ASSESSMENT OF GENETIC DIVERSITY IN COWPEA (*Vigna unguicalata)* USING SDS-PAGE, RANDOM AMPLIFIED POLY-MORPHIC DNA (RAPD) AND INTER-SIMPLE SEQUENCE RE-PEAT (ISSR) MARKERS

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Cowpea (*Vigna unguicalata*) is a diploid species (2n=22) belonging to the genus *Vigna*, tribe phaseoleae and the family *Fabaceae*. Cowpea is an important legume throughout the tropics and subtropics covering Africa, Asia and Central South America, as well as parts of Southern Europe and United States (Singh *et al*., 1997; Kaga *et al*., 2000). The seed protein contents range from 23 to 32% of seed weight rich in lysine and tryptophan, and a substantial amount of mineral and vitamins (Hall *et al*., 2003). Also, the crop fixes 80% of its nitrogen requirement for growth from the atmosphere (Asiwe *et al.*, 2009), thereby reducing nitrogen fertilizer demand and costs of crop production. Several parts of cowpea are used in human consumption and animal feeding (Diouf, 2011). Asparaginase found in all cowpea cultivars is therapeutically important protein used in combination with other drugs in the treatment of acute lymphotic leukemia, Hodgkin's disease and melanosarcoma (Verma *et al*., 2007). Cowpea contains a low amount of fat and high level of fiber which can prevent heart disease by reducing the low-density lipoprotein (Phillips *et al*., 2003).

The development of genetic studies in cowpea will be promoted by its relatively small genome (Takeda and Matsuoka, 2008). The genetic diversity in cowpea seems to be narrow, in spite of substantial variation in seed color, seed proteins, and pod type and seed size among cultivated cowpea (Vaillancourt *et al*., 1993). Morphological traits may not be significantly distinct and usually require growing plants to maturity prior to identification. Moreover, morphological characters may be unstable due to environmental influences (Ghalmi *et al.*, 2010). Over the years, the methods for detecting and assessing genetic diversity have extended from analysis of discrete morphological traits to biochemical and molecular traits (Badr *et al.*, 1998; Zannou *et al.*, 2008; Badiane *et al*., 2012; Badr and Halawa, 2012; Barakat *et al.*, 2013).

SDS protein marker was used extensively to identify and study the genetic characters and relationships of many plants (Kakaei and kahrizi, 2011; Maged and Shawkat, 2012). Many authors recommended the use and applications of SDS-protein as rapid method to identify and characterize cowpea (Cerderia *et al*.,

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1985; Fotso *et al*., 1994; Freitas *et al*., 2004 and Oppong - Konadu *et al*., 2005).

Molecular markers have proved to play a considerable role in assessing genetic diversity between and within different species and individuals. Random amplified of polymorphic DNA (RAPD) and inter- simple sequence repeat polymorphic DNA (ISSR) have been widely used for genetic diversity studies (Ajibade *et al*., 2000; Lakhanapaul *et al*., 2000; Ba *et al*., 2004; Lavanya *et al*., 2008; Nkongolo *et al*., 2009; Saini *et al*., 2010; Huaqiang *et al*., 2013). RAPD and ISSR markers are advantageous over other markers because they are easier to use, less expensive, faster and involve non-radioactive substances (Nagaoka and Ogihara, 1997; Nicola and Valeria, 2002; Dikshit*et al*., 2007; Adnan and Katsuhiko, 2011; Malviya *et al*., 2012). RAPD analysis detects nucleotide sequence polymorphisms using a single primer of arbitrary nucleotide sequence (Williams *et al*., 1990). ISSR permits detection of polymorphisms in inter-microsatellite loci, using a primer designed from dinucleotide or trinucleotide simple sequence repeats. Different studies showed that inter simple sequence repeats markers have a high potential to identify polymorphism and determine genomic diversity across species as compared to other random primers (Souframanien and Gopala, 2004).

Since knowledge of genetic diversity is essential for evolving systemic breeding and conservation strategies, this work aims to: 1) study the genetic variation among Egyptian four cowpea cultivars using SDS-PAGE, RAPD and ISSR-PCR techniques 2) investigate the utility of biochemical and molecular approaches to establish genetic fingerprint for these cultivars.

