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Variability of morphological characters, protein patterns and random amplified polymorphic DNA (RAPD) markers in some *Pisum* genotypes

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The present study involved characterization of variability of qualitative morphological characters, protein patterns and random amplified polymorphic DNA (RAPD) markers in some *Pisum sativum* genotypes. 17 different *Pisum* genotypes were used in this study. They are: Seven *P. sativum* L. cultivars (Vectory Freezer, Master, Lincoln, Little Marvel, Montana, Local and Billinder), three wild taxa of *P. sativum* L., five accessions of *P. sativum* L. and two accessions of *P. fulvum* L. The results demonstrate a high potential and resolving power of DNA-based methods (RAPD markers) for discrimination between *Pisum* genotypes. The two accessions of *P. fulvum* L. were successfully separated on the basis of RAPD markers, while the morphological characters and protein patterns could not be distinguished between them. Wild taxa collected from the north coastal region of Egypt were separated from other genotypes, suggesting different genetic pool. RAPD markers indicated that four *P. sativum* L. cultivars (Vectory Freezer, Master, Lincoln and Little Marvel) and two accessions *P. fulvum* L. are grouped in one cluster, suggesting that these cultivars may be originated from the *P. fulvum* L. gene pool. Data indicated 46 RAPD markers specific to ten genotypes, and one marker specific to *P. fulvum* L. The three wild taxa of *P. sativum* L. showed high number of specific RAPD markers, reflecting similarities among these taxa and their differences from other genotypes. The use of RAPD markers linked to qualitative morphological characters in breeding programs makes the selection for these characters easy and possible at the young seedling stage without reaching the maturation stage.

Key words: *Pisum sativum*, seed storage proteins, random amplified polymorphic DNA (RAPD) markers, numerical analysis.

INTRODUCTION

Pisum is a genus of the family Fabaceae, native to southwest Asia and northeast Africa. It contains one to five species, depending on the taxonomic interpretation. The International Legume Database (ILDIS) accepts three of the *Pisum* specie: *P. abyssinicum*, *P. fulvum* and *P. sativum* (ssp *elatius* and ssp *sativum*). Jing et al. (2010) strongly supported the model of independent domestications for *P. abyssinicum* (syn. *P. sativum* ssp *abyssinicum*) and *P. sativum* ssp *sativum*.

Pisum sativum L. (the field or garden pea), is domesticated as a major human food crop. The increasing demand for protein-rich raw materials for

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Table 1. Cultivars, locations, origin and accession number of the studied *Pisum* genotypes.

S/N	*Pisum* species	Genotype			
		Cultivars	**Taxa location**	**Accession origin**	**Accession number**
1	*P. sativum* L.	Vectory Freezer			
2	*P. sativum* L.	Master			
3	*P. sativum* L.	Lincoln			
4	*P. sativum* L.	Little Marvel			
5	*P. sativum* L.	Montana			
6	*P. sativum* L.	Local			
7	*P. sativum* L.	Billinder			
8	*P. sativum* L.		Ghazala		
9	*P. sativum* L.		Wadi El-Ramel		
10	*P. sativum* L.		El-Dabba		
11	*P. sativum* L.			Turkey	1023
12	*P. sativum* L.			Greece	1945
13	*P. sativum* L.			Cyprus	1749
14	*P. sativum* L.			Syria	2811
15	P. *sativum* L.			Algeria	876
16	*P. fulvum* L.			Syria (1)	2916
17	*P. fulvum* L.			Syria (2)	2917

animal feed or intermediary products for human nutrition have led to a greater interest in this crop as a protein Source (Santalla et al., 2001). Pea cultivars have a relatively narrow gene pool. Zong et al. (2009), suggested four gene pools for the genus *Pisum*. These are *fulvum*, *abyssinicum*, *arvense* and gene pool *sativum*. Information about genetic diversity among pea cultivars is critical for designing optimal breeding strategies in order to obtain a continuous progress in pea improvement.

The heavy use of a small number of varieties as parents by competing breeding programs have led to low genetic diversity among pea cultivars (Simioniuc et al., 2002; Baranger et al., 2004). Many researchers indicated that molecular markers are useful complements to morphological characters because they are plentiful, independent of tissue, and allow cultivar identification in the early stages of development. They revealed that polymorphism at DNA level is a very powerful tool for characterization and genetic diversity estimation (Tar'an et al., 2005; Espo´sito et al., 2007; Sarikamis et al., 2010).

