Biochemical and molecular genetic studies using SDS-protein, isozymes and RAPD-PCR in some common bean (*Phaseolus vulgaris* L.) cultivars

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ABSTRACT

SDS-protein and isozymes (esterase, glutamate oxaloacetate transminase, peroxidase and polyphenol oxidase) banding patterns and RAPD-PCR markers were used to genetically identify five Egyptian common bean (Phaseolus vulgaris L.) cultivars, which are grown in Egypt. The results showed a wide range of variation in total protein content, isozyme activities and SDS-protein banding patterns. However, low levels in the banding patterns of isozymes were observed. For RAPD-PCR analysis, seven random arbitrary primers were used. Twenty-five cultivar-specific markers (13 positive and 12 negative) were detected indicating that they can be used as markers for the five Phaseolus cultivars used in the present study. The phylogenetic relationships between the five common bean cultivars were determined by RAPDistance software package, version 1.04. The dendrogram tree showed that Coby and Samantha cultivars are very close (similarity of 85.5%), while Julia was genetically far from other cultivars (similarity of 75.5%). In conclusion, the biochemical and molecular genetic analysis used in the present study successfully distinguished among different common bean cultivars.

Key words: *Genetic fingerprinting, protein, isozymes, RAPD-PCR, Phaseolus.*

INTRODUCTION

Common beans (*Phaseolus vulgaris* L.) are usually harvested and stored as seed. When this crop is tested agronomically, or included in breeding programs, the distinction and identity of samples need to be established prior to agronomic evaluation and breeding. Seed morphology has traditionally formed the basis for commercial grading, but morphological differences are not always sufficient to differentiate among closely related cultivars. Sister lines are particularly difficult to identify correctly because of their common genetic background. A relatively rapid and accurate method of cultivar identification would benefit breeding programs and facilitates classification of seed samples for agronomic studies, seed certification and germplasm management.

In *Phaseolus vulgaris*, (Bassiri and Adams, 1978; Weeden, 1984; Jaaska and Jaaska, 1988; Prestamo and Manzano, 1993; Becerra-Velasquez and Gepts, 1994) used isozyme activity levels and isozyme banding patterns for the detection of genetic diversity

among cultivars, localized virus infection (Wagih, 1993) and the construction of genetic maps were also determined (Garvin and Weeden, 1994; Vallejos *et al.*, 1992).

For studying the genetic relationships among cultivars and landraces of *Phaseolus lunatus*, RAPD analyses are used (Nienhuis *et al.*, 1995; Kaga *et al.*, 1993). Similar studies were performed in faba bean and soybean (Hussein *et al.*, 2000; 2001).

The objective of this study is to use isozyme banding patterns and RAPD-PCR markers to genetically identify different cultivars of *Phaseolus vulgaris* and to study phylogenetic relationships among them.

MATERIALS AND METHODS

Seed material

Five cultivars of *Phaseolus vulgaris* L., namely; Coby, Bronco, Samantha, Giza-6 and Julia were obtained from the Institute of Vegetable Crops, Ministry of Agriculture, Giza, Egypt. These cultivars included white beans (Coby, Bronco, Samantha and Giza-6) and black bean (Julia). The cultivar Coby has a short stem and grows well under low and high temperatures, in winter and summer, and has a moderate vegetative growth. It is used as green pods as well as dry seeds. The cultivar Bronco has thin pale-green pods with a good vegetative growth, highly sensitive to fluctuations in temperature during growing season and highly susceptible to rust. The cultivar Samantha has very thin pods, with a good vegetative growth and highly susceptible to rust. The cultivar Giza-6 ripens very early, used as dry seed and highly tolerant to BCMV virus and rust. The cultivar Julia has very short stem, moderate vegetative growth, and it is for green use and it has very thin pods and seeds.

Germination conditions of seeds

All seeds were surface sterilized with 0.25% (v/v) sodium hypochlorite solution for 5 minutes and washed 7 times with sterile water. The seeds were germinated in petri dishes and incubated at room temperature in the dark. Germinated seeds were collected when the seedling had developed to a stage at which coleoptile had expanded to 5-7 cm. The germinated seeds were stored at -20 °C for further analysis.

