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Molecular markers and genetic variation revealed by RAPD-PCR in seven cowpea (*Vigna unguiculata* (L.) Walp.) cultivars

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Abstract

Cowpea is one of the most important protein rich legumes worldwide. This study used the Random Amplified Polymorphic DNA (RAPD) assays to reveal the positive and negative DNA markers in seven cultivars of cowpea, which could use for cultivars identification and discrimination, for future breeding programs and derivation of novel genotypes. Also, to evaluate the proportion of genetic relationship among the cultivars by detecting the genetic polymorphism at molecular level and correlate the obtained results with some morphological traits and source of cultivars. RAPD Results showed that a total number of 56 DNA fragments were amplified, ranged in size between 1637 bp and 163 bp, while the total polymorphic bands number was 45 and the percentage of polymorphism was 80.3 %. Five cultivars (Cream-12, Chinese red, Blackeye crowder, Brown crowder, and Cream-7) appeared to have whether positive or negative markers with different molecular weight, while Azmerly and Dokki-331 cultivars did not produce any specific bands. The dendrogram of RAPD data showed that the 7 cultivars of cowpea were grouped in 6 clusters based on genetic similarities; the highest value of similarity was 86.6 % between Azmerly and Dokki-331, while the lowest was between Cream-12 and Blackeye crowder with a value of 54.2 %. The obtained results suggest that RAPD markers were better linked to the source of the cultivar and to the extent of seed crowding in pod trait, while the seed colour and the growth habit traits might or might not show correlation with the molecular data.

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Introduction

The genus *Vigna* has many cultivated species, but cowpea is the most economically important species among all these species (Ajibade *et al.*, 2000). Cowpea is one of the most important grain legumes in Africa (Diouf and Hilu, 2005), it grown extensively in the tropics and sub-tropics of savannah regions, considered as a major food in many areas especially in western and central African countries where almost every part of the plant is eaten (Ouédraogo *et al.*, 2002), in addition to parts of north and South America "cultivated widely in California, Puerto Rico and Brazil", and Asia as well. Nigeria is the largest producer of cowpea (Udensi *et al.*, 2016) with 3, 027, 596 tonnes (FAO, 2016), however Niger ranked as the largest in terms of cultivated area with 5,192,100 hectares (FAO, 2016), cowpeas seeds considered a rich source of proteins (Khan *et al.*, 2015) carbohydrate as well as minerals and vitamins. Cowpea also used as an animal fodder in many areas worldwide (Nagalakshmi *et al.*, 2017).

In Egypt cowpea considered as one of the major legume crops, consumed as green bods and dry seeds. The area of cultivated cowpea in Egypt was 1968 hectares for dry seeds, with estimated production 7162 tonnes, and Average production 3.6 tonnes per hectare (FAO, 2016). Also, Egypt considered as one of important countries in exporting cowpea especially green pods as a fresh crop and semi-processed ready to consume frozen green pods.

Generally RAPD-PCR protocols is one of the widely used tool to estimate the genetic variation among and within plant cultivars (Kumar and Gurusubramanian, 2011;Kumari and Thakur, 2014; Obiadalla-Ali *et al.*, 2015). In cowpea a small number of investigation have been done via RAPDs (Ba *et al.*, 2004). According to Ali *et al.*, (2015)RAPD technique is used widely to estimate the genetic diversity among legumes. RAPD-PCR protocol is considered as a potential technique to determine the bezel of cultivar purity and providing genetic bases for the genetic mapping as an assisting marker in plant improvement either by plant breeding or plant biotechnology methods. The aims of this investigation are concerning about revealing of molecular markers and evaluate the proportion of genetic relationship among seven cultivars of cowpea "local and foreign", which could also play many important roles as an assistant-marker in correlation with other investigation among and within these cultivars.

