

Analysis of Genetic Diversity among a Population of Canola Genotypes As Reveled By ISSR-PCR and Their Associations to Seed Yield and Oil Content

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Abstract

A Population of four canola variants and their derived gamma rays- induced mutant lines were used to examine genetic diversity among them based on ISSR- PCR analysis. The study was extended to find if any ISSR variants were related to seed yield and oil percentage. The evaluation of mutant lines for three consecutive years at two different locations (Inshas and Ras Sudr) for seed yield and its attributes showed that lines 92 and 63 possessed highly significant performance than the other mutants and their parents for seed yield /plant in both locations. Thirteen ISSR primers were used in this study. All primers generated 1064 DNA bands, with an average of 81.84 bands per primer, 980 bands of them were polymorphic. All primers detected unique bands (84 bands); no monomorphic bands were observed in this study. The profiles generated by primer ISSR2 contained the highest percentage of polymorphic bands (97.16%). Results showed that lines 92 and 63 generated from Evita and Pactol cultivars possessed the highest seed yield /plant in Inshas and Ras Sudr locations, respectively. Those lines also showed the highest number of unique, bands which could be used as candidate molecular markers for high seed yield. Data showed that line 92 and 63 are excellent performing new genotypes which could be introduced in breeding program to obtain new Egyptian canola varieties. The primers ISSR2, ISSR4, ISSR6 and ISSR9 could be considered specific primer for canola genotypes in searching for the discovery of molecular markers associated high seed yield/ plant and consequently oil percentage.

Key words: Canola cultivars, Genetic diversity, ISSR-PCR, Seed yield, Oil content

Introduction

Oil crops of various plants, especially family of *Brassicaceae* such as species of Brassica had a great deal of research in recent years. Canola (rapeseed; *Brassica napus* L. genome AACC, $2n = 38$) arises from spontaneous hybridization between turnip (*Brassica rapa*) (AA, $2n = 20$) and cabbage (*Brassica oleracea*) (CC, $2n = 18$). It is now the second largest oilseed crop over the world after soybean (*Glycine max*) providing 13% of the world's supply (Abbas *et al.*, 2009).

Availability of genetic variability is the prerequisite for any breeding programme. Induced mutation has been extensively used for developing new genetic variation in crop plants. Literature revealed that more than 2200 mutant varieties of different crops with improved agronomic traits have been developed and released to the farmers for general cultivation all over the world (Maluszynski *et al.*, 2000).

Molecular tools facilitate the identification of genomic locations linked to traits of interest and help in indirect selection of such complex traits without the need for difficult phenotypic measurements. In the last few decades, new DNA molecular markers, based on the PCR technique, such as RAPD-PCR and ISSR among others, have become excellent tools for plant breeders (Williams *et al.* 1990).

Inter simple sequence repeat (ISSR) technique is a PCR based method, which involves amplification

of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites, usually 16–25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter- SSR sequences of different sizes. The microsatellite repeats used as primers can be di-nucleotide, tri-nucleotide, tetranucleotide or penta-nucleotide. The primers used can be either unanchored (Gupta *et al.*, 1994; Wu *et al.*, 1994) or more usually anchored at 3' or 5' end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz *et al.*, 1994).

Therefore, the present study aimed to determine the genetic diversity among four canola cultivars and their derived gamma rays- induced mutant lines using ISSR-PCR and their associations to seed yield and oil content.

Materials and Methods

Plant materials

Four cultivars Serow 4, Serow 6, Pactol and Evita and their derived mutant lines (Farrag *et al.*, 2012) were used in this study.

Seed yield and oil percentage measurement

Seeds of the best five plants from each mutant line of nine mutants and their parents were sown in experimental design with three replicates at two locations (Inshas & Ras Sudr) for three successive seasons, 2014, 2015 and 2016.

Average mean of seed yield (g) / plant for three seasons and Oil percentage according to McCabe *et al.*, 1993 (during the last season) were measured.