Total protein and enzyme activity

Protein was extracted from germinated seeds of each cultivar. Concentration was determined according to Bradford (1976). The enzyme activities of esterase (Est), glutamate oxaloacetate transminase (GOT), peroxidase (Prx) and polyphenol oxidase (POD) were determined according to Gillard and Dennid (1974), Reitman and Frankel (1957), Chance and Maehly (1955); Prestamo and Manzano (1993) and Baaziz *et al.* (1994), respectively. One unit of esterase activity was the amount of enzyme, which liberates one µmol of ρ-nitrophenol per minute. One unit of GOT activity was the amount of enzyme which converts one µmol α-ketoglutarate to L-glutamate per hour. One unit of peroxidase activity was the amount of enzyme, which causes one O.D. change at 470 nm per minute under assay conditions. Lastly one unit of polyphenol oxidase activity was the amount of enzyme, which causes one O.D. change at 570 nm per hour.

Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for total proteins of the five cultivars according to the method of Laemmli (1970), as modified by Studier (1973). SDS-denatured bovine serum albumin (66,000 Dalton), ovalbumin (45,000 Dalton), glyceraldyde-3-phosphate dehydrogenase

(36,000 Dalton), carbonic anhydrase (29,000 Dalton), trypsinogen (24,000 Dalton) and soybean trypsin inhibitor (20,000 Dalton) were used for the calibration curve. Subunit molecular weight was estimated as described by Laemmli (1970).

Electrophoresis of isozymes was performed under non-reducing conditions in 10% (w/v) acrylamide slab gel according to Davis (1964), using Tris-glycine buffer (pH 8.3). Esterase isozymes were localized on the gel using α-naphthyl acetate and fast blue RR (Gottlieb, 1974). GOT isozymes were detected on the gel using L-aspartic acid, α-ketoglutaric and fast blue BB (Scandalios, 1969). Peroxidase isozymes were localized on the gel using guiacol and hydrogen peroxide according to Shaw and Prasad (1970). Polyphenol oxidase isozymes were detected according to Baaziz *et al.* (1994), in which the gel was immersed in a solution containing 0.1% 1-dihydroxyphenyl alanine solubilized in 0.05 phosphate buffer pH 7.5. Relative band mobility was measured in relation to the dye front and indicated by Rf values.

RAPD-PCR

DNA was extracted according to EL-Fiky (2001). Seven Operon random primers (A13-16 and 18-20, Table 1) were applied. PCR reactions were conducted according to Williams *et al.* (1990). The reaction conditions were optimized and mixtures (25 µl total volume) were composed of dNTPs (200 µM), MgCl2 (1.5 mM), 1X buffer, primer (0.2 µM), DNA (100 ng), Promega Taq DNA polymerase (2 units). Negative control was included in which all the ingredients were present except template DNA. Amplification was carried out in a thermocycler (UNO II, Biometra) programmed for 95°C for 5 min (one cycle); followed by 94°C for 1 min, 36°C for 1 min and 72°C for 2 min (45 cycles); 72°C for 5 min (one cycle), then 4°C (infinitive). Amplification products (7 µl) were mixed with 3 µl loading buffer and separated on 1.5% agarose gel and stained with 0.5 µg/ml ethidium bromide, and visualized with ultraviolet light and photographed. DNA fragment sizes were determined by comparisons with the 1 Kb DNA ladder marker (Promega Inc.).

Marker nomenclature

Each RAPD-PCR marker was named by the primer used and DNA fragment size in base pairs (bp). For example, A14-1900 refers to a marker with a band size of 1900 bp amplified against primer A14.

Table (1): Seven Operon random primers used and sequences.

Primer	Sequence 5`--------- 3`
A13	CAGCACCCAC
A14	TCTGTGCTGG
A15	TTCCGAACCC
A16	AGCCAGCGAA
A18	AGGTGACCGT
A19	CAAACGTCGG
A20	GTTGCGATCC

Data Analysis

RAPD patterns were scored for each cultivar and genetic distances were calculated according to Sokal and Sneath (1963) by using RAPDistance software package, version 1.04 (Armstrong *et al.*, 1994).