Materials and methods

Plant material

Seven of cowpea (*Vigna unguiculata* (L.) Walp.) cultivars (Azmerly, Cream-12, Dokki-331, Chinese red, Blackeye crowder, Brown crowder and Cream-7) were used in the present study (Tabel 1).

The cultivars were collected from two sources and they vary in the growth habits, seed colour and extent of seed crowding in pod "pod-filing", as described in table (1). The cultivars were planted on April 1st, 2017, in the open field of vegetable experimental farm, Faculty of Agriculture, Assiut University.

Genomic DNA extraction

Few grams of fresh young (6 weeks) leaves were harvested from five plants of each cultivar. The cultivars leaves were then used for the extraction of the DNA using the CTAB method described by (Murray and Thompson, 1980; and Saghai-Maroof *et al.*, 1984) with some modifications. Plant tissues were grounded in 1 ml of extraction buffer (100mM Tris-HCl, 1.4M NaCl, 20mM EDTA, 2% CTAB) and then incubated at 65°C(with frequent gentle shaking) for 30 min. Then 50 µl β-mercaptoethanol was added to the samples. Mixture of chloroform: isoamyl alcohol (24:1) were then added to the samples (An equal volume for each tube), then samples were hand-blended by light inversion in order to form an emulsion. Samples were centrifuged at 13000rpm for 10 minutes at 15°C. The supernatants were then transferred to new Eppendorf tubes, then equal volumes of cold (-20°C) Ethanol: isopropanol (1:1) were added to the supernatant. Samples were then placed in the fridge and left overnight at 4°C to assure precipitation. Next day, samples were centrifuged at 13000 rpm at 4°C for 5 minutes. The supernatants

were removed from the tubes and the pellets were then washed with 75% ethanol (v/v) and centrifuged afterwards at 10000 rpm at 4°C for 2 minutes, this step was repeated twice. The pellets were then air-dried by left overnight at room temperature. Afterwards the pellets were dissolved in 50µl distilled sterile water; these DNA dilutions were performed to adjust the optimum concentration for RAPD-PCR analysis.

RAPD markers and the PCR conditions

To study the genetic differences (Genetic fingerprinting) among those cultivars, the random amplified polymorphic DNA (RAPD) assays described by Williams *et al*., (1990) were used.

This technique is based on the polymerase chain reaction (PCR) of random sites spread all over the genomic DNA using random short (ten-mer) primers. Seven random oligonucleotide primers (OPAB-4, OPA-8, OPA-12, OPE-5, OPN-3, OPP-2 and r-1302.1) were used in the present study. Their codes and sequences are shown in table (2). The PCR mixture was prepared by mixing 2 µL of DNA sample, 2.5 µL 10x PCR buffer, 0.5 µl of 10 mM dNTP stock (10 mM of each dNTP) and 0.2 µl of 10 µM primer stock and 1 U of Taq DNA polymerase in a final volume of 25 µL. The thermocycler was programmed as follows: initial denaturation for 5 minutes at 95°C, then 35 cycles of 1 minute at 95°C, 1 minute at 33°C and 2 minutes at 72°C, then followed by 5 minutes at 72°C and a final hold at 10°C. The amplified PCR products were then analyzed by standard gel electrophoresis using 0.8 % agarose gel, and a Bioline-Hyperladder 100 bp plus as molecular weight marker. Following electrophoresis, gels were stained with ethidium bromide (10 mg/ml) and visualized under U.V. light.

Data analysis

The Gene Profiler 4.03 computer software program was used to analyze the agarose gel photos to detect the presence of the bands and calibrating them for size. A binary data matrix recording the presence (1) or the absence (0) of bands was made.

The software package MVSP (Multi-Variate Statistical Package) was used to calculate the genetic similarities using the Dice coefficient of similarity (Nei and Li, 1979). The results were then represented as a dendrogram for each primer and allover primers.

Results and discussion

RAPD-PCR protocol was preceded and the agarose electrophoresis derived from RAPD-PCR products of the seven cowpea cultivars are shown in figure (1).