The utility of Random Amplified Polymorphic DNAs (RAPDs) in detecting genotype specific markers has been shown by Kongkiatngam et al. (1996). Kovesa et al. (2001) identified a polymorphic 750-bp fragment, RAPD marker, specific to particular pea genotypes. In addition, linkage between RAPD markers and qualitative morphological characters was indicated by Bahieldin and Ahmed (1994). The use of RAPD markers in breeding programs makes the selection for morphological characters easy and possible at the young seedling stage without need to reach the maturation stage. This type of selection is called marker-assisted selection (MAS) indicated by Michelmore et al. (1991). Nisar and Ghafoor (2011) identified an RAPD marker linked with powdery mildew resistance in pea.

The aim of this study is to assess the variability among wild and cultivated genotypes belonging to two species of the genus *Pisum* based on qualitative morphological characters, protein patterns and RAPD markers. One objective is to examine the potential of each method for discrimination between *Pisum* genotypes. Other objective is to identify genotype-specific markers, particularly those linked to morphological characters.

MATERIALS AND METHODS

Genotype sampling

Seeds representing 17 samples belonging to two species of *Pisum* (*P. sativum* L. and *P. fulvum* L.) were obtained from different origins. Seven *P. sativum* L. cultivars (Vectory Freezer, Master, Lincoln, Little Marvel, Montana, Local and Billinder) were obtained from the Horticultural Research Institute, Giza, Egypt. Three wild taxa of *P. sativum* L. were collected from different locations at the North West coastal region of Egypt. These locations are Ghazala, El-Dbba and Wadi El-Ramel (150 km, 100 km eastern and 20 km western Matrouh city). The three wild taxa were identified at the Cairo University herbarium. Seven accessions of *P. sativum* L. and *P. fulvum* L. were obtained from the International Center for Agriculture Research in Dry Areas (ICARDA), Aleppo, Syria (Table 1). *Pisum* samples were grown in a randomized complete block design with three replicates under greenhouse conditions. Qualitative morphological characters were scored from 10 individuals of each genotype as shown in results and discussion.

SDS-PAGE of seed storage proteins

Characterization and molecular mass determination of seed storage

Table 2. Codes and sequences of arbitrary 10-mer primers.

Code	Sequence	Code	Sequence
OPB-01	GTTTCGCTCC	OPB-11	GTAGACCCGT
OPB-02	TAGTCCCTGG	OPB-12	CCTTGACGCA
OPB-03	CATCCCCCTG	OPB-13	TTCCCCCGCT
OPB-04	GGACTGGAGT	OPB-14	TCCGCTCTGG
OPB-05	TGCGCCCTTC	OPB-15	GGAGGGTGTT
OPB-06	TGCTCTGCCC	OPB-16	TTTGCCCGGA
OPB-07	GGTGACGCAG	OPB-17	AGGGAACGAG
OPB-08	GTCCACACGG	OPB-18	CCACAGCAGT
OPB-09	TGGGGGACTC	OPB-19	ACCCCCGAAG
OPB-10	CTGCTGGGAC	OPB-20	GGACCCTTAC

proteins was carried out using one-dimensional SDS-polyacrylamide gel electrophoresis. Samples were prepared for electrophoresis by extracting proteins from 0.5 g seed powder in 1 ml of 0.2 M Tris/HCl, pH 8, and 1 mM phenylmethylsulphonyl fluoride (PMSF) for 2 h in a refrigerator. The extract was centrifuged at 5000 g for 20 min and proteins in the supernatant were precipitated with 5 volumes of cold acetone at -20°C for 2 h. Pellets obtained after centrifugation at 7500 g for 20 min were dissolved in 20 µl of sample buffer (0.125 M Tris/HCl, pH 6.8, 2% m/v SDS, 10% m/v sucrose, 1% v/v β-mercaptoethanol, 0.1% m/v bromophenol blue) and denatured by heating at 80°C for 3 to 5 min, 17% SDS polyacryamide gel slabs were prepared as described by Laemmli (1970). Equal amounts of proteins were loaded per track. Electrophoresis was carried out in Tris/glycine - SDS running buffer (0.25 M Tris, 1.88 M glycine, 0.1% SDS) using a vertical gel electrophoresis unit (Mini-Protein Cell, *BioRad*, USA) at 140 V for the first 15 min followed by 150 V until the indicator dye reached the bottom of the gel. Gels were stained overnight in 20 ml of 0.25% kenacid blue, 50% (v/v) methanol, 7% (v/v) glacial acetic acid and destained by shaking overnight in 50% methanol and 7% glacial acetic acid.