MATERIALS AND METHODS

Plant materials

Four cowpea cultivars (Kareem 7, Dokki 331, Kaha 1 and Kafer El-Sheikh 1) were used in this investigation. Seeds of these cultivars were kindly supplied from Horticulture Research Institute, Agriculture Research Center (ARC), Giza, Egypt.

SDS-PAGE

Characterization of proteins profiles was carried out using one dimensional sodium dodecyle sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide slap gel (12%) was prepared according to Laemmli (1970). For each cowpea cultivar, ten dry seeds were milled together to a fine powder, and then 0.2 mL of sample buffer (0.2 M Tris-HCl pH 6.8, 2% SDS) was added to 0.02 g of seed meal and stored overnight at 4°C. Centrifugation was performed at 9000 rpm for 6 minutes and the 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% mercapto ethanol) in 1:1 ratio and denatured by heating at 90°C for 3 minutes. Equal amounts of

samples were 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 h. Protein bands were visualized by staining the gel using 0.25% coomassie brilliant blue (R-250). Molecular weights of different bands were calibrated with Sigma wide range molecular marker.

DNA isolation

Genomic DNA was extracted from the young leaves of the four cowpea cultivars by using DNA extraction kits (Kit DNase Qiagene). The concentration and purity of the extracted DNA was determined. Concentrations were adjusted at 6ng for all samples using TE buffer pH 8. Purity of DNA for all samples was between 90-97% and the ratio between 1.7-1.8 depicting high purity of the isolated DNA.

RAPD-PCR

Amplification of RAPD fragments was performed according to Williams *et al*. (1990). Ten primers (10 bp oligonucleotide) were used. The sequences of the used primers are shown in Table (1). PCR reactions were performed in 25 µl total volume, using 3µl from the extracted DNA and 3µl of each primer for amplification reaction. The polymerase chain reactions mixtures contain the entire necessary reagents except primer and the template DNA which were add to it. Amplification protocol was carried out using PCR System 9700 (Perkin Elmer, England) programmed for initial denaturation step at 94°C for 5 min, followed by 45 cycles each at 94°C for 30 sec, annealing at 36°C for 1 min and extension at 72°C for 2 min. and final extension at 72°C for 1 min.

ISSR-PCR

Ten ISSR primers were used; their names and sequences are shown in Table (1). PCR analysis was performed in 25 µl reaction as RAPD conditions and amplification was 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 50°C for 1 min, and an extension step at 72°C for 2 min, followed by an extension cycle for 7 min at 72°C in the final cycle. The products of both RAPD and ISSR-based PCR analyses were detected using agarose gel electrophoresis (1.2% in 1X TBE buffer), stained with ethidium bromide (0.3 µg/ml). PCR products were visualized on UV light; photographed and analyzed using a gel documentation system (Bio Rad Gel Doc-2000).

Data analysis

The RAPD and ISSR reproducible bands were scored as present (1) or absent (0), each of which was treated as independent locus regardless of its intensity. By comparing the banding patterns of genotypes for a specific primer, genotype-specific bands were identified. Faint or unclear bands were not considered. Band size was estimated by comparing with 1-kb ladder DNA (In vitrogen, USA). Ge-

netic similarity among cultivars was calculated according to Dice similarity coefficient and a dendrogram using unweighted pair group method with arithmetic average (UPGMA) was constructing.

RESULTS AND DISCUSSION

SDS-PAGE

Table (2) and Fig. (1) demonstrate the SDS-protein profiles of four cowpea cultivars used in this study. A maximum of 18 bands were detected with molecular weights ranging from 250 to 14 kDa. Only four polymorphic bands were recored with a percentage of 22.22%. One positive unique band at the molecular weight of 121 KDa was observed in the cultivar Kareem 7. On the other hand, three negative unique bands with the molecular weight of 96, 72, and 28 KDa were detected in all cultivars except cultivar Kareem 7, Kaha-1 and Kafer-El-Sheikh-1, respectively.