RESULTS AND DISCUSSION

Total protein and enzyme activity

Total protein and isozyme activity levels were used as biochemical markers for the characterization of *Phaseolus vulgaris* cultivars. Data presented in Table (2) show the total protein content and the activity levels of esterase, GOT, peroxidase and polyphenol

oxidase enzymes in the five *Phaseolus* cultivars included in the present study. It is obvious that the 5 cultivars show highly significant variations in the total protein content, (ranging from 89 to 151 mg/g). With regard to the isozyme activities the Coby cultivar has the highest esterase (190 unit g^{-1}) and GOT (110 unit g^{-1}) activities, while Julia cultivar has the lowest esterase (108 unit g^{-1}) and GOT (75 unit g^{-1}) activities. In case of peroxidase activity, Julia cultivar has the highest level (1600 unit g^{-1}) and Samantha cultivar has the lowest level (1100 unit g^{-1}). The polyphenol oxidase activity level of Bronco cultivar was the highest (325 unit g^{-1}) and Coby cultivar was the lowest (217 unit g^{-1}). These data reflect that the different cultivars are genetically divergent.

Protein and isozyme banding patterns

Tables (3 & 4) and Figure (1) show the electrophoretic separation of SDS protein banding patterns as well as esterase, GOT, peroxidase and polyphenol oxidase isozymes. It is shown that the 5 cultivars had different protein profiles, which reflect their genetic diversity. The isozyme patterns of esterase showed disappearance of a different one band for cultivars Bronco, Samantha, Giza-6 and Julia with (Rf of 0.41, 0.29, 0.11 and 0.25, respectively). On the other hand, the banding patterns of GOT isozyme showed unique bands for the cultivars Coby and Julia (Rf of 0.23 and 0.09, respectively). In case of peroxidase and polyphenol oxidase banding patterns, they showed disappearance of one band (Rf of 0.7) for the cultivars Coby, Bronco and Julia and a band (Rf of 0.39) for the cultivars Coby, Bronco and Samantha, respectively.

Based on the obtained data for total protein, isozyme activity levels and banding patterns from the five cultivars of *Phaseolus* vulgaris, it is concluded that there is a variation in protein content and activity levels for all isozyme between different cultivars. The banding patterns of protein revealed high levels of polymorphism (or specific markers). However, banding patterns of esterase and GOT isozymes revealed slightly higher levels of polymorphism than peroxidase and polyphenol oxidase. These data were supported by Brown *et al.* (1982) and Bassiri and Adams (1978), who indicated that low levels of isozyme variation were observed among *Phaseolus vulgaris* cultivars due to the recovery of low band numbers.

Table (2): The activity levels of different enzymes for five cultivars of Phaseolus vulgaris.

Cultivar	Total protein (mg g^{-1})	EST activity (unit g^{-1})	GOT activity (unit g^{-1})	Prx activity (unit g^{-1})	POD activity (unit g^{-1})
Coby	151 ± 8	190 ± 10	110 ± 7	1500 ± 75	217 ± 14
Bronco	128 ± 5	170 ± 5	80 ± 4	1300 ± 50	325 ± 12
Samantha	89 ± 7	135 ± 4	100 ± 5	1100 ± 66	310 ± 20
Giza-6	112 ± 5	156 ± 6	85 ± 4	1250 ± 43	285 ± 10
Julia	123 ± 5	108 ± 6	75 ± 5	1600 ± 76	233 ± 12

Table (3): Banding patterns and molecular weight (MW) of SDS proteins for five cultivars of Phaseolus vulgaris.

