

Full Length Research Paper

Assessment of genetic diversity of nine hybrid combinations of canola using SDS-PAGE, RAPD and ISSR markers

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Genetic variations of nine hybrid combinations of canola were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) marker systems. The protein profile did not show any polymorphism. The RAPD and ISSR primers recorded a high degree of polymorphism among generations of the three canola hybrids. A maximum of 48 DNA bands were detected as polymerase chain reaction (PCR) products for the six RAPD primers from which 42 were polymorphic with mean polymorphism percentage of 82.6%. The four ISSR primers produced 25 bands, of which 23 were polymorphic. The percentage of polymorphism using ISSR primers ranged from 66.6 to 100.0% with an average of 85%. Using cluster analysis, the 9 canola generations were categorized into single generation, F3 (P1 × P3) formed a separate operational taxonomic units (OTU) in cluster and one main cluster that was further divided into three sub clusters. The results show high variation within and among hybrids.

Key words: Canola, generations, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), genetic diversity.

INTRODUCTION

Canola (rapeseed; *Brassica napus* L. genome AACC, 2 = 38) is an important oil crop in the world. It is now the second largest oilseed crop over the world after soybean (*Glycine max*), and can provide 13% of the world's supply (Abbas et al., 2009). Diallel mating procedure has been extensively employed to evaluate parental genotypes before taking any decision concerning the breeding system to be used. In this respect, canola hybrid is already produced by means of self-incompatibility mechanism (Odenbach and Beschonarner, 1995). Several canola investigators studied the combining ability, gene actions and performance of economical traits of canola and detected variability in these parameters. The findings are helpful in the choice of parents suitable for developing superior hybrids as a group for exploiting hybrid vigor and/or building genotypes to be used in following breeding program (Sharaan et al., 2006). But, the success in breeding programs of a crop species largely relies on the presence of sufficient genetic diversity in the germplasm

and the knowledge about the characteristics of the genotypes and their genetic relationship. The genetic diversity is a statistical concept referring to the variance among alleles at individual gene loci, among several loci or gene combinations, between individual plants within population, and between populations or cultivars. The methods of measuring the genetic diversity include classical genetic analysis to evaluate variation in single genes, multivariate analysis to analyze variation in polygenic traits, genealogical analysis, molecular analysis to detect genetic diversity with biochemical and molecular markers (Mahasi and Kamundia, 2007). Other methods have also been applied for this purpose including biochemical techniques especially sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). It is widely used due to its simplicity and effectiveness for describing the genetic structure of crop germplasm. Seed storage proteins have been used as genetic markers in four major areas: (1) analysis of genetic diversity within and between acces-

Table 1. Pedigree and origin of the four canola parents.

Symbol	Parent	Pedigree	Origin
P1	35/9	C103/SIDO -2C103 9C-6SU-1SU-13SW - 2SW0SW	Egypt
P2	26/18	18C-21SU-4SW-15SW-1SW- 0SW	Egypt
P3	Duplo	Variety	Germany
P4	Drakkar	Variety	Germany

Table 2. List of RAPD and ISSR primers and their nucleotide sequences.

Method	Primer code	Sequence
RAPD	OP 09	5-TCCCACGCAA-3
	OP 10	5-TCAGAGCGCC-3
	OP 11	5-GACAGGAGGT-3
	OP 12	5-CAGTGCTGTG-3
	OP 13	5-GTCAGAGTCC-3
	OP 14	5-AGCATGGCTC-3
ISSR	HB 09	GTGTGTGTGTGTGG
	HB 11	GTGTGTGTGTGTCC
	HB 13	GACGACGACGC
	HB 14	CTCCTCCTCGC

sions, (2) founding genome relationships, (3) plant domestication in relation to genetic resource conservation and breeding, and (4) as a tool in crop improvement. SDS-PAGE of seed protein is considered to be a practical and reliable method, because seed storage proteins are largely independent of environmental fluctuations (Stoyanova and Boller, 2010; Takac et al., 2011). On the other hand, DNA based technologies are more suitable in estimating genetic similarity of cultivars. However, the estimated levels of polymorphism of the varieties widely varied with techniques used. The value of RAPD analysis for efficient germplasm management in plants is already known (Young, 2000; Jaroslava et al., 2002). The technique is quick, easy and required less time. This detects nucleotide sequence polymorphisms using a single primer of arbitrary nucleotide sequence (Welsh and McClelland, 1990; Williams et al., 1990). However, ISSR or microsatellite are currently becoming the preferred technique for the molecular characterization of different plant species, because of higher repeatability, co dominant nature, specificity and having multiple alleles (Plieske and Struss, 2001; Halton et al., 2002).