Genomic DNA extraction

Genomic DNA for each genotype was extracted using Plant DNeasy Mini Kit (Qiagen, Santa Clarita, CA) according to the manufacturer's instructions. RNase A (100 mg/ml, Sigma, USA) was added to the DNA solution to remove RNA contamination, incubation at 37°C for 30 min is required. Quantification of the DNA concentration in different samples was done by measuring the optical density at 260 nm according Beer-Lambert equation: Concentration ($\mu\text{g/ml}$) = $\text{OD}_{260} \times 50 \times \text{dilution factor}$. The quality of DNA was determined using agarose gel (0.9%) electrophoresis.

ISSR amplification

Thirteen ISSR primers were used in this study for generating reproducible and reliable amplicons for different canola mutant lines and their parents. The names and sequences of the selected primers are shown in Table (1). PCR reaction was performed in 25 μl reaction volume containing 30 ng template DNA, 2.5 μL of 1X PCR buffer, 1.5 μL of 25mM MgCl_2 , 2.5 μL of the dNTPs mix, 30 pmol of ISSR primer, 1.0 U Taq DNA polymerase (Promega, WI, USA). The amplification was performed in a thermal cycler (Applied Bio Systems, USA) programmed for initial denaturation for 5 min at 94°C; 40 cycles of 2 min denaturation at 94°C, 45 Sec. annealing at 50°C and 2 min extension at 72°C, followed by extension cycle for 7 min at 72°C in the final cycle.

Table 1. List of ISSR primers used in this study.

Primer Name	Sequence 5'→3'
ISSR- 1	AGAGAGAGAGAGAGAGYC
ISSR- 2	AGAGAGAGAGAGAGAGYG
ISSR- 3	ACACACACACACACACYT
ISSR- 4	ACACACACACACACACYG
ISSR- 5	GTGTGTGTGTGTGTGTYG
ISSR- 6	CGCGATAGATAGATAGATA
ISSR- 7	TCTCTCTCTCTCTCTCA
ISSR- 8	AGACAGACAGACAGACGC
ISSR- 9	GATAGATAGATAGATAGC
ISSR- 10	GACAGACAGACAGACAAT
ISSR- 11	ACACACACACACACACYA
ISSR- 12	ACACACACACACACACYC
ISSR- 13	AGAGAGAGAGAGAGAGYT

Detection of PCR products

The ISSR-based PCR products analyses were detected using 1.5% agarose gel electrophoresis in 1X TBE buffer, stained with 0.3 $\mu\text{g/ml}$ ethidium bromide and then visually with UV-transilluminator, documented in Gel-Doc XR (Bio-Rad) and photographed. The size of the amplicons was determined using 100 bp plus DNA ladder.

Data Analysis

The ISSR bands were scored as present (1) or absent (0), each of which was treated as independent. By comparing the banding patterns of all genotypes for a specific primer, genotype -specific bands were identified. Generated data 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 100bp ladder (Invitrogen, USA). Genetic similarity among genotypes was calculated according to Dice coefficient (Dice, Lee R. 1945). A dendrogram was generated by clustering analysis method (Rokach, Lior, and Oded Maimon. 2005) using Statistica software.

Results and Discussion

Seed yield and seed oil content

Average means of seed yield /plant, during three successive generations was calculated (Figure 1). At both locations Inshas and Ras Sudr all mutant lines achieved significant difference in seed yield compared to their parents except line 38 which had no significant difference compared to Serow 6 cultivar at Inshas location. Among mutant lines, Line 92 followed by line 75 and line 74 showed the highest seed yield/plant in Inshas location whereas line 8, line 66 and line 78 showed the lowest seed yield / plant in the same location. In Ras Sudr, mutant lines 63 and 66 showed the highest seed yield/plant, whereas line 78 and line 38 showed the lowest seed yield/plant. Oil percentage of seeds for the four cultivar and their mutant lines were estimated according to McCabe, and Smith, (1956). The line 75 had the highest value of oil percentage than the other genotypes while line 11 showed the lowest value of oil percentage (Figure 2). Canola plants have a high seed oil content (up to 45%), high protein content (30 to 35%) and easy to cultivate in newly reclaimed lands with relatively low costs (Afiah *et al.*, 2007; Sharaan *et al.*, 2006).

The previous results were similar to those obtained by Malek *et al.*, (2012), where they found

that the overall performance of the two selected mutants, MM-10-04 and MM-08-04, was better than the popular mother variety. Also Javed *et al.*, (2003)

found that mutant lines RM-005 and RM-025 had the high seed yield which was significantly higher than the control variety.