Band number	MW (KD)	Cultivars				
		Coby	Bronco	Samantha	Giza-6	Julia
1	136	+	-	-	-	-
2	127	-	-	-	-	+
3	124	-	-	-	+	+
4	120	-	-	-	-	+
5	116	-	-	+	-	-
6	108	-	-	+	-	-
7	106	-	-	-	-	+
8	99	-	-	-	-	+
9	91	-	-	-	-	+
10	78	+	-	-	-	-
11	77	-	-	-	+	-
12	75	-	-	+	-	-
13	71	-	-	-	+	-
14	69	-	+	-	-	-
15	67	+	-	-	+	-
16	65	-	-	-	-	+
17	61	+	-	+	-	-
18	59	-	-	-	+	-
19	58	+	-	-	-	-
20	55	-	+	-	-	-
21	54	-	-	+	-	-
22	50	-	-	-	+	+
23	49	-	-	+	-	-
24	48	-	+	-	-	-
25	47	+	-	-	-	-
26	46	-	-	-	+	-
27	45	+	-	-	-	-
28	43	-	-	+	-	+
29	42	-	+	-	-	-
30	41	-	-	-	+	-
31	40	+	-	-	-	-
32	39	-	-	-	-	+
33	38	-	-	+	+	-
34	37	+	-	-	-	-
35	36	-	+	-	-	-
36	34	-	-	-	-	+
37	31	-	-	+	-	-
38	30	-	+	+	+	+
39	29	+	-	-	-	-
40	28	-	-	+	+	-
41	27	-	+	+	-	+
42	26	-	-	-	-	+
43	24	-	+	-	+	-
44	23	+	+	+	+	+
45	22	-	+	+	-	-
46	21	-	-	-	+	+
47	20	+	+	+	-	-

Identification of RAPD-PCR molecular markers

A total number of 62 DNA bands were detected as generated by the 7 random primers for the five cultivars used in the present study, in which 37 (about 60%) were useful as RAPD-PCR markers (Figure 2 and Tables 5 & 6). The least number of polymorphic bands was detected for primer A16 (one out of 5 amplified bands), while the largest number of polymorphic bands was detected for primer A20 (8 out of 11 bands). However, 25 bands were common (monomorphic) for all cultivars.

Cultivar specific markers

The specific markers for *Phaseolus* cultivars generated from RAPD-PCR analysis are shown in Tables (6 & 7). Twenty-five out of the 62 (about 40%) RAPD-PCR markers were found to be useful as cultivar-specific markers. The largest number of RAPD-PCR specific markers was scored for Julia (8 markers), while the lowest (3 markers) was scored for Samantha. A number of 13 specific markers was scored for the presence of unique bands for a given cultivar (positive marker), while 12 were scored for the absence of a common band (negative marker). In the meantime, the largest number of RAPD-PCR cultivar-specific markers was generated by primer A8 (13 markers), followed by primer A17 (10 markers). On the other hand, the least number of RAPD-PCR specific markers was generated by primer A18 (5 markers), followed by A20 (6 markers).

In conclusion, all primers used in the present study allowed enough distinction between the five *Phaseolus* cultivars. Overall comparison among cultivars across the 7 primers revealed the power of RAPD-PCR analysis in distinguishing among closely related *Phaseolus* cultivars. These cultivar-specific markers may be used in subsequent experiments to detect molecular markers for polymorphic genes with economic importance among these and other *Phaseolus* cultivars.

Table (4): Banding patterns and relative mobilities (Rf) of different isozymes for five cultivars of Phaseolus vulgaris.

Isozyme	Rf	Cultivars					Total no. bands
		Coby	Bronco	Samantha	Giza-6	Julia	
Est	0.11	+	+	+	-	+	4
	0.25	+	+	+	+	-	
	0.29	+	+	-	+	+	
	0.41	+	-	+	+	+	
GOT	0.09	-	-	-	-	+	3
	0.13	+	+	+	+	+	
	0.23	+	-	-	-	-	
Prx	0.07	-	-	+	+	-	2
	0.16	+	+	+	+	+	
POD	0.05	+	+	+	+	+	4
	0.24	+	+	+	+	+	
	0.39	-	-	-	+	+	
	0.48	+	+	+	+	+	