Agronomic parameters are employed in most cases to discriminate among generations in canola. However, studies on the capability of molecular genetic methods are very limited. So, this study aimed at assessing the genetic variations of nine canola combinations belonging to three hybrids by using SDS-PAGE, RAPD and ISSR markers.

MATERIALS AND METHODS

Plant

The seeds of the nine generations (F2, F3 and F4) belonging to three hybrids H1 (P1 × P3), H2 (P2 × P3) and H3 (P3 × P4) of canola were supplied by Agronomy Department (Plant Breeding Section), Faculty of Agriculture, Fayoum University, Fayoum, Egypt. The pedigree and the origin of the parents of these generations are shown in Table 1.

SDS-PAGE

SDS-PAGE was performed in 14% acrylamide slab gels following the system of Laemmli (1970). Protein extraction was conducted by mixing 0.1 g of the seeds of each generation with an equal weight of pure, clean, sterile fine sand. The seeds were then ground to fine powder using a mortar and pestle and were homogenized with 1.5 M Tris-HCl buffer, pH 8.8 in clean Eppendorf tube and were left in refrigerator overnight (Badr, 1995). Then, 20 µl of each sample supernatant was loaded in the gel. After the run was completed, the gel was stained, destained and photographed.

Molecular analysis

Two polymerase chain reaction (PCR)-based techniques; RAPD and ISSR were used. Leaves from nine generations three weeks old greenhouse cultivated plants per generation were pooled together for DNA isolation. DNA extraction was performed using protocols of Dellaporta et al. (1983). For RAPD analysis, six 10-mer random DNA oligonucleotide primers (Operon Technologies, Inc, USA) of arbitrary sequences were independently used in PCR reactions as described by Williams et al. (1990). Codes and sequences of these primers are listed in Table 2. Amplifications were performed in 25 µl reaction volume containing: 1.0 µl deoxyribonucleotide triphosphates (dNTPs; 8 mM), 0.2 µl Taq DNA polymerase (5 U/µl), 2.5 µl 10X buffer with 15 mM MgCl₂, 1.0 µl Primer (10 mM), 1.0 µl template DNA (50 ng/µl) and up to 25 µl H₂O. Each of the reaction mixtures was overlaid with a drop of light mineral oil per sample. Amplification was carried out in Biometra Robocycler. The optimal conditions for PCR amplification were as follows: an initial 4 min denaturation step at 94°C followed by 40 cycles of 90 s at 94°C, 90 s at 37°C and 150 s at 72°C, with a final extension step at 72°C for 7 min. A volume of 25 µl of the RAPD products were electrophoresed in agarose (1.2%) ultra pure used for resolving the PCR products. The run was performed for 1 h at 100 V using Biometra gel electrophoresis submarine (20 × 10 cm). For ISSR analysis, four primers as shown in Table 2 was carried out in a total reaction of 30 µl containing: 2.5 µl dNTPs (8 mM), 0.3 µl Taq DNA polymerase (5 U/µl), 3.0 µl 10X buffer with 15 mM MgCl₂, 2.0 µl Primer (10 mM), 2.0 µl template DNA (50 ng/µl) and up to 30 µl H₂O. PCR amplification was programmed to fulfill 30 cycles after an initial denaturation cycle for 2 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 30 s, an anne-