Table 1. Cowpea Cultivars' growth habits, seed colours, seed crowding in pods and sources.

No.	Cultivar	Growth habit	Seed colour	~~Extent of~~ Seed crowding in pod	Source
1	Azmerly	Determinate	White with black eye	Non-crowder	Agriculture research centre, Egypt.
2	Cream-12	Determinate	Yellowish-white	Non-crowder	Dr.Miller, Texas A&M University, USA.
3	Dokki-331	Determinate	White with black eye	Non-crowder	Agriculture research centre, Egypt.
4	Chinese Red	Indeterminate	Red	Non-crowder	Dr.Miller, Texas A&M University, USA.
5	Blackeye crowder	Determinate	White with black eye	crowder	Dr.Miller, Texas A&M University, USA.
6	Brown crowder	Determinate	Dark Brown	crowder	Dr.Miller, Texas A&M University, USA.
7	Cream-7	Determinate	Yellowish-white	Non-crowder	Agriculture research centre, Egypt.

Then data were used to come across with the results of RAPD-PCR analysis obtained from all the primers as shown in table (**3**) with the total number of bands (a), Size range (bp), number of polymorphic bands (b) and polymorphism percentage (b/a x 100). A total no. of 56 DNA fragments were amplified ranged in size from 1637 bp to 163 bp while the total polymorphic bands number was 45 and the percentage of polymorphism was 80.3 % (Table 3). Unique DNA fragments (positive and negative markers) of cowpea

cultivars and their molecular weight (bp) detected by the different employed primers are shown in table (**4**), whilst the genetic similarity percentage (%) calculated from the DNA fragments among the seven cowpea cultivars are represented in table (**5**). The dendrogram demonstrating the relationship among the seven cowpea cultivars based on data recorded from the RAPD markers polymorphism are revealed in figure (2).

Table 2. Primers codes and sequences and GC percentages (%).

Primer code	Sequence (5' to 3')	GC(%)
OPAB-4	5'- GGCACGCGTT -3'	70
OPA-8	5'- GTGACGTAGG -3'	60
OPA-12	5'- TCGGCGATAG -3'	60
OPE-5	5'-TCAGGGAGGT-3'	60
OPN-3	5'- GGTACTCCCC-3'	70
OPP-2	5'- TCGGCACGAC -3'	70
r-1302.1	5'- GGAAATCGTG -3'	50

Molecular markers

Unique DNA fragments with different molecular weight were detected in some cultivars but not in the others (Table 4). It could be used as positive DNA markers for the cultivars identification and discrimination. As well as the absence of a common band in a specific cultivar is referred to be a negative marker (non-amplified fragment) and could be also used as negative DNA markers which is might be useful for future breeding programs and derivation of plant lines (Saker, 2005).

Table 3. RAPD polymorphism obtained from the seven cowpea cultivars.

Primer	Total number of bands (a)	Size range (bp)	Number of polymorphic bands (b)	Polymorphism b/a x 100 (%)
OPAB-4	12	1637-224	6	50
OPA-8	11	1625-163	11	100
OPA-12	9	1564-254	9	100
OPE-5	8	1477-277	5	62.5
OPN-3	6	1484-496	6	100
OPP-2	7	1399-665	6	85.7
r-1302.1	3	893-713	2	66.6
Total	56	1637-163	45	80.3

According to the positive and negative markers in Table (4), 5 cultivars (Cream 12, Chinese red, Blackeye crowder, Brown crowder, and Cream-7) appeared to have whether positive or negative markers with different molecular weight.

On the other hand, the other two cultivars (Azmerly & Dokki-331) did not show any specific bands (positive or negative marker) with all the seven used primers, since all the produced bands in both Azmerly & Dokki-331 cultivars where common bands with other cultivars.