DNA extraction and RAPD-PCR

Total genomic DNA was isolated from 1 g of *Pisum* leaves using cetyltrimethyl ammonium bromide (CTAB) buffer (2% CTAB, 100 mM Tris/HCl, pH 8, 20 mM EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol and 1% polyvinyl pyrrolidoine PVP) as described by Giannino et al. (1989). DNA was precipitated in equal volume of isopropanol and pellets were resuspended in 20 µl TE buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA). The concentration of DNA was determined spectrophotometrically and adjusted to 50 µl. PCR reactions were conducted according to Williams et al. (1990) to detect RAPD markers using 20 arbitrary 10-mer primers (Kit B) obtained from Operon Technologies, Alameda, California (Table 2). Reaction conditions were optimized using some combinations of reaction constituents to fit for the RAPD analysis of *Pisum*. PCR reactions were performed in 50 µl total volume of 10 mM Tris/HCl, pH 9, 50 mM KCl, 2.5 mM $MgCl_2$, and 200 µM of each of dATP, dGTP, dCTP and dTTP (Promega), containing 100 pmol primer, 1.25 U *Taq* DNA polymerase (Promega) and 50 ng template DNA overlaid with a drop of mineral oil. Amplification was carried out in a DNA thermocycler (Perkin Elmer Cetus 480, Norwalk, CT) programmed for 40 cycles, after initial denaturation for 3 min at 94 °C, with the following temperature profile: 1 min at 94°C, 1 min at 37°C and 2 min at 72°C, followed by a final extension for 5 min at 72°C. Negative controls lacking template DNA were included for each reaction mixture. Amplification products were visualized along with a DNA marker (λ phage DNA digested with BstEII) and 100 bp DNA ladder on 1.2% agarose gel with 1X TAE buffer (10 mM Tris/acetic, pH 7.4 and 1 mM EDTA) and detected by staining with ethidium bromide (10 µg/µl) for 30 min (Sambrook et al., 1989). Gels were then destained in deionized water for 10 min and photographed on Polaroid films under UV light.

Data analysis

The presence or absence of each protein and RAPD band was treated as a binary character. Data from qualitative morphological characters, protein patterns and RAPD-PCR were processed for cluster analysis performed using the program NTSYS PC (numerical taxonomy and multivate analysis system) as described by Rohlf (1993).

RESULTS AND DISCUSSION

Morphological characters

Six qualitative morphological characters were recorded from 10 individuals of each genotype and showed high polymorphism among the studied *Pisum* genotypes (Table 3). Seed shape, coat colour and cotyledon colour varied among the genotypes. Hilum colour which is a monogenic character (Blixt, 1974) indicated that all cultivars were homogenous in having colourless hilum, while the wild taxa have black hilum. The shape of cotyledons was wrinkled for all cultivars and the two accessions of *P. fulvum* L. All cultivars showed white flower colour, while wild taxa indicated purple flower colour.

The dendrogram generated using the data from qualitative morphological characters separated the examined genotypes into three main clusters (Figure 1). The first cluster included four cultivars (Vectory Freezer, Master, Lincoln +and Little Marvel) and the two *P. fulvum* L. accessions. The two cultivars (Montana and Billinder) were included with two *P. sativum* L. accessions from Cyprus and Algeria in the second cluster. The wild taxa and cultivar (Local) were included with some accessions from ICARDA in a separate cluster. Complete similarity

Table 3. Different states of qualitative morphological characters of studied *Pisum* genotypes as numbered in Table 1.