Protein profiles could be used as a general biochemical fingerprint for cowpea cultivars. In this investigation, low level of protein polymorphism was detected with the percentage of 22.22% which attributed to conservative nature of the seed protein (Bonfitto *et al*., 1999). Low level of protein polymorphism was reported in peach cultivars (Mansour *et al*., 1998), in mung bean cultivars (Hassan, 2001) and accessions belong to *Apiaceae* family (Sayed *et al*., 2012).

Seed protein of cowpea is known to contain 2 main kinds of proteins (globulins and albumins). Globulins are the major protein component in cowpea (Murray *et al*., 1983). Three major globulins are known which is responsible for the nutritional value of seed. α-vignin a minor globulin is composed by one main type of subunit 22 kDa while β-vignin is a glycosylated globulin, composed of two main polypeptides 55 and 60 kDa (Freitas *et al*., 2004).

RAPD-PCR

RAPD-PCR is one of the most widely used molecular techniques to detect polymorphism among cultivars of different plant species. This is due to its technical simplicity and low cost, compared to other molecular markers. The fingerprints generated by these primers revealed characteristic profiles of each cultivar.

In this study, 10 random arbitrary oligonucleotide primers were used to differentiate between the four cowpea cultivars and out of 10 primers, only seven primers generated strong amplification profiles with distinct bands that revealed extensive DNA polymorphism to cowpea cultivars (Table 3 and Fig. 2). The ten primers detected a total of 72 fragments (Table 3), with an average of 7.2 fragments per primer. The total number of amplified fragments varied from 3 (E-07) to 11 (B-10) primers.

Of the 72 amplified bands, 23 were polymorphic, with an average of 2.3 polymorphic bands per primer. The percentage of polymorphism ranged from 16.67%

with the primer (D-16) to 55.56% with the primer (A-19) with an average of 26.37%. Nine unique bands were identified out of the polymorphic ones. The number of bands detected by each primer depends on primer sequence and the extent of variation in specific cultivar.

From the previous results, it can be concluded that, the ten utilized primers generate relatively polymorphism within the studied cowpea cultivars (26.37%). The primer (A-19) and (C-20) were more successful in cultivar identification. The former primer generated 3 unique bands, while the latter produced 2 unique bands. The primer (A-19) produced clear unique banding patterns for 3 cowpea cultivar (Kareem-7, Dokki-331 and Kafer-EL-Sheikh-1) and can be used to distinguish between them (Table3). In this respect, Zannou *et al.* (2008) studied the potential application of RAPD techniques in determining genetic diversity among cultivated cowpea varieties and recorded a total of 32 amplified bands.

ISSR-PCR

ISSR-PCR technique was applied to numerous plant genetic studies as it overcomes most limitations of other markers. ISSR covers a large portion of the genome, because microsatellites are abundant throughout the genome. The ISSR fingerprinting pattern revealed by ten primers is shown in Fig. (3) and Table (4). ISSR primers amplified a total number of 110 bands, of which 32 were polymorphic fragments and 19 unique markers. The number of bands ranged from 8 (U_2, U_4, U7 and U_{27}) to 15 (U_9). Average number of polymorphic bands per primer was 3.2 and the percentage of polymorphism ranged between 16.67% (U_{18}) and 75% (U_{27}) with an average of 28.27%. The results presented here show that ISSR primers are able to reveal variability among the cowpea cultivars. This polymorphism has three main functions as a mean of identifications, to detect genetic erosion and to reveal genetic relationships.

Primers with higher polymorphic bands are more efficient in studying genetic diversity. In the present investigation, the percentage of polymorphism of RAPD and ISSR markers recorded 26.37% and 28.27%, respectively. This indicates that the efficiency of ISSR markers in terms of amplification of a large number of polymorphic fragments as compared with protein and RAPD (Tables 2, 3 and 4). Variation in the level of polymorphism detected by RAPD or ISSR assays could be due to different primers used (Malviya, 2012).