Results in Table (4) also showed that the Chinese red cultivar had 5 markers, they were all positive markers. They were produced from 4 primers (1165, 224 bp by OPAB-4; 945 bp by OPA-8; 948 bp by OPA-12; 713 bp by r-1302.1). These bands should be

further investigated to know if they were correlated to whether the indeterminate growth habit or to the red seed colour. Blackeye crowder, Cream-12 and Cream-7 cultivars appeared to have only negative markers. OPA-8 primer produced 3 negative markers with molecular weight of 1050, 465, 390 bp when applied on the Blackeye crowder cultivar. Cream-12 have 3 negative markers with molecular weight of 1025, 933, 803 bp when the OPE-5 primer was used, while Cream-7 got only one negative marker with molecular weight of 1322 bp produced by the OPN-3 primer. The Brown crowder cultivar got 2 positive markers with primers OPA-8, and OPP-2 and the amplified DNA fragments were at molecular weight of 646 and 665 bp respectively (Table 4).

Table 4. Unique DNA fragments (positive and negative markers) of cowpea cultivars and their molecular weight (bp) detected by the different employed primers.

Primer	Cultivar	Positive marker (amplified fragment) (bp)	Negative marker (non-amplified fragment) (bp)
OPAB-4	Chinese red	1165	-
		224	-
OPA-8	Chinese red	945	-
	Blackeye crowder	-	1050
		-	465
		-	390
	Brown crowder	646	-
OPA-12	Chinese red	948	-
OPE-5	Cream-12	-	1025
		-	933
		-	803
OPN-3	Cream-7	-	1322
OPP-2	Brown crowder	665	-
r-1302.1	Chinese red	713	-

Genetic similarity matrix and cluster analysis.

Results of the presence / absence of DNA fragments were analysed using MVSP program of Nei and Li, (1979), and pair-wise comparisons between the tested cultivars were used to calculate the genetic similarity (Table 5), and to draw the dendrogram (Fig. 2). The data in figure (2) showed that the 7cultivars of cowpea were grouped in 6 clusters based on genetic similarities given in table (5).

Table 5. Genetic similarity percentage (%) calculated from the DNA fragments amplified from the seven cowpea cultivars using seven random primers.

	Azmerly	Cream-12	Dokki-331	Chinese red	Blackeye crowder	Brown crowder	Cream-7
Azmerly	100						
Cream-12	71.2	100					
Dokki-331	86.6	73.3	100				
Chinese red	66.7	61.8	68.4	100			
Blackeye crowder	75.8	54.2	62.7	72.0	100		
Brown crowder	69.4	67.7	68.5	76.5	77.8	100	
Cream-7	63.3	64.2	65.6	69.6	66.7	72.7	100

The highest value of similarity was of 86.6 % between Azmerly and Dokki-331 Cultivars. Both Azmerly and Dokki-331 formed the 1st cluster that was distinct from other cultivars (Fig. 2). This distinctive highest value of similarity could be due to several reasons; their seeds are morphologically similar in the colour (white seeds with black-eye); they're local cultivars and were collected from the same site (Agriculture research centre, Egypt); and finally, they have the same growth habit (Determinate).