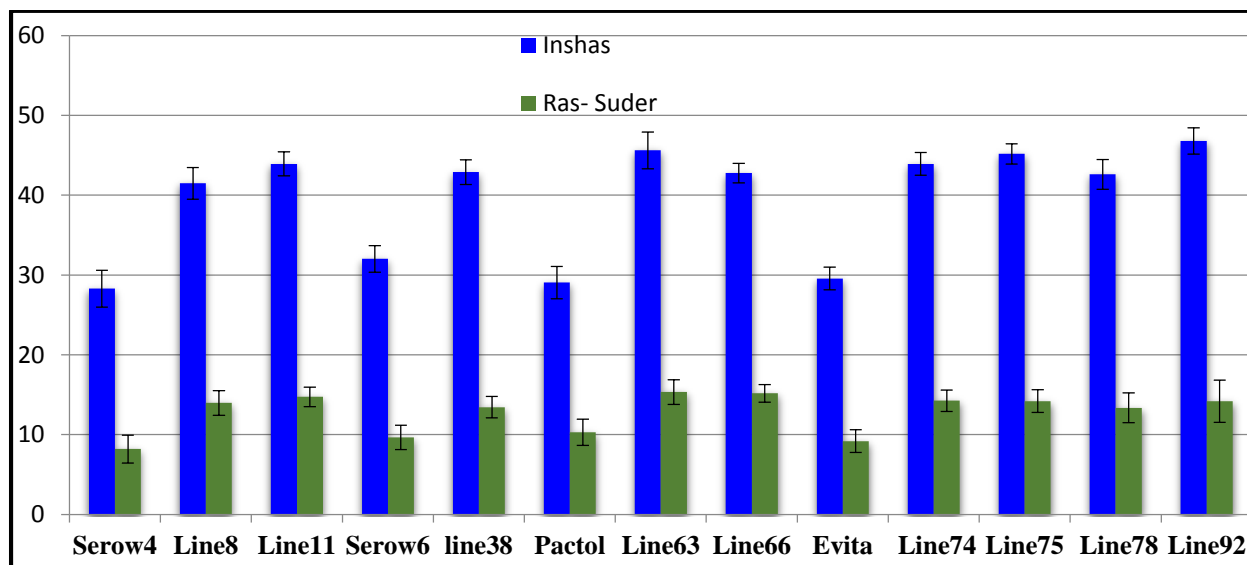


Figure 1: Seed yield/plant (g) of nine mutant lines and four parents at two different locations (Inshas & Sudr). The presented data are the average mean \pm standard error.