Character	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Seed shape																	
Spherical					+	+	+	+		+	+	+	+	+	+		
Oval-elongate													+	+			
Square	+	+	+	+		+			+	+	+	+		+	+	+	+
Irregular	+	+		+												+	+
Coat colour																	
Green	+	+		+				+		+	+	+				+	+
Cream	+		+	+	+		+						+		+	+	+
Pigmentated brown						+		+	+	+	+	+		+	+		
Speckled						+		+	+	+	+	+		+			
Pigmentated purple						+					+	+					
Hilum colour																	
Colourless	+	+	+	+	+	+	+						+		+	+	+
Black								+	+	+	+	+		+			
Cotyledon colour																	
Cream	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Green	+	+		+										+		+	+
Cotyledon shape																	
Smooth								+	+	+	+	+	+	+	+		
Wrinkled	+	+	+	+	+	+	+									+	+
Flower colour																	
White	+	+	+	+	+	+	+						+		+	+	+
Purple								+	+	+	+	+		+			

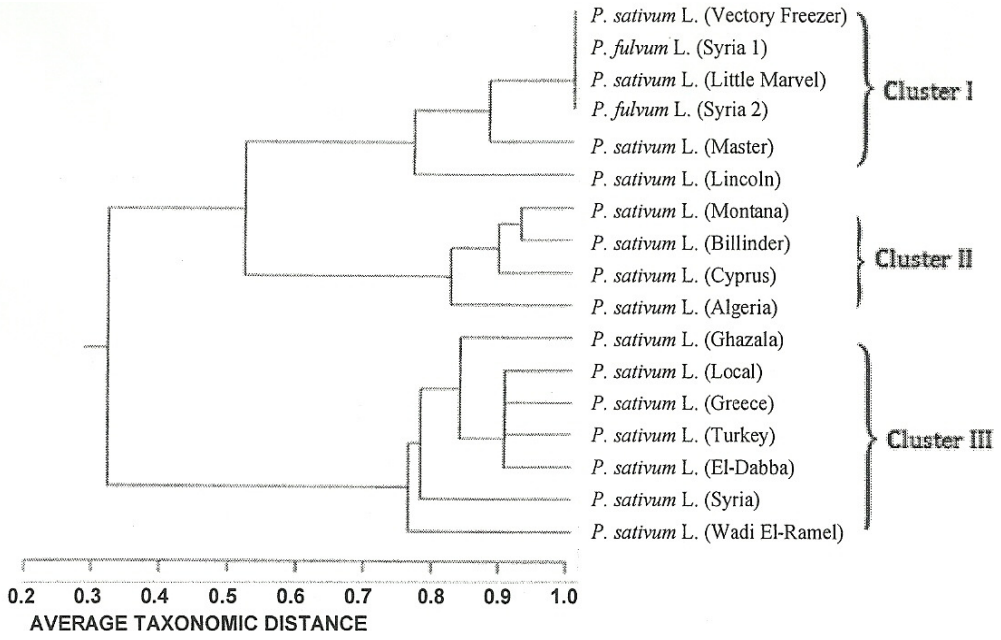

Figure 1. Dendrogram based on markers of qualitative morphological characters illustrating the relationships of studied *Pisum* genotypes.