Genetic relationships based on RAPD and ISSR profiles

Knowledge of genetic relationships among genotypes is useful in plant breeding programs. Genetic similarity was calculated from the dice similarity index value for the 4 cultivars of cowpea considering RAPD and ISSR approaches individually as well as together. In this study, the investigated cowpea genotypes showed a genetic similarity using RAPD profiles ranged from 0.92 to 0.69 (Table 5). The highest genetic similarity was between

kareem 7 and Dokki331, while the lowest was between genotypes Dokki 331 and Kafer El-Sheikh 1. On the other hand, ISSR markers showed genetic similarity ranged from 0.92 (Dokki 331 and Kaha 1), to 0.78 (Dokki 331 and Kafer EL-Sheikh 1). Based on combined data high genetic similarity (0.89) was observed between Kareem 7 and Dokki 331, while the lowest genetic similarity of 0.74 was between Dokki 331 and Kafer EL-Sheikh 1 (Table 5). These results indicate that the two genotypes, Dokki 331 and Kafer El-Sheikh 1 are the most diverse type. Cultivars showing the lowest genetic similarity are of great concern to plant breeders to be further selected as parents. Weising *et al*. (2005) mentioned that it is mandatory that genetically divergent parents could be chosen which exhibit sufficient polymorphism, but are not so distant as to cause sterility of the progeny. In the present investigation, the results revealed that different types of profiles expressed different levels of genetic similarity among the four cultivars. This could be due to the different mechanisms of polymorphism detection by the different bands type. The accuracy of genetic similarity estimates based on molecular data depends on several variable factors such as the number of bands analyzed, their distribution over the genomes and the accuracy in scoring them.

A dendrogram obtained from UPGMA cluster analysis based on protein analysis classified the 4 cultivars into two groups (Fig. 4). Only cultivar Kareem 7 was isolated in a single group, while the second group contained the other three cultivars. Dendrogram based on the ten RAPD profiles revealed two main genetic clusters (Fig. 4). The first cluster comprises the cultivar Kareem 7 and Dokki 331, while the second cluster includes Kaha 1 and Kafer El-Sheikh 1. On the other hand, a dendrogram based on the ten ISSR data revealed two main genetic clusters. Cultivar Kafer El-Sheikh 1 separated in a single group, while the second cluster includes the other three cultivars (Kaha 1, Dokki 331 and Kareem 7). The second cluster was divided into two subgroups. The first subgroup comprises cultivar Kareem 7, whereas the second subgroups include cultivars Dokki 331 and Kaha 1. Dendrogram obtained from combined data are in agreement with that of ISSR dendrogram in which cultivar Kafer El-Sheikh-1 was separated in a single group, while the SDS-PAGE and RAPD dendrogram showed some variations in the clustering of cowpea cultivars.

Considering all the gained data, it is evident that molecular markers RAPD and ISSR are good tools in assessing genetic variation, germplasm identification among the four cultivars of cowpea. These markers provide interesting tools for breeding new varieties in Egyptian cowpea.

SUMMARY

Genetic diversity of four cultivars of Egyptian cowpea (*Vigna unguicalata*) was studied by SDS-PAGE, RAPD and ISSR-PCR. SDS-PAGE recorded a low degree of polymorphism among the four

cultivars of cowpea. Only four polymorphic bands were recorded with a percentage of 22.22%. On the other hand, RAPD-PCR generated polymorphism among the DNA samples of the studied cultivars. The ten primers generated a sum of 23 polymorphic bands in the cultivars under study. Nine unique bands were identified out of the polymorphic ones. The percentage of polymorphism ranged from 16.67% to 55.56% with an average of 26.37%. ISSR bands generated higher level of polymorphism than RAPD among the DNA samples of the studied cowpea cultivars. The ten ISSR primers generated a sum of 32 polymorphic bands in the cultivars under study. Nineteen unique bands were identified out of the polymorphic ones. The percentage of polymorphism ranged between 16.67% and 75% with an average of 28.27%.