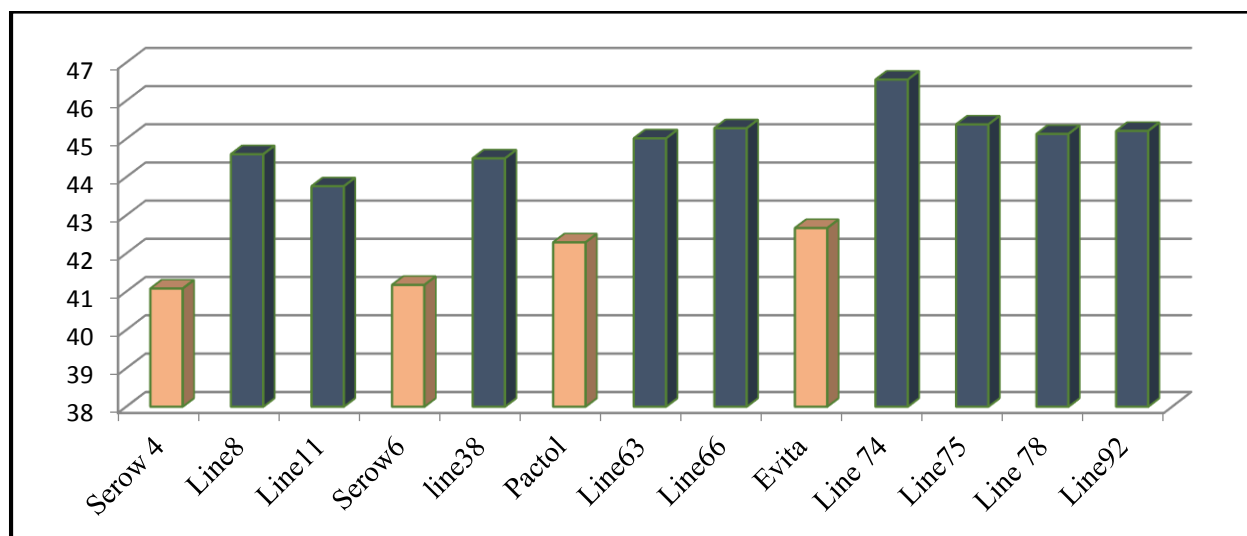


Figure 2: Seed oil content (%) of nine mutant lines and four parents estimated during the last season.

Polymorphisms detected by ISSR markers

High level of polymorphism was observed among the studied *Brassica napus* genotypes (Tables 2). Thirteen ISSRs primers were used to characterize the genetic divergence of the four canola cultivars and their mutants. The number of amplified bands, the number of polymorphic bands, the number of unique bands and the polymorphism percentage are shown in Table 3. All primers successfully generated reproducible polymorphic products except ISSR7 primer that did not produce any products neither with serow 4 and its mutants nor with serow 6 and its mutant, ISSR8 primer did not produce any products with serow 4 and its mutants, ISSR12 primer did not produce any products neither with serow 4 and its

