه الدراسة إلى تقييم الإختلافات الوراثية ودرجات القرابة بين ثمانية أصناف من السورجم بإستخدام كل من تحليلات الـ RAPD والبيروكسيدز و البروتين. تم إستخدام 13 بادىء عشوائى أعطوا 163واسم يتراوح وزنهم الجزيئى مابين 202 إلى 2787 زوج قواعد مما يعكس إختلافات كبيرة. و تتراوح عدد الواسمات لكل بادىء مابين تسعة واسمات للبادىء OP-G14 إلى 20 واسم للبادىء OP-A4 و كان عدد الواسمات الكلية التى تم تسجيلها 163منهم 121 واسم متباين أعطوا نسبة تباين وراثى 74%. تم تحديد الواسمات الجزيئية المحددة للصنف وأوضحت النتائج أن الصنف ICSR-93004 سجل 4 معلمات ايجابيه يتبعه صنف جيزة 15 الذى أعطى 13 واسم . فى حين أن باقى الأصناف أعطت واسم واحد فقط فيما عدا صنف ICSR-93003. و بالرغم من أن المصفوفات الوراثية تعتمد على شجرة القرابة ألا إنها أوضحت مستويات مختلفة من التشابه الوراثي . أوضحت نتائج التحليل أن عدد شرائط البروتين الكلية كانت 9 من بينهم 5 اشرطة متباينة بنسبة (55,5%) . أظهرت النتائج أن إنزيم البيروكسيدز أعطى 4 شرائط و كان الشريط الثانى هو الذى يمكن استخدامه فى تمييز الصنف جيزة 15 عن باقى الاصناف.