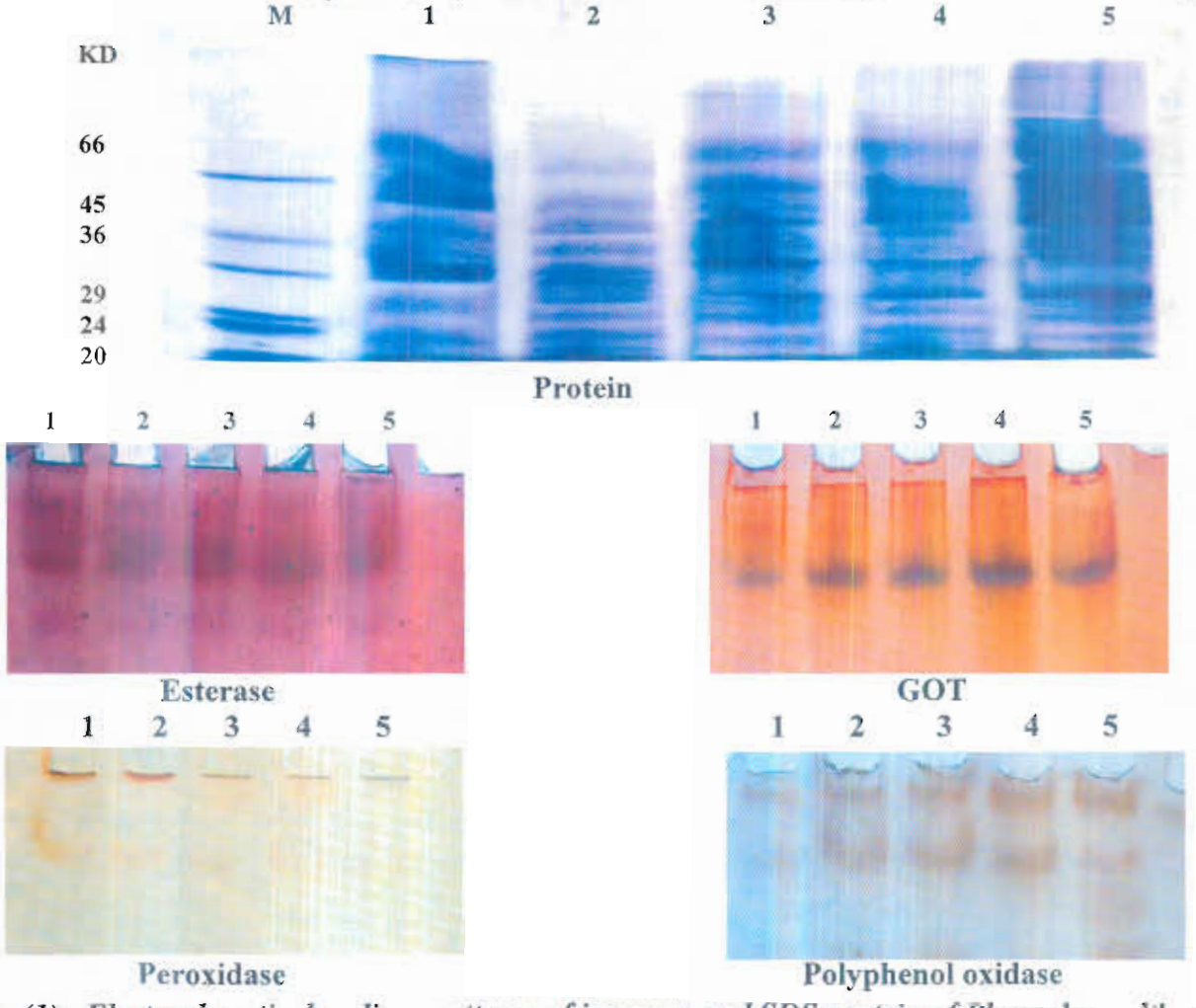

Fig. (1): Electrophoretic banding patterns of isozymes and SDS-protein of Phaseolus cultivars. (Lanes 1-5 represent Koby, Bronco, Samantha, Giza-6, Julia, respectively). M = MW standard SDS-protein.

Phylogenetic relationships

The dendrogram tree and the similarity indices among the five *Phaseolus vulgaris* cultivars utilizing RAPD-PCR markers (Figure 3 and Table 8) were detected by RAPDistance software package 1.4 according to Sokal and Sneath matrix (1963). The analysis was based on the number of markers that were different between any given pair of cultivars. The strongest relationship was scored between white seed cultivars Coby and Samantha (similarity index of 85.5%), while cultivar Julia and cultivar Bronco were shown to be the most genetically distant cultivars (similarity index of 70.8%), when compared with the other three cultivars. In addition, pairs of cultivars Samantha/Bronco showed a similarity index of 84%, followed by cultivars Bronco/Coby with a similarity index of 84.6%. From the dendrogram tree, the black seed cultivar Julia appeared to be a unique cultivar with a similarity index of 75.5% when compared with the other four cultivars (Figure 3). The white seed cultivar Giza-6 was also shown to be genetically distant when compared with the other three white seed cultivars Bronco, Samantha and Coby.