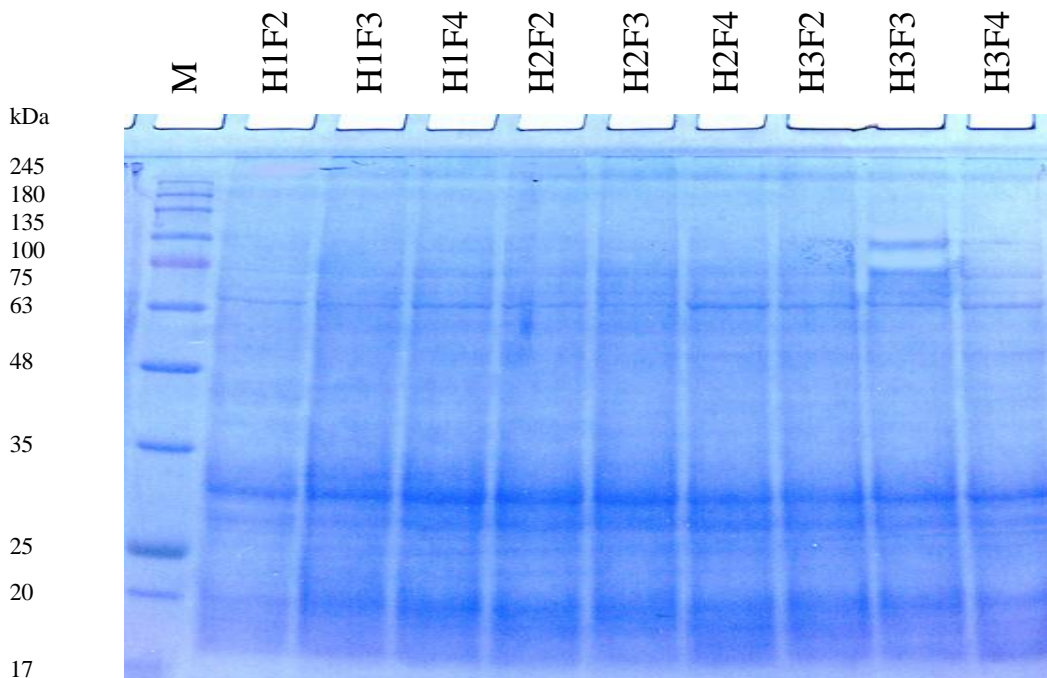


Figure 1. The produced seed protein profile of nine generations belonging to three canola hybrids using SDS-PAGE technique. M: Standard protein marker, kDa: kilo Dalton.

ling step at 44°C for 45 s and an elongation step at 72°C for 90 s. The primer extension segment was done for 7 min at 72°C in the final cycle. PCR-product of 15 µl was resolved in 1.5% agarose gel electrophoresis with 1 X Tris-acetate-ethylenediaminetetraacetic (TAE) running buffer. The run was performed at 80 V for 180 min using Biometra gel electrophoresis submarine (20 × 10 cm).

Data analysis

Bands of RAPD and ISSR techniques were visualized on ultraviolet (UV)-transilluminator and photographed by gel documentation system (Biometra Bio Doc Analyze 2000). Differences in bands intensity among profiles of the different samples were not considered. The binary data generated were used to estimate levels of polymorphism by dividing the polymorphic bands by the total number of scored bands. Band size was estimated by comparing with 1 kb ladder (Invitrogen, USA) using gel analyzer version 3 program. Data generated by RAPD and ISSR primers were used to compile a binary matrix for cluster analysis (NTSYSpc Ver. 2.2). Genetic similarity among accessions was calculated according to Dice similarity coefficient (Dice, 1945) and was used to construct a dendrogram using unweighted pair group method with arithmetic average (UPGMA) and sequential hierarchical and nested clustering (SHAN) routine (Rohlf, 2005).

RESULTS

The produced SDS-protein profile of the nine generations is shown in Figure 1. A maximum number of 25 bands were detected at approximately molecular weights ranging between 135.27 and 14.85 kDa. The protein profile did not show any polymorphism among different genera-

tions.

Photos of the produced banding patterns by application of RAPD and ISSR techniques on the nine studied generations of canola are shown in Figures 2 and 3, respectively. The number and types of the amplified DNA bands and percentage of the total polymorphism are given in Tables 3 and 4, respectively. The six RAPD primers scored 42 polymorphic bands out of 48 bands. The number of amplified fragments with RAPD primers ranged from 5 to 14, with the size of the band ranging from 225.4 to 2842.1 bp. The polymorphism ranged from 60.0 to 100.0%, with an average of 82.6% (Figure 2 and Table 3). Twelve unique bands detected in the six RAPD profiles; six in F2 and F3 of hybrid (P1 × P3), two in F2 of hybrid (P2 × P3), four in F2, F3 and F4 of hybrid (P3 × P4), these bands distinguished the nine generations and could be considered as molecular markers for them as shown in Table 3. A total of 25 fragments were generated by the four used ISSR primers of this study (Table 4). Primer HB 09 yielded the highest number of products (12 amplicons), whereas primer HB11 recorded the least (3 amplicons). ISSR primers produced a higher level of polymorphism (85%) than those of RAPD through 2 monomorphic bands and 23 polymorphic ones as shown in Table 4. Three unique bands characterized F2 and F4 of hybrid (P1 × P3) at 621.4, 627.3 and 432.8 bp, and F2 and F3 of hybrid (P2 × P3) at 372.2, 200.3 and 248.3 bp, while only one unique band had distinguished F2 of hybrid (P3 × P4) with molecular size 221.9 bp.