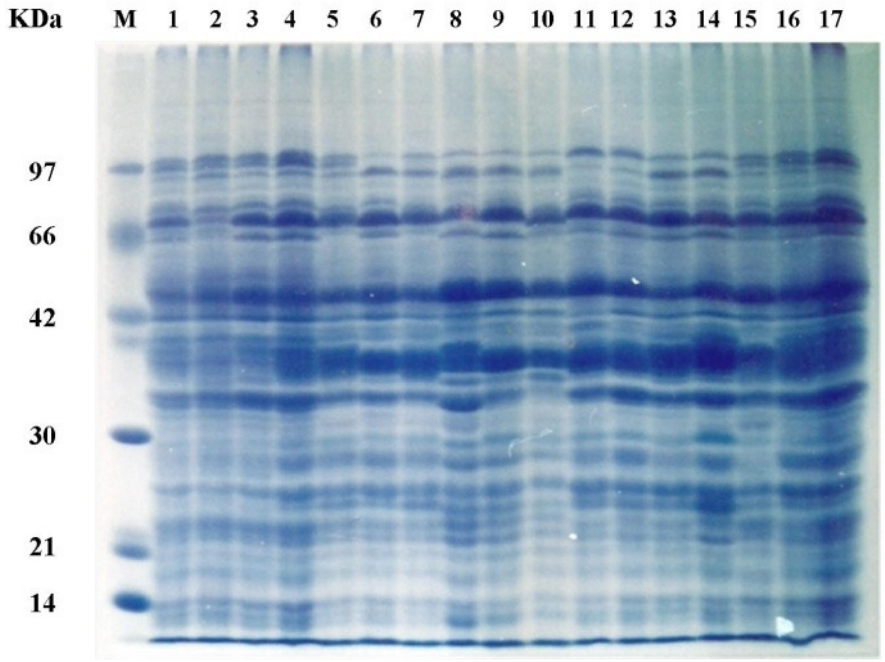

Figure 2. Electrophoretic profile product by SDS-PAGE of seed storage proteins of studied *Pisum* genotypes as numbered in Table 1. M: marker proteins.

was recorded among the two *P. sativum* L. cultivars (Vectory Freezer and Little Marvel) and the two *P. fulvum* L. accessions, which indicates that these four genotypes are identical at this level of evaluation. Marx (1977) suggested that the differences between *P. fulvum* L. and other *Pisum* species are little, to consider the first as a distinct species. Smýkal et al. (2008) studied 164 accessions representing pea (*P. sativum* L.) using nine qualitative and eight quantitative morphological parameters. They were able to identify five to seven clusters in the dendrogram generated from morphological characters.

Protein pattern

The SDS-PAGE patterns of seed storage proteins (Figure 2), shows that among 32 electrophoretic protein bands, only 15 bands were recorded to be polymorphic. Similar results were obtained by Suska and Stejskal (1992) by electrophoretic analysis of seed storage proteins in 13 cultivars of *P. sativum* L. They indicated that among 41 electrophoretic protein bands, only 15 bands were polymorphic. However, Baranger et al. (2004) was able to characterize 121 polymorphic protein bands when they studied 148 accessions of *Pisum*. The electrophoretic profile presented in Figure 2, shows a certain degree of polymorphism among the studied genotypes, except for the two *P. fulvum* L. accessions which showed identical patterns. Similar study by (Przybylska et al., 1982) indicated that *P. fulvum* L. could be clearly distinguished from other *Pisum* species.

The dendrogram generated using protein patterns indicated two main clusters as seen in Figure 3. The first cluster was divided into two subclusters. The first included only the *P. sativum* L. accession from Algeria, while the second subcluster can be divided into two subgroups. The first included four cultivars (Vectory Freezer, Master, Lincoln, Little Marvel) and the two accessions of *P. fulvum* L., while the second included the other accessions provided by ICARDA. The second cluster included cultivars (Montana and Billinder) in addition to the wild taxon collected from El-Dabba. The two accessions of *P. fulvum* L. showed 100 identities. The dendrogram delimited the two accessions of *P. fulvum* L. and the four cultivars (Vectory Freezer, Master, Lincoln, and Little Marvel) as two separate subgroups. These data agree with the conclusion of Ben-Zeév and Zohary (1973) who stated that *P. fulvum* L. is a fully divergent species from *P. sativum* L.

RAPD-PCR analysis

The 17 studied *Pisum* genotypes were compared using 20 arbitrary 10-mer primers in RAPD-PCR. One primer (OPB-19) resulted in no PCR products for all studied genotypes (Figure 4). The other 19 primers generated a total of 216 PCR bands, 196 of them were polymorphic, reflecting high level of polymorphism (90.7%). This is consistent with Samec and Nasinec (1996) who obtained 149 polymorphic bands when they used 8 random