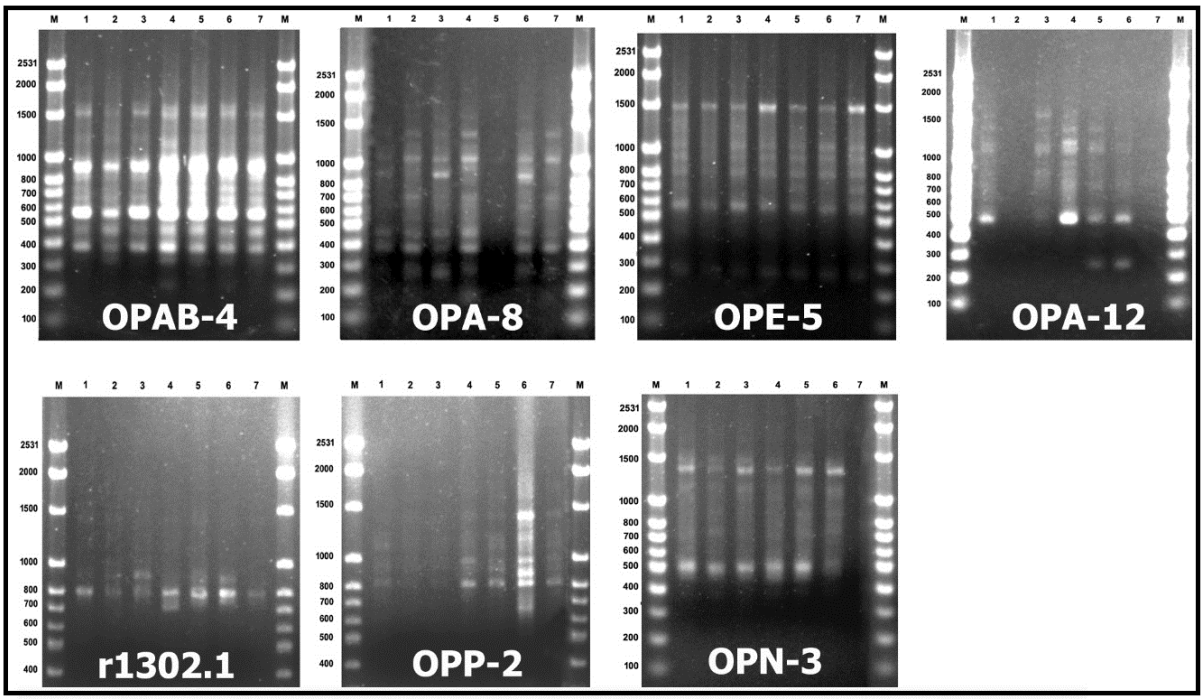

Fig. 1. Agarose electrophoresis of the seven different RAPD-PCR products on the seven cowpea cultivars.

On the other hand, the lowest genetic similarity was observed between Cream-12 and Blackeye crowder cultivars with a value of 54.2 % (Table 5). This lowest genetic similarity between Cream-12 and Blackeye crowder was expected because not only the morphological difference of the Seed colour between the two cultivars (Table 1), but also the differences found in some agronomic traits screened by Damarany (1994) such as: Pod length, No. of seeds/pod, pod-filling, and weight of 100-seeds.

Despite the differences in the seed colours, and according to the results shown in table (5) and the 2nd formed cluster in figure (2) the highest value of genetic similarity for Blackeye crowder found with the Brown crowder with a value of 77.8%. This could be because of both cultivars belong to the same morphological-group based on the extent of seed crowding in the pod (Table 1), in addition to they were collected from the same source.

The illustrated results in figure (2) showed that the3rd cluster includes: Brown crowder, Blackeye crowder and Chinese red cultivars which were collected from the same source. It could be noticed that the degree of similarity within this cluster ranged between 72.0 % and 77.8 % as in Table (5).

The genetic similarity values within this cluster are high compared to the other values of clusters which contain more than 2 cultivars. These high values were unexpected, because Chinese red cultivar differs in the seeds colour, growth habit and extent of seed crowding in the pod, than Brown crowder and Blackeye crowder (Table 1).

The 4th formed cluster in figure (2) indicated that Cream 12 has the lowest value of genetic similarity cultivar comparing with other cultivars within those cluster, and this could be due that Cream 12 is the only foreign cultivar within this cluster. Same explanation might been concluded in the 5th cluster in figure (2) which illustrated that Cream 7 (the only local cultivar within this cluster) showed the lowest value of genetic similarity in comparison with other cultivars within this cluster. Both Cream-12 and Cream-7 cultivars which have a Yellowish-white seed colour did not produce any bands when the OPA-12 primer was applied. Thus, further investigation should be applied to find if there is a relation between the absence of the amplification by the OPA-12 primer and the Yellowish-white seed colour.