Genetic similarity was calculated from the dice simila-

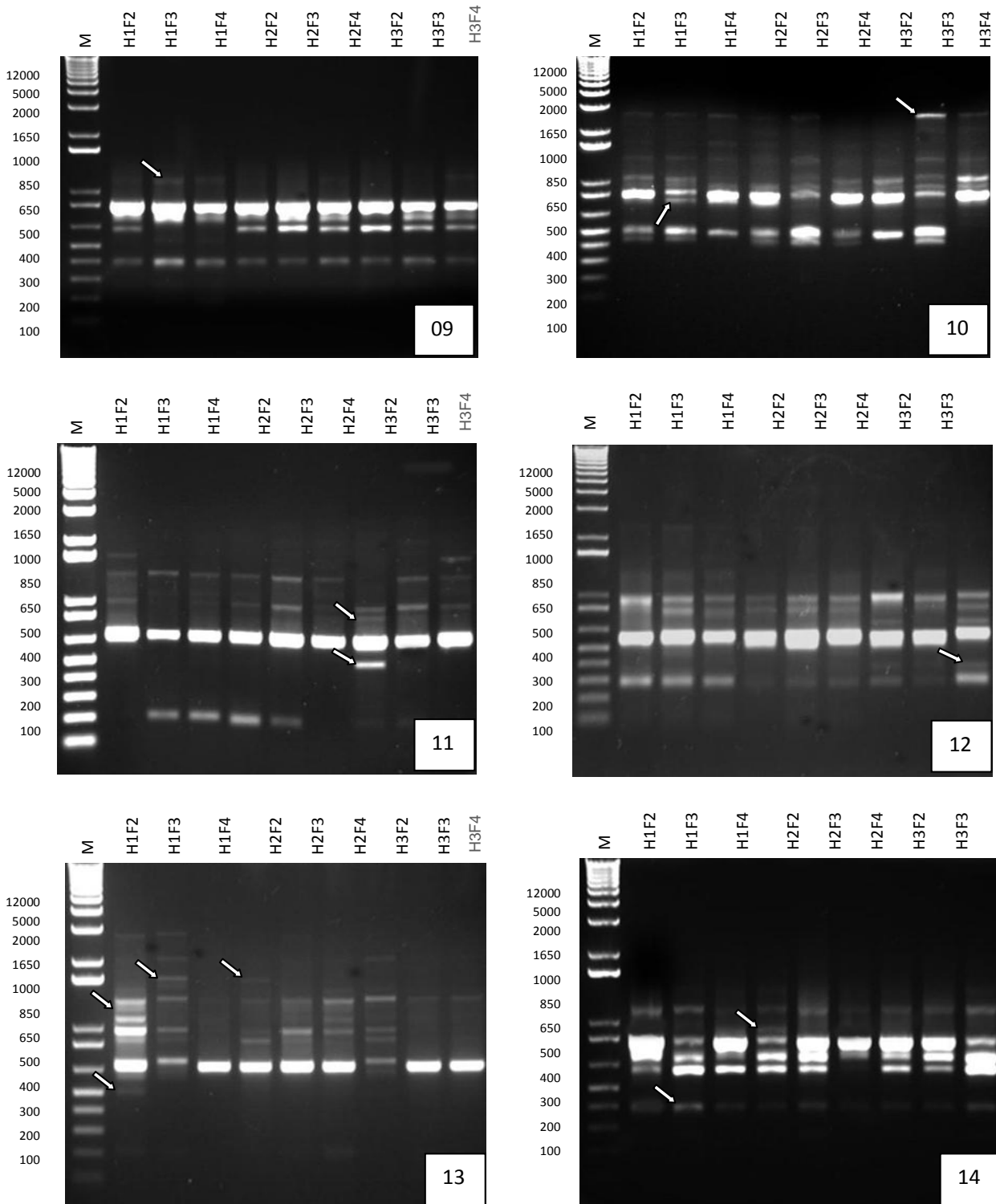


Figure 2. RAPD profiles of the nine generations belonging to three canola hybrids generated by the OP 09, 10, 11, 12, 13 and 14 primers; M = Marker. Arrows indicate unique bands.