A dendrogram obtained from UPGMA cluster analysis based on protein analysis classified the four cultivars into two groups. Only cultivar Kareem 7 was isolated in a single group, while the second group contained the other 3 cultivars. Dendrogram based on the ten RAPD primers revealed two main genetic clusters. The first cluster comprises the cultivar Kareem 7 and Dokki 331, while the second cluster includes Kaha 1 and Kafer El-Sheikh 1. On the other hand, a dendrogram based on the ten ISSR data revealed two main genetic clusters. Cultivar Kafer El-Sheikh 1 separated in a single group, while the second cluster includes the other three cultivars. Dendrogram obtained from combined data are inagreement with that of ISSR dendrogram in which cultivar Kafer El-Sheikh-1 was separated in a single group, while the SDS-PAGE and RAPD dendrograms showed some variations in the clustering of cowpea cultivars.

Considering all the gained data, it is evident that molecular detection of RAPD and ISSR are suitable tools than SDS-PAGE in assessing genetic variation among the four cultivars of cowpea. These markers provide interesting tools for breeding new varieties of Egyptian cowpea.

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Table (1): RAPD and ISSR primers used for identification of four cultivars of cowpea.

Serial	RAPD		ISSR	
	Primers	Primer sequence 5'→3'	Primers	Primer sequence 5'→3'
1	(A-19)	CAAACGTCGG	U2	$(AG)_8YC$
2	(B-10)	CTGCTGGGAC	U4	$(AG)_8YG$
3	(C-16)	CACACTCCAG	U7	$(AG)_8T$
4	(C-20)	ACTTCGCCAC	U8	$(AG)_8C$
5	(D-01)	ACCGCGAAGG	U9	$(AG)_8G$
6	(D-16)	AGGGCGTAAG	U10	$(GA)_8T$
7	(D-18)	GAGAGCCAAC	U11	$(GA)_8C$
8	(E-03)	CCAGATGCAC	U15	$(CT)_8G$
9	(E-07)	AGATGAGCCG	U18	$(CA)_8G$
10	(G-03)	GAGCCCTCCA	U27	$(TG)_8A$

Table (2): The presence (+) and absence (-) of seed protein bands of four cowpea cultivars.

Band No.	MW KDa.	Lane-1 Kareem-7	Lane-2 Dokki-331	Lane-3 Kaha-1	Lane-4 Kafer-El-Sheikh-1
1	250	+	+	+	+
2	197	+	+	+	+
3	121	+	-	-	-
4	96	-	+	+	+
5	92	+	+	+	+
6	85	+	+	+	+
7	72	+	+	-	+
8	66	+	+	+	+
9	50	+	+	+	+
10	40	+	+	+	+
11	36	+	+	+	+
12	30	+	+	+	+
13	28	+	+	+	-
14	26	+	+	+	+
15	22	+	+	+	+
16	18	+	+	+	+
17	16	+	+	+	+
18	14	+	+	+	+
Total	18	17	17	16	16
% of polymorphism		22.22%			

Table (3): Percentage of polymorphism and number of total, polymorphic and unique bands generated by ten RAPD primers with the four cowpea cultivars (1: Kareem-7, 2: Dokki-331, 3: Kaha-1, 4: Kafer El-Sheikh-1).

Primers	Total No. of bands	No. of polymorphic band		Unique band		Polymorphism %
		unique	Non unique	size (bp)	cowpea cultivar	
(A-19)	9	3	2	486 225 180	(4) (1) (2)	55.56%
(B-10)	11	1	3	1073	(1)	36.36%
(C-16)	4	0	0	0.00	0.00	0.00
(C-20)	10	2	2	678 447	(4) (4)	40.00%
(D-01)	9	1	1	182	(2)	22.22%
(D-16)	6	1	0	405	(3)	16.67%
(D-18)	7	0	3	0	0	42.86%
(E-03)	5	0	0	0	0	0.00
(E-07)	3	0	0	0	0	0.00
(G-3)	8	1	3	225	(3)	50.00%
Total	72	9	14			Average = 26.37%

Table (4): Percentage of polymorphism and number of total, polymorphic and unique bands generated by ten ISSR primers with the four cowpea cultivars (1: Kareem-7, 2: Dokki-331, 3: Kaha-1, 4: Kafer El-Sheikh-1).