mutants nor with Evita and its mutants, and finally ISSR13 primer that did not produce any products with Evita and its mutants (Figure 3). The percentage of polymorphism ranged from 78% (ISSR 13) to 97.16% (ISSR 2) with an average of 87.58%. A total of 1064 bands were amplified and 980 (92.1%) of them were shown to be polymorphic. It was found that 84 of them were unique bands, representing 7.9% of all amplified bands. The average of total amplified bands per primer was 81.84 (Table 3).

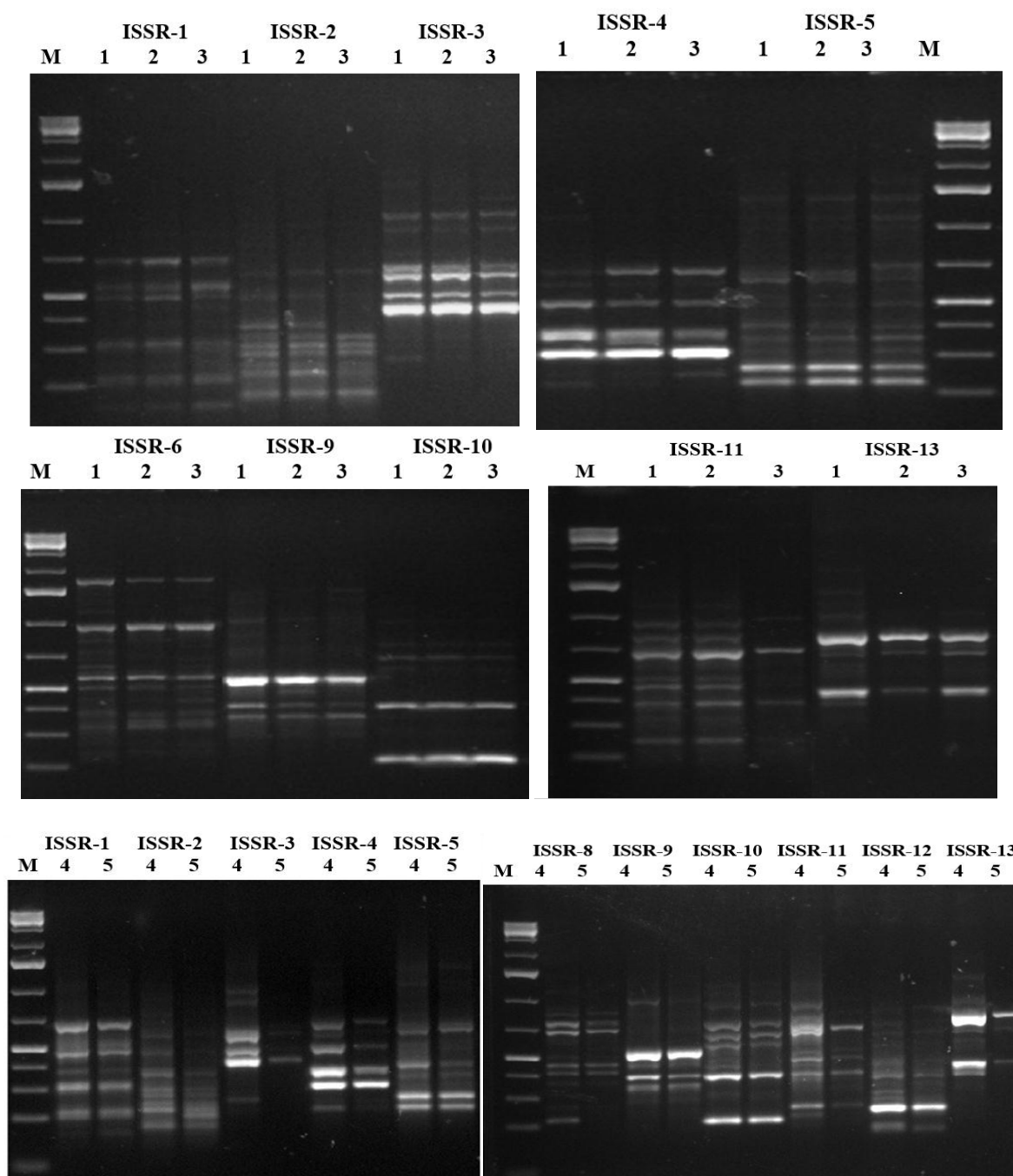
The number of bands detected by each primer depended on primer, sequence and the extent of variation in specific cultivar. The number of amplified fragments varied from 33 (ISSR 12) to 113 (ISSR 5) and the amplicon size varied from 46 bp