urity index value for all the nine generations of canola. Based on RAPD and ISSR markers, the maximum genetic similarity was 0.78 between F3 (P2 × P3) and F3 (P3 × P4), while the lowest genetic similarity of 0.52 was bet-

ween F2 and F3 of the hybrid (P1 × P3) (Table 5). The phylogenetic relationships among nine generations of canola were analyzed by UPGMA method (Figure 4). The cluster result indicated that all generations could be

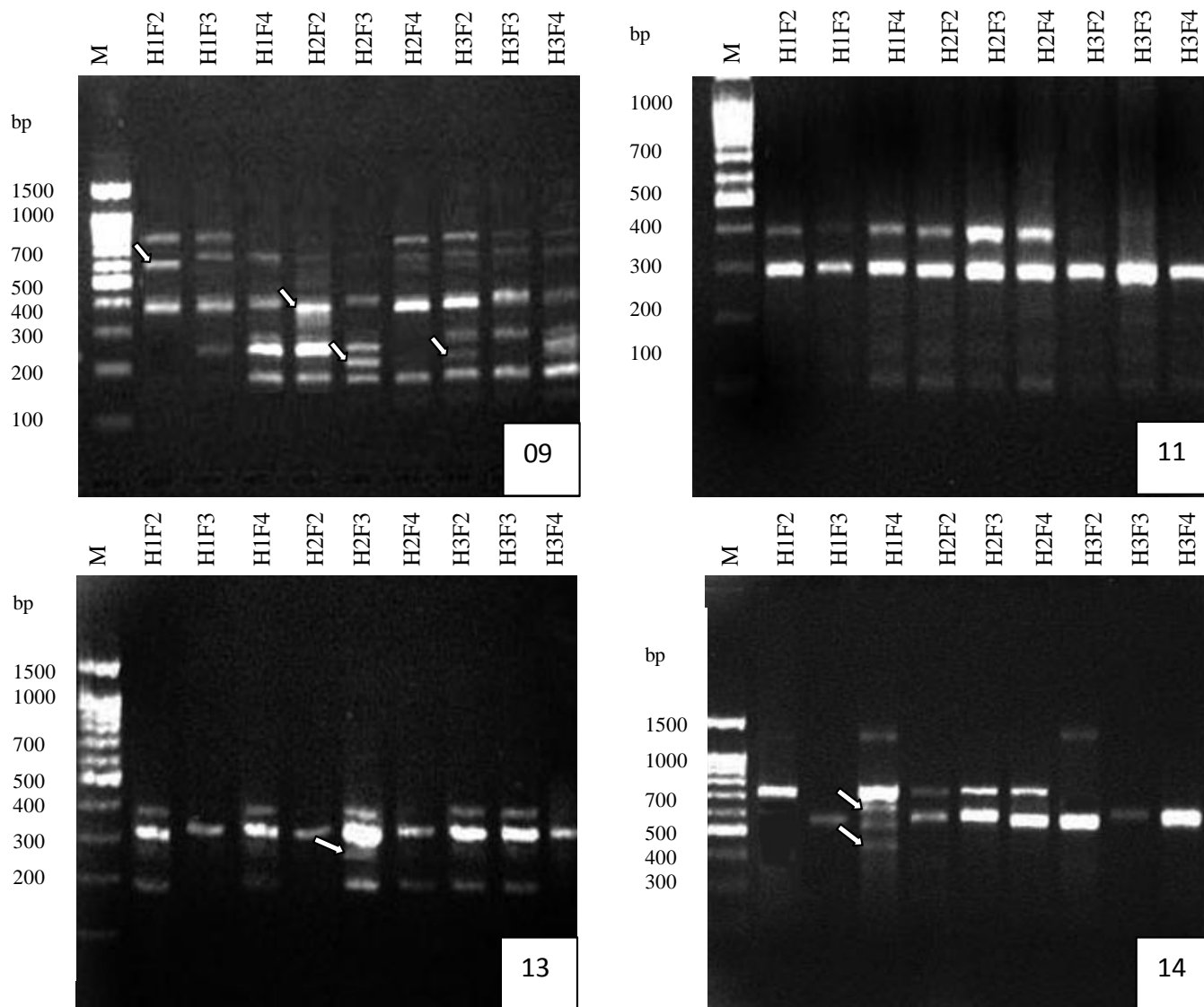


Figure 3. ISSR profiles of the nine generations belonging to three canola hybrids generated by the HB 09, 11, 13 and 14 primers; M = marker. Arrows indicate unique bands

Table 3. Number and types of the amplified DNA bands as well as the percentage of the total polymorphism generated by six RAPD primers in nine canola generations.