Primers	Total No. of bands	No. of polymorphic		Unique band		Polymorphism%
		unique	Non unique	size (bp)	cowpea cultivar	
U2	8	00	0	0.00	0.00	0.00
U4	8	0	0	0.00	0.00	0.00
U7	8	2	0	379 420	(1) (2)	25.00%
U8	13	3	1	486 1119 1219	(4) (1) (2)	30.77%
U9	15	6	0	212 239 261 565 606 1410	(1) (1) (1) (2) (4) (1)	40.00%
U10	14	3	1	218 904 988	(4) (3) (3)	28.57
U11	12	0.00	5	0	0	41.67%
U15	12	3	0	342 634 728	(4) (4) (4)	25.00%
U18	12	0	2	0.00	0.00	16.67%
U27	8	2	4	379 420	(1) (2)	75.00%
Total	110	19	13			Average = 28.27%

Table (5): Similarity coefficient of the four cowpea cultivars.

RAPD data analysis				
	Kareem-7	Dokki-331	Kaha-1	Kafer-El-Sheikh-1
Kareem-7	1.00			
Dokki-331	0.92	1.00		
Kaha-1	0.73	0.75	1.00	
Kafer-El-Sheikh-1	0.72	0.69	0.84	1.00
ISSR data analysis				
	Kareem-7	Dokki-331	Kaha-1	Kafer-El-Sheikh-1
Kareem-7	1.00			
Dokki-331	0.86	1.00		
Kaha-1	0.88	0.92	1.00	
Kafer-El-Sheikh-1	0.80	0.78	0.80	1.00
Combined data				
	Kareem-7	Dokki-331	Kaha-1	Kafer-El-Sheikh-1
Kareem-7	1.00			
Dokki-331	0.89	1.00		
Kaha-1	0.81	0.84	1.00	
Kafer-El-Sheikh-1	0.76	0.74	0.82	1.00

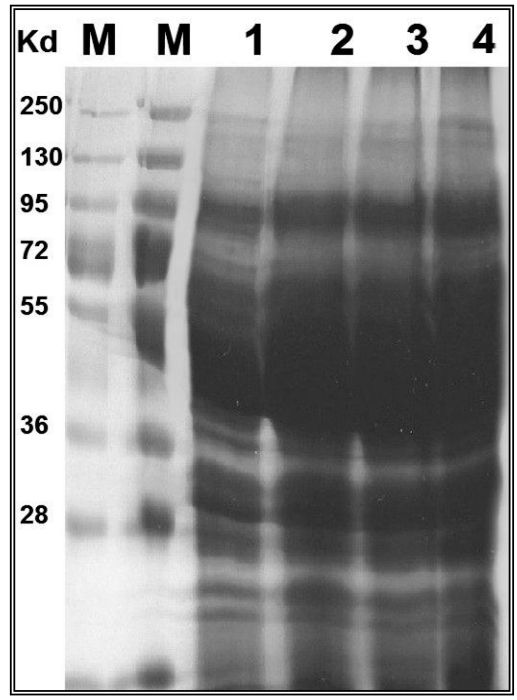

Fig. (1): SDS-protein banding patterns of seeds of four cowpea cultivars.
M= Molecular marker
Lane 1: Kareem 7
Lane 2: Dokki 331
Lane 3: Kaha 1
Lane 4: Kafer El-Sheikh 1