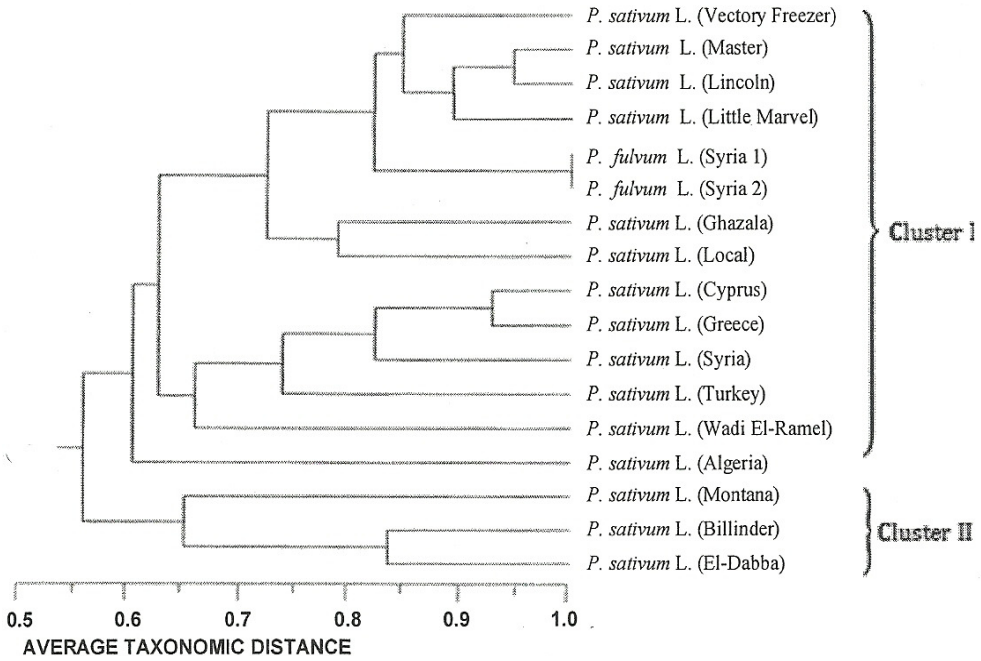

Figure 3. Dendrogram based on markers of SDS-PAGE of seed storage proteins illustrating the relationships of studied *Pisum* genotypes.

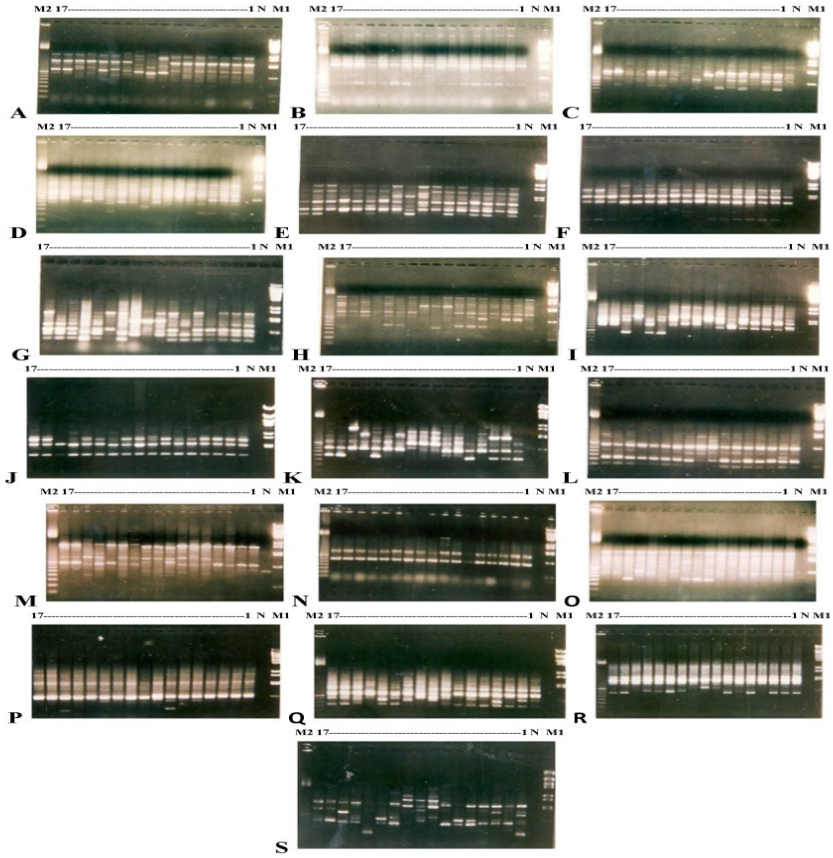

Figure 4. Electrophoretic pattern of RAPD markers of studied *Pisum* genotypes as numbered in Table 1, using Operon B kit primers: OPB-O1 (A) – OPB-20 (S). OPB-19 is not shown. M_1: (λ phage DNA digested with BstEII), M_2: (100 bp DNA ladder), and N: negative control.