Primer code	Monomorphic band	Polymorphic bands		Total band	Polymorphism (%)
		Unique	Shared		
OP 09	2	1	2	5	60
OP 10	0	2	8	10	100
OP 11	1	2	4	7	86
OP 12	2	1	3	6	67
OP 13	0	4	10	14	100
OP 14	1	2	3	6	83

distinguished by RAPD and ISSR markers, respectively. A dendrogram based on UPGMA analysis grouped the 9

generations into one main cluster and single generation F3 (P1 × P3) formed a separate operational taxonomic

Table 4. Number and types of the amplified DNA bands as well as the percentage of the total polymorphism generated by four ISSR primers in nine canola generations.

Primer code	Monomorphic band	Polymorphic band		Total band	Polymorphism (%)
		Unique	Shared		
HB 09	0	4	8	12	100
HB 11	1	0	2	3	66.6
HB 13	1	1	2	4	75
HB 14	0	2	4	6	100

Table 5. Dice similarity coefficient of nine canola generations based on RAPD and ISSR data analysis.

Generation	H1F2	H1F3	H1F4	H2F2	H2F3	H2F4	H3F2	H3F3	H3F4
H1F2	1.00								
H1F3	0.52	1.00							
H1F4	0.53	0.54	1.00						
H2F2	0.62	0.57	0.74	1.00					
H2F3	0.71	0.64	0.69	0.74	1.00				
H2F4	0.69	0.55	0.72	0.72	0.67	1.00			
H3F2	0.55	0.61	0.57	0.54	0.53	0.55	1.00		
H3F3	0.68	0.63	0.61	0.68	0.78	0.59	0.63	1.00	
H3F4	0.61	0.59	0.67	0.67	0.68	0.68	0.68	0.70	1.00

units (OTU) in cluster showing less similarity coefficient (0.58) with the other generations (Figure 4). Generations within main cluster II were further divided into three subclusters (IIa, IIb and IIc). Subcluster IIa comprised F2(P1xP3) with F3(P2xP3) and F3(P3xP4). Subcluster IIb comprised F4(P1xP3) with F2(P2xP3) and F4(P2xP3). Within cluster IIc, F2 and F4 belonging to (P3 x P4) appeared to be closer to each other with similarity coefficient of 0.68.

DISCUSSION

The produced SDS-PAGE of seed protein profile (Figure 1) did not show any polymorphism among different generations. The results were not similar with the reports of Ahmed and Afiah (2008), who have detected a slight variation in protein banding pattern of nine lines of two ancestors of canola investigated under three environmental conditions. The low level of protein polymorphism could be attributed to the conservative nature of the seed protein. This conclusion is in accordance with Nisar et al. (2007) and Sultana and Ghafoor (2008).

Two marker systems, ISSR and RAPD, are used in this study to evaluate genetic diversity of canola generations. Six RAPD primers scored 42 polymorphic bands out of 48 bands. The polymorphism ranged from 60.0 to 100.0%, with an average of 82.6%. On the other hand, four ISSR primers produced a higher level of polymorphism (85%) than those of RAPD through 2 monomorphic bands and 23 polymorphic bands. These results are

in agreement with the study of Abdelmigid (2012) who indicated the presence of wide genetic variability as a result of the high polymorphism among some genotypes of *B. napus*. The significant level of polymorphism has previously been reported in canola (Seyis et al., 2003; Shengwu et al., 2003; Shiran et al., 2004; Marjanovic-jeromela et al., 2009; Moghaddam et al., 2010), chickpea (Sudupak, 2004) and common bean (Marotti et al., 2007). This high level of polymorphism could be attributed to their pedigree information (Agrama and Tuinstra, 2003).