Table (5): Banding patterns of RAPD-PCR for five cultivars of Phaseolus vulgaris.

Primer	Band number	Cultivar				
		Coby	Bronco	Samantha	Giza-6	Julia
A13	1	-	-	+	-	-
	2	+	+	+	+	+
	3	-	-	-	+	-
	4	-	-	-	+	-
	5	+	+	+	+	+
	6	+	+	+	+	-
	7	+	+	+	+	+
	8	+	+	+	+	+
	9	+	+	+	+	+
	10	+	+	+	+	+
A14	1	+	-	-	-	-
	2	+	+	+	+	+
	3	+	+	-	+	+
	4	-	+	-	+	-
	5	+	+	+	+	+
	6	-	+	-	-	-
	7	-	+	-	-	-
	8	+	-	-	+	-
	9	-	-	+	-	+
A15	1	-	-	+	-	+
	2	+	+	+	+	+
	3	+	-	+	-	+
	4	+	+	+	+	+
	5	+	+	+	+	+
	6	+	+	+	-	+
	7	+	+	+	+	+
A16	1	+	+	+	+	+
	2	+	+	+	+	+
	3	+	+	+	+	+
	4	+	+	+	+	+
	5	+	-	+	+	+
A18	1	+	+	+	+	+
	2	+	+	+	+	+
	3	+	-	+	+	+
	4	+	+	+	-	-
	5	+	+	+	-	-
	6	+	+	+	+	-
	7	+	+	+	+	+
	8	-	-	+	-	-
	9	+	+	+	+	-
	10	+	-	-	-	-
A19	1	+	+	+	+	+
	2	-	-	-	+	-
	3	+	-	-	-	+
	4	+	-	-	-	-
	5	+	+	+	+	+
	6	+	-	-	-	-
	7	-	-	-	+	+
	8	+	+	+	+	-
	9	+	-	-	-	+
	10	+	+	+	+	+
A20	1	+	+	+	+	+
	2	+	-	+	+	+
	3	+	+	+	-	-
	4	+	+	+	-	+
	5	-	-	-	-	+
	6	+	+	+	+	-
	7	+	+	+	+	+
	8	-	-	+	+	+
	9	-	-	-	-	+
	10	+	+	+	+	-
	11	+	+	+	+	+

Table (6): Number of amplified fragments and specific markers of five Phaseolus cultivars based on RAPD-PCR analysis.

			Cultivar										
			Coby		Bronco		Samantha		Giza-6		Julia		
Primer	TAF	PB	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	TSM
A13	10	4	7	0.0	7	0.0	8	1	9	2	6	1	4
A14	9	7	5	1	6	2	3	1	5	0.0	4	0.0	4
A15	7	3	6	0.0	5	0.0	7	0.0	4	1	7	0.0	1
A16	5	1	5	0.0	4	1	5	0.0	5	0.0	5	0.0	1
A18	10	7	9	1	7	1	9	1	6	0.0	4	2	5
A19	10	7	8	2	4	0.0	4	0.0	6	1	6	1	4
A20	11	8	8	0.0	7	1	9	0.0	7	1	8	4	6

TAF = Total amplified fragment, PB = Polymorphic bands, AF = Amplified fragments,
SM = Specific marker, including either the presence or absence in a band in specific cultivar, TSM = Total no. specific markers across cultivars.

Table (7): Cultivar-specific markers in five Phaseolus cultivars resulting from RAPD-PCR analysis.

Cultivar	Marker		Total
	Positive	Negative	
Coby	A14-1900, A18-300, A19-1800, A19-1040.	---------	4
Bronco	A14-690, A14-610.	A16-530, A18-1700, A20-1250	5
Samantha	A13-1050, A18-500.	A14-1190	3
Giza-6	A13-900, A13-730, A19-2500.	A15-750, A20-1100	5
Julia	A20-875, A20-420.	A13-520, A18-750, A18-375, A19-750, A20-730, A20-250.	8
Total	13	12	25

In conclusion, the biochemical and molecular genetic analysis used in the present study successfully distinguished among different common bean cultivars. The study recommends the use of RAPDs as a rapid and accurate method of identification to facilitate classification of seed samples for agronomic studies, seed certification and germplasm management and breeding programs in *Phaseolus vulgaris*.