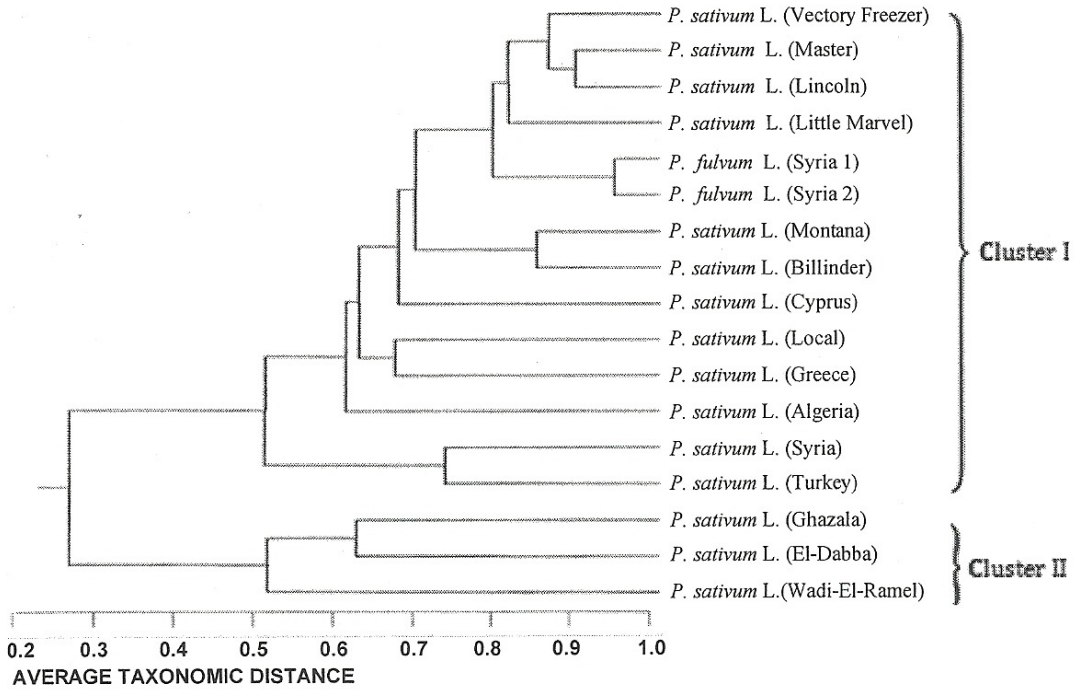

Figure 5. Dendrogram based on RAPD markers illustrating the relationships of studied *Pisum* genotypes.

primers to identify 42 genotypes of *P. sativum* L.

Choudhury et al. (2007) obtained high level of polymorphism (74.8%) when they studied 24 varieties of Indian peas. The average number of RAPD markers per genotype was 67.7. The highest number of RAPD markers (79) was found for *P. fulvum* L. accession from Syria (2), while *P. sativum* L. accession from Syria showed the lowest number (50).

The dendrogram generated using RAPD markers indicated two main clusters (Figure 5). Wild taxa were separated in one cluster. The second cluster included all cultivars and all accessions provided by ICARDA. The highest degrees of similarity were found between the two accessions of *P. fulvum* L. (0.926), the two cultivars Master and Lincoln (0.914), and between the two cultivars Montana and Billinder (0.872). The two accessions of *P. fulvum* L. were successfully separated on the basis of RAPD markers, while the morphological characters, protein patterns and isozymes could not distinguish between them. RAPD markers indicated that cultivars (Vectory Freezer, Master, Lincoln and Little Marvel) and *P. fulvum* L. accessions were still less than one group, suggesting that these cultivars may be originated from the *P. fulvum* L. gene pool.