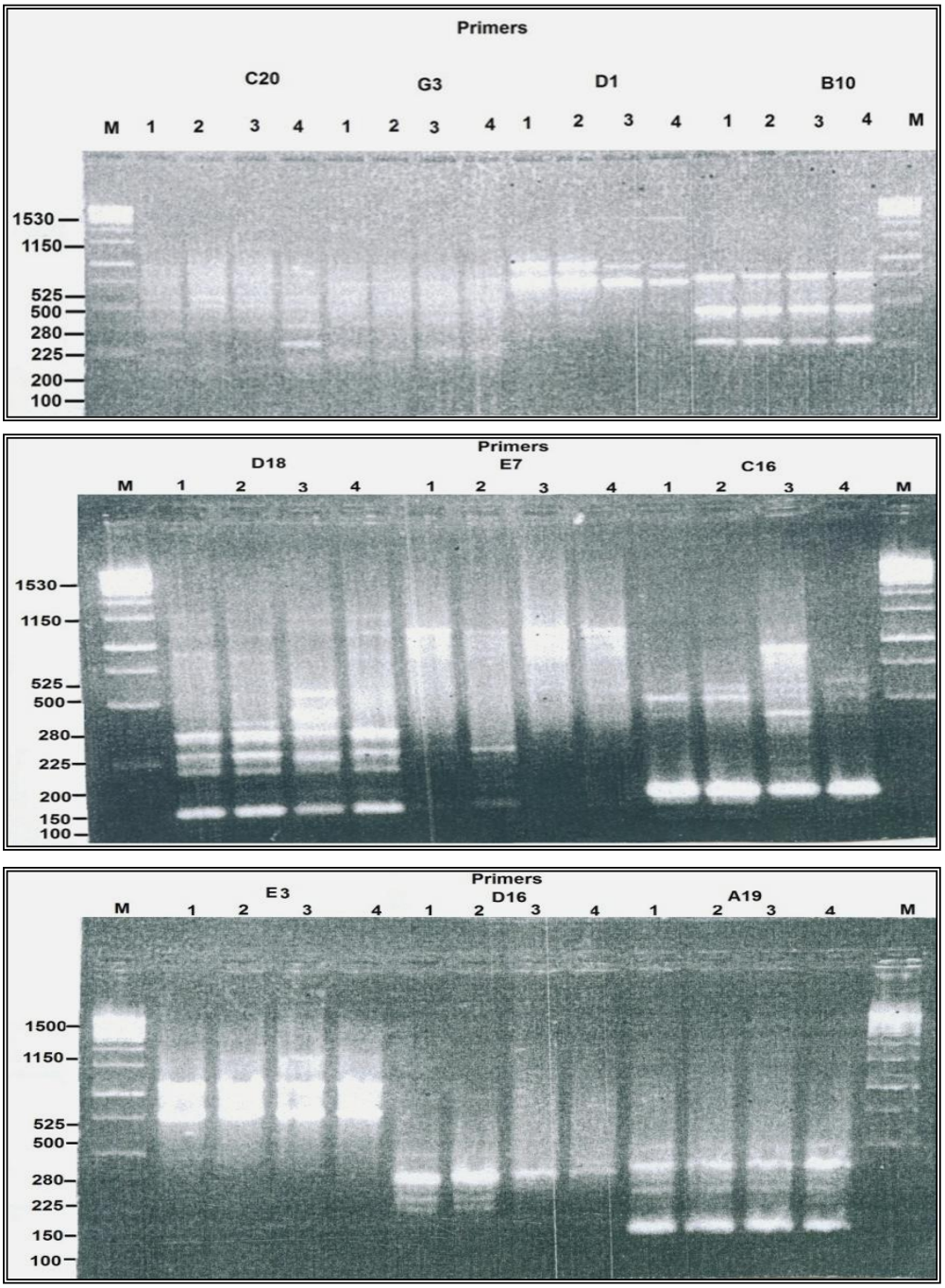

Fig. (2): RAPD profiles of the four cowpea cultivars using ten primers: (C20, G3, D01, B10, D18, E7, C16, E3, D16 and A19). M refers to DNA ladder marker 1 Kb, Lane 1: Kareem 7, Lane 2: Dokki 331, Lane 3: Kaha 1 and Lane 4: Kafer El-Sheikh 1.