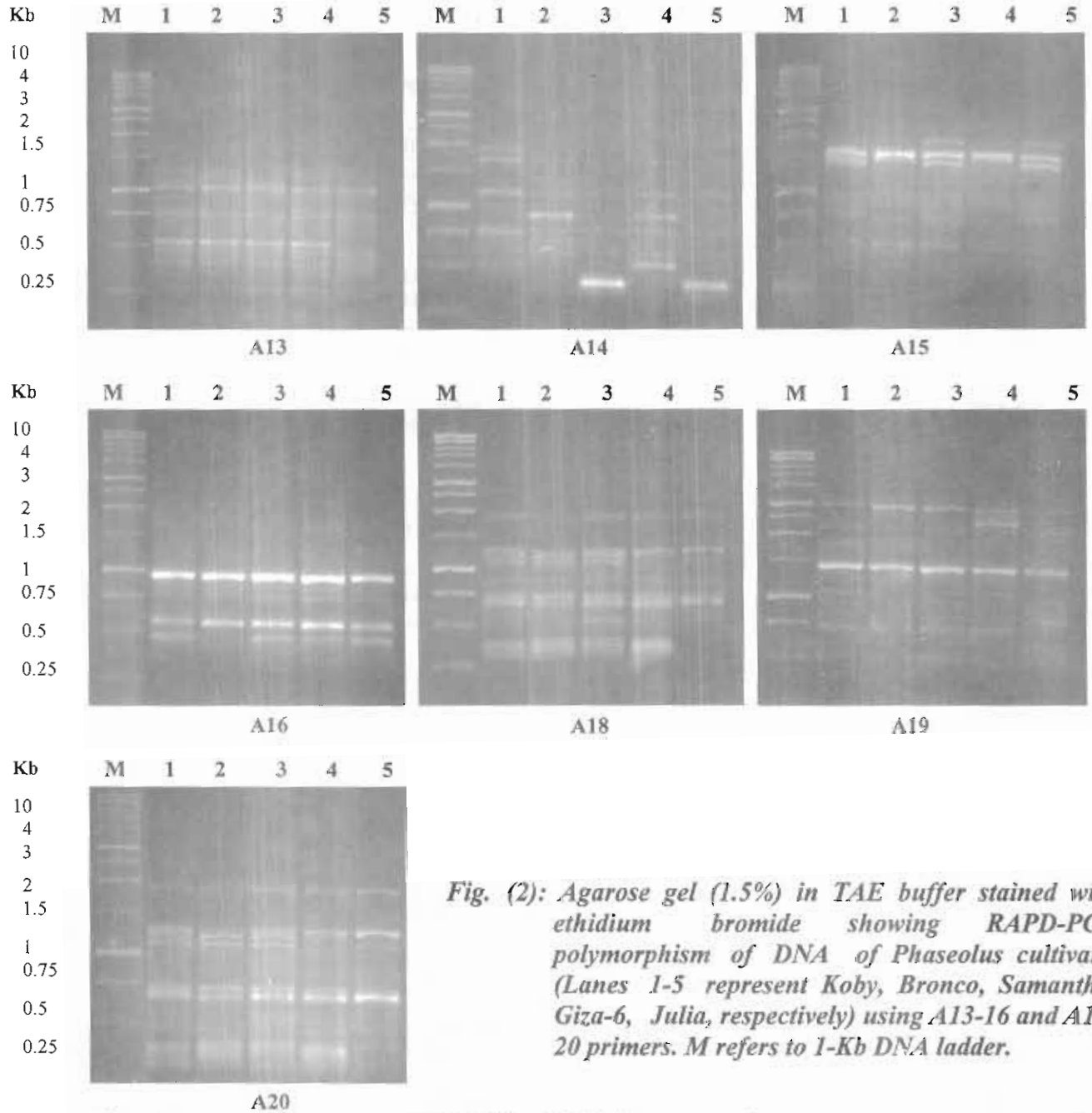

Fig. (2): Agarose gel (1.5%) in TAE buffer stained with ethidium bromide showing RAPD-PCR polymorphism of DNA of Phaseolus cultivars (Lanes 1-5 represent Koby, Bronco, Samantha, Giza-6, Julia, respectively) using A13-16 and A18-20 primers. M refers to 1-Kb DNA ladder.