These results indicated that RAPD dendrogram is more efficient and different from those generated using morphological characters and protein pattern. This is consistent with the results of Wachira et al. (1995), James and Ashburner (1997) and Weder (2002) who indicated that analysis using RAPD markers provide a more reliable genetic variation which was not measurable using morphological or isozyme methods. Cheghamirza et al. (2002), Kovesa et al. (2005) and Samatadze et al. (2008) indicated that RAPD-PCR analysis revealed high genomic polymorphism among pea (*P. sativum* L.) varieties.

The dendrogram generated using all types of markers (morphological characters, protein patterns and RAPD markers) were similar to that generated using RAPD markers (data not shown). This is due to the high number (196 polymorphic bands) of RAPD markers compared with those of morphological characters (17) and protein patterns (15).

In the present study, 60 specific RAPD markers, out of 196 polymorphic bands were scored (Table 4). No specific markers were scored at the level of morphological, seed storage proteins. The utility of RAPDs in detecting high percentage of specific markers has previously been shown by Kongkiatngam et al. (1996). Kovesa et al. (2001) identified a polymorphic 750-bp fragment, RAPD marker, specific to particular pea genotypes. Also, 46 RAPD markers were specific to ten genotypes, one marker (R-B12-475) was specific to *Pisum fulvum* L. The three wild taxa of *P. sativum* L. collected from the north coastal region of Egypt shared 13 specific RAPD markers. In addition, each of them indicated its own specific markers. The taxon collected from Ghazala showed 13 specific markers and the taxon collected from Wadi El-Ramel showed 12 specific markers, while the taxon collected from El-Dabba showed only 5 specific markers. The was a high number of taxa-specific markers reflecting similarities among these taxa

Table 4. Specific markers for *Pisum* species and genotypes.

Species-specific markers	
***Pisum fulvum* L.**	R-B12-475
Wild taxa-specific markers	R-B01-1650, R-B05-1600, R-B06-1400, R-B06-1225 R-B07-1050, R-B08-1450, R-B09-1375, R-B11-850 R-B13-1700, R-B13-1300, R-B13-950, R-B16-550 R-B20-1600
Genotypes-specific markers	
P. sativum L. (cv. Master)	R-B12-450
P. sativum L. (cv. Local)	R-B13-1150, R-B16-450, R-B18-1100
P. sativum L. (cv. Billinder)	R-B15-800, R-B16-400, R-B17-1150
P. sativum L. (taxon Ghazala)	R-B03-1000, R-B06-950, R-B07-900, R-B07-650, R-B08-1325, R-B10-1150, R-B11-1100, R-B12-1000, R-B13-850, R-B14-2500, R-B16-1000, R-B16-800, R-B16-650
P. sativum L. (taxon Wadi El-Ramel)	R-B01-2300, R-B01-800, R-B01-600, R-B04-500, R-B04-450, R-B05-600, R-B07-850, R-B09-450, R-B15-1100, R-B17-1350, R-B17-800, R-B18-900
P. sativum L. (taxon El-Dabba)	R-B01-2500, R-B04-1350, R-B05-750, R-B07-1900,R-B17-1500
P. sativum L. (Turkey)	R-B12-2400, R-B11-650, R-B13-2500, R-B20-2050
P. sativum L. (Greece)	R-B17-1000
P. sativum L. (Syria)	R-B13-775
P. sativum L. (Algeria)	R-B11-1700, R-B16-350, R-B20-900

Table 5. Markers linked to qualitative morphological characters.

Morphological characters	**Specific markers**
Colourless hilum	R-B08-1225, R-B20-1400
Cream cotyledon	*R-B12-450
Wrinkled cotyledon	R-B04-350, R-B20-600
White flower	R-B08-1225, R-B20-1400

*Negative marker.

and their distance from other genotypes. It also shed light on the possible use of these markers in detecting different wild germplasm conferring resistance to environmental stress conditions.

RAPD markers linked to qualitative morphological characters indicated that colourless hilum and white flowers are linked to the same markers (R-B08-1225, R-B20-1400) as shown in Table 5. The use of these RAPD markers in breeding programs makes the selection for these characters easy and possible at the young seedling stage without reaching the maturation stage. This type of selection is called marker-assisted selection (MAS) indicated by Michelmore et al. (1991). Nisar and Ghafoor (2011) identified a RAPD marker linked with powdery mildew resistance in pea.

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