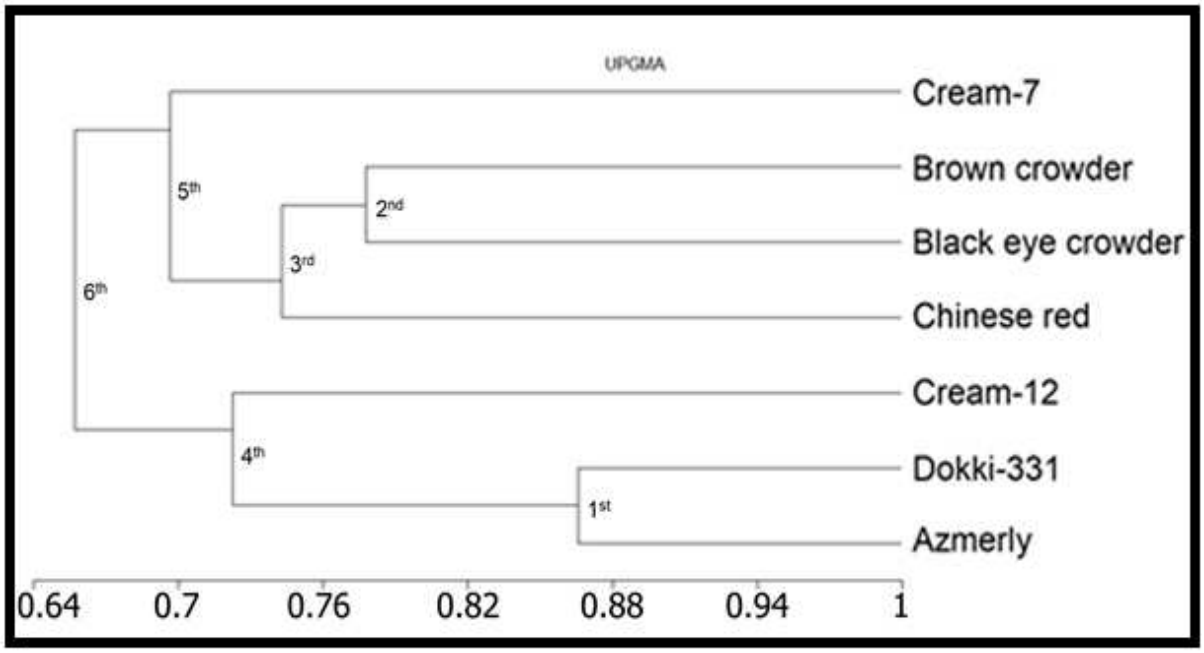

Fig. 2. Dendrogram demonstrating the relationship among the seven cowpea cultivars based on data recorded from the RAPD markers polymorphism.

From all the above results, it may be concluded that there is a probability of correlation between the RAPD profile and the source, from which cultivars were collected.

These results are in agreement with the results of Udensi *et al.*, (2016) when different cowpea cultivars obtained from Asia and from Africa were investigated, and the same results were also obtained by Ghalmi *et al.*, (2010) who tested different cultivars of cowpea belonging to different geographical origins.

On the other hand, our results documented the absence of correlation between the growth habits and clustering system since the Chinese red (the only indeterminate cultivar) did not show the lowest genetic similarity value when compared to the other cultivars as expected. The RAPD data and the dendrogram profile showed that there is a correlation between the RAPD data and the extent of seed crowding in pod trait, since both the Blackeye crower and Brown crowder cultivars were clustered together. While in the other morphological trait like the seeds colour, might or might not show correlation with the molecular data. These results were in agreement with Ghalmi *et al.*, (2010) who tested the correlation between some morphological traits and both RAPD and ISSR makers.

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