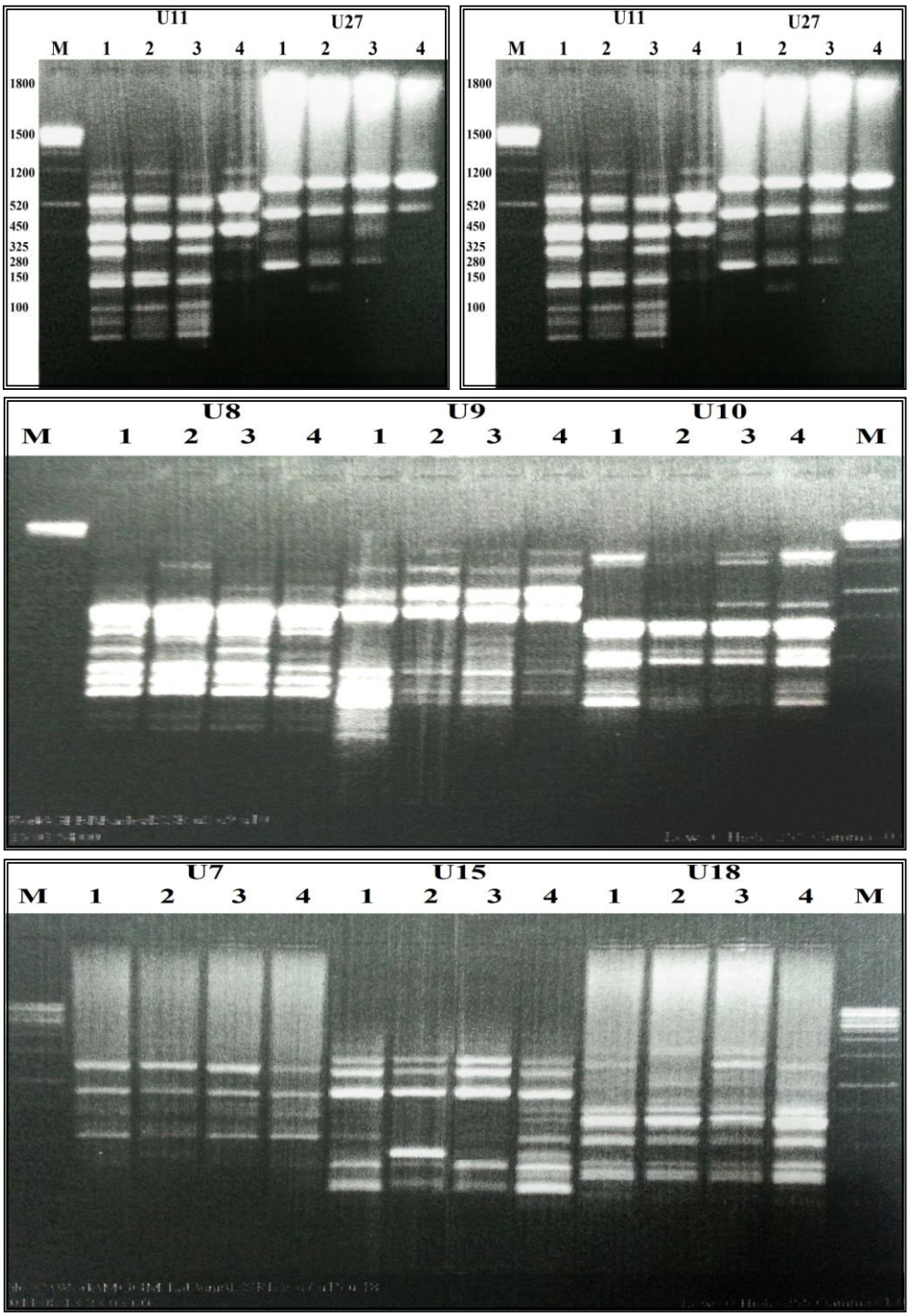

Fig. (3): ISSR profile of four cowpea cultivars using ten primers: (U11, U27, U8, U9, U10, U2, U4, U7, U15 and U18). M refers to DNA ladder marker 1 Kb, Lane 1: Kareem 7, Lane 2: Dokki 331, Lane 3: Kaha 1 and Lane 4: Kafer El-Sheikh 1.

(a) SDS- PAGE (b) RAPD

(c) ISSR (d) Combined data (ISSR, RAPD, Protein)

Fig. (4): UPGMA dendrogram among cowpea cultivars (a) SDS-PAGE (b) RAPD (c) ISSR (d) combined data (protein, ISSR and RAPD) 1 = Kareem 7, 2 = Dokki 331, 3 = Kaha 1 and 4 = Kafer El-Sheikh 1.