Nineteen unique bands (Figures 2 and 3 and Tables 3 and 4) produced by ten primers of RAPD and ISSR are considered as molecular markers and succeeded in discriminating the nine canola generations. The recorded molecular variations among canola generations in this study supported the results of Kaiser and Sadaqat, (2004) who indicated that various generations; P1, P2, F1, F2, BC1, and BC2 of three crosses of *B. napus* have significant difference for all the morphological traits and oil contents under both normal and drought conditions, and Sharaan et al. (2006) who revealed highly significant differences among the parents and their F₁ and F₂ crosses for all studied plant and yield traits of canola.

Using cluster analysis, the 9 canola generations are categorized into one main cluster and single generation F3 (P1 x P3) formed a separate OTU in cluster. The main cluster II is further divided into three sub clusters. The results of genetic similarity analyses (Figure 4 and Table 5) showed that extensive genetic diversity existed in 9 canola generations used during this study. The detection of moderate to high level of genetic diversity in this study

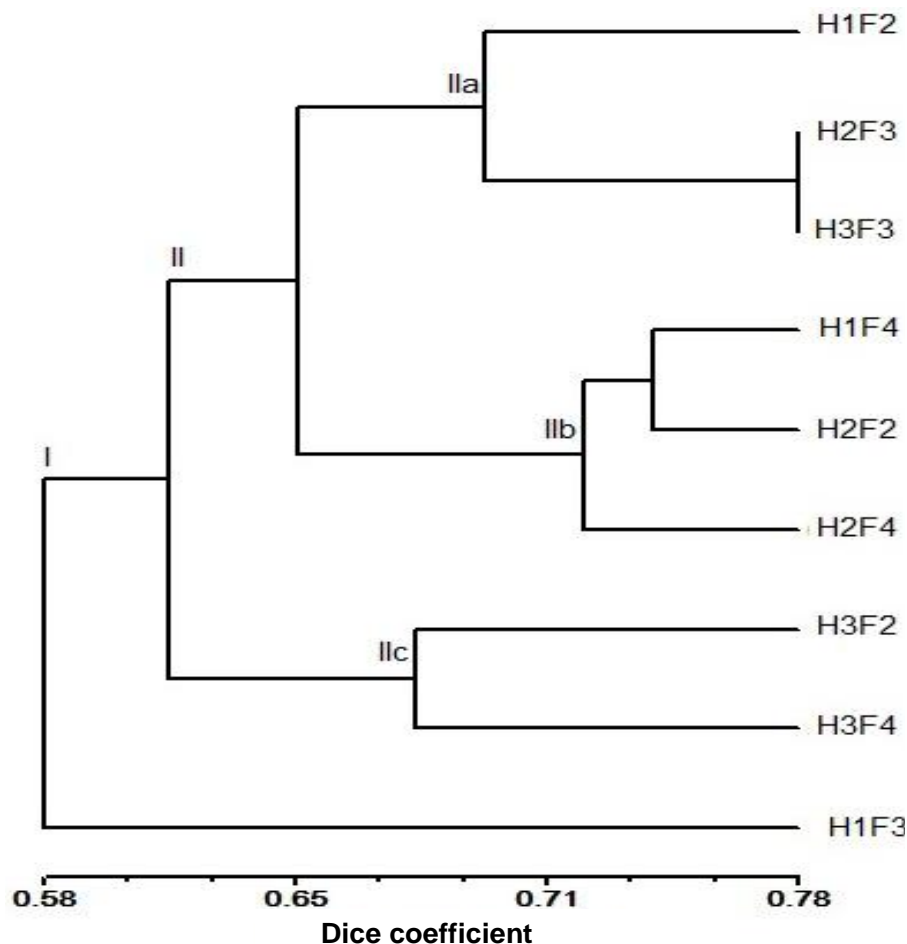


Figure 4. UPGMA phenogram showing genetic diversity of the nine canola generations based on combination of RAPD and ISSR characters.

is in agreement with previous reports where molecular markers detected high level of genetic polymorphism (Welsh and McClelland, 1990; Dos Santos et al., 1994; Chen et al., 2000; Abbas et al., 2008; Abdelmigid, 2012) in different crop species. For better understanding of the presence of genetic variability in canola generations and hybrids and consequently more efficient utilization of existing variability for improvement of the crop in Egypt, more biochemical and molecular data is required. Conclusively, this study is used to identify suitable genetic markers that could be used in crop breeding through marker assisted selection (MAS) and confirmed that RAPD and ISSR markers provided useful information for understanding the intra- and inter-specific variations and genetic relationships of canola hybrids through different generations for selecting the best for quality and yield improvements.

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