Table (8): Similarity indices calculated by RAPDistance package among five cultivars of Phaseolus vulgaris.

Cultivar	Coby	Bronco	Samantha	Giza-6	Julia
Coby	1				
Bronco	0.846	1			
Samantha	0.855	0.840	1		
Giza-6	0.789	0.788	0.800	1	
Julia	0.774	0.708	0.784	0.752	1

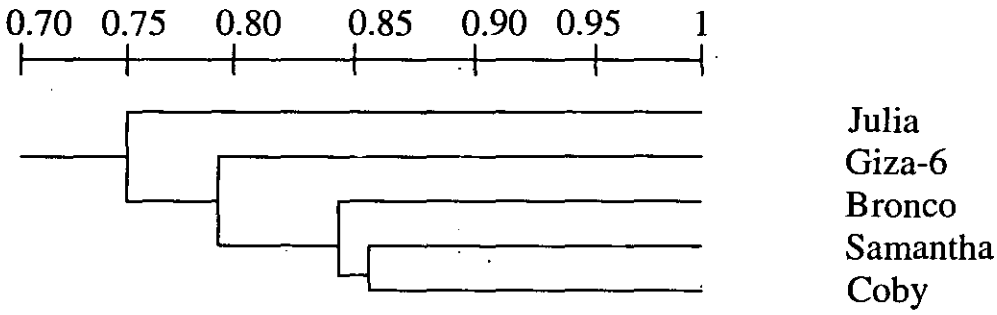

Fig. (3): Phenogram demonstrating the relationships among five Phaseolus vulgaris cultivars based on a compiled data set.

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الملخص العربى

تحديد البصمات الوراثية لبعض أصناف الفاصوليا المزروعة فى مصر باستخدام البروتينات و مشابهات الانزيمات وشرائط الـ د.ن.أ

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تم تحديد البصمات الوراثية لخمسة أصناف من الفاصوليا (كوبى، برونكو، سامنتا ، جيزة-٦ وجوليا) والتى تزرع فى مصر ، تم الحصول عليها من معهد بحوث الخضر مركز البحوث الزراعية . وتم استخلاص البروتينات الكلية وكذلك د.ن.أ من البادرات (عمر أسبوعين) وتم تقدير المحتوى الكلى للبروتين وكذلك نشاط مشابهات أربعة انزيمات (الاستريز ؛ الجلوتاميت أكسالو أسيتيت ترانسمينيز؛ البيروكسيديز والبولىفينول أكسيديز) . وقد أستخدمت طرق التفريد الكهربى باستخدام الـ (PAGE) لتحديد البصمات الوراثية لكل من البروتين ومشابهات الانزيمات وكذلك تم استخدام طريقة تفاعل البلمرة المتسلسل RAPD-PCR لتحديد البصمة الوراثية لل د.ن.أ باستخدام ٧ بوادئ عشوائية .

وأظهرت النتائج وجود تباينات واضحة فى المحتوى الكلى للبروتين ونشاط مشابهات الانزيمات وكذلك بصمات البروتين بالرغم من وجود مستويات محدودة من بصمات مشابهات الانزيمات . وأظهرت بصمة ال د.ن.أ وجود ٢٥ واسما متخصصا لتحديد الاختلافات الوراثية بين الاصناف . وقد أستخدمت النتائج المتحصل عليها فى عمل سجل قرابة Dindrogram بين الاصناف الخمسة باستخدام برنامج RAPDistance . وظهر وجود درجة قرابة عالية (٨٥،٥%) بين الصنفين كوبى و سمنتا وأقل درجة قرابة (٧١%) بين الصنفين برونكو وجوليا . وظهر أن الصنف جيزة-٦ هو أبعد الاصناف بيضاء البذور قرابة بينما الصنف جوليا (أسود البذور) كان أبعدها فى القرابة من كل الاصناف . وتوضح الدراسة أن استخدام الطرق الوراثية البيوكيماوية والجزيئية هى وسائل كفوءة للتمييز بين أصناف الفاصوليا المختلفة.