(ISSR 6) to 14200 bp (ISSR 1). ISSR 2, ISSR 4, ISSR 6, and ISSR 9 produced the highest percentage of polymorphism in the present study. Primers with higher polymorphic bands are more efficient in studying genetic diversity and discrimination of the genotypes (Roman *et al.*, 2004; Moghaddam *et al.*, 2010).

ISSR results showed that line 92 that showed the highest seed yield/plant in Inshas location gave 9 unique bands, also line 63 that showed the highest seed yield/plant in Ras Sudr location gave 14 unique bands with all used primers, which mean that those bands must be used for selecting high yield canola genotypes.

Our finding with ISSR molecular markers tested pointed to some distinguish bands and unique bands and could be used as selection tool for high number of pods/plant and high weight(g) of 100 seeds.

The ISSR marker used in this study have also been used as effective tools to determine genetic diversity and to shed light on the phylogenetic relationships in Brassica (Marjanovic-jeromela *et al.*, 2009), Ricinus (Gajera *et al.*, 2010). These studies have given important clues in understanding the origin and relationships between species, which will assist in developing breeding strategies.



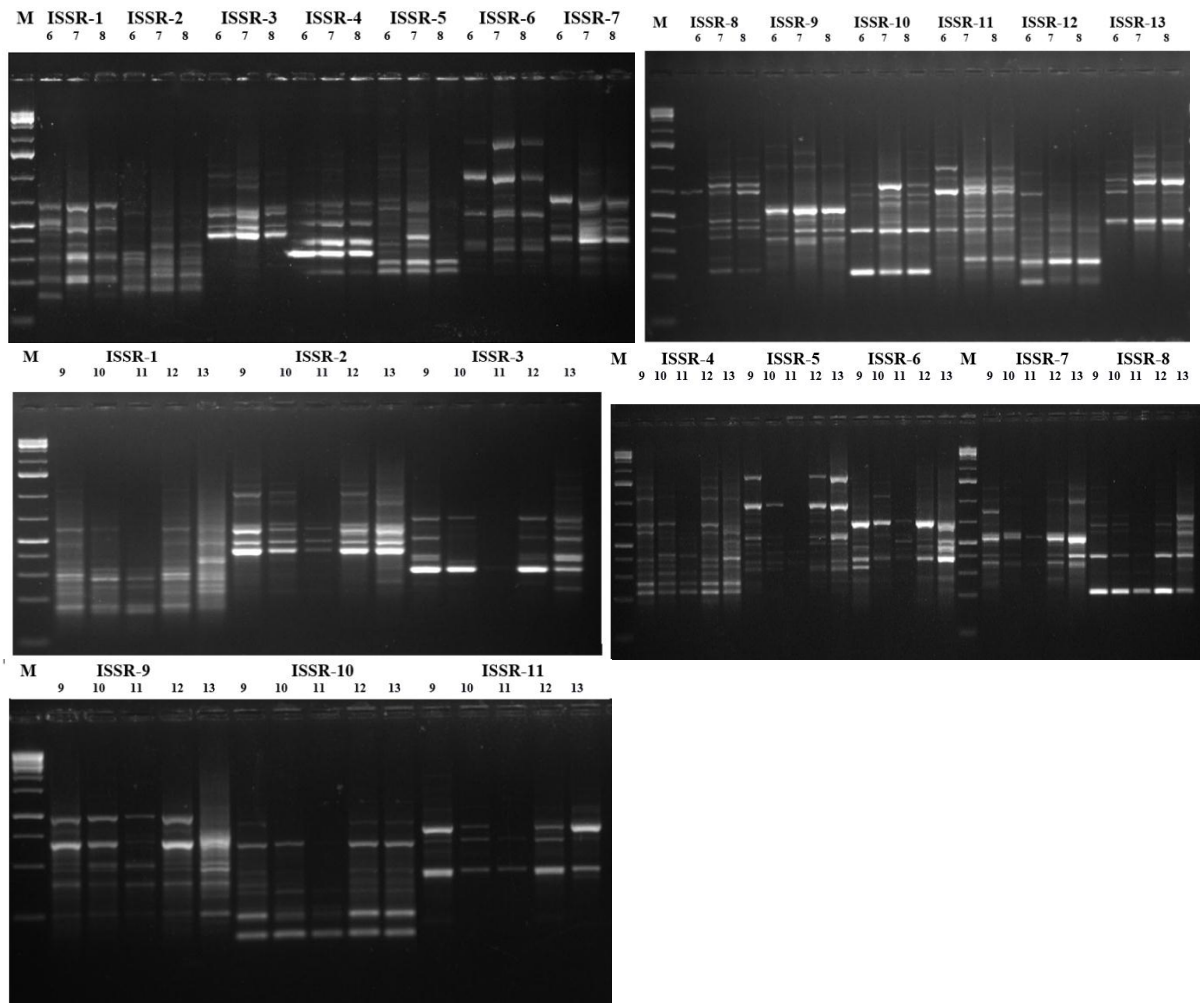


Figure 3: ISSR-PCR products of 13 genotypes of canola produced with thirteen primers. Lane M is 100bp ladder and lanes 1 to 13 represent different canola accessions Serow 4, Line 8, Line 11, Serow 6, Line 38, Pactol, Line 63, Line 66, Evita, Line 74, Line 75, Line 78, and Line 92 respectively.

Table 2. List of canola cultivars and their derived mutant lines and their origin.

Cultivar/ Mutant	Origin
Serow 4	Egypt
Line 8	Mutant line from Serow 4
Line 11	Mutant line from Serow 4
Serow 6	Egypt
Line 38	Mutant line from Serow 6
Pactol	French
Line 63	Mutant line from Pactol
Line 66	Mutant line from Pactol
Evita	Germany
Line 74	Mutant line from Evita
Line 75	Mutant line from Evita
Line 78	Mutant line from Evita
Line 92	Mutant line from Evita

Table 3. Amplified DNA fragments (bands) after using ISSR primers on canola genotypes and their derived mutants.

primer	Sequence 5'-3'	Total bands	Polymorphic bands	Unique bands	Polymorphism (%)
ISSR1	AGAGAGAGAGAGAGAGYC	103	97	6	94.17
ISSR2	AGAGAGAGAGAGAGAGYG	106	103	3	97.16
ISSR3	ACACACACACACACACYT	70	65	5	92.85
ISSR4	ACACACACACACACACYG	110	106	4	96.36
ISSR5	GTGTGTGTGTGTGTGYG	120	113	7	94.16
ISSR6	CGCGATAGATAGATAGATA	71	68	3	95.77
ISSR7	TCTCTCTCTCTCTCA	45	36	9	80
ISSR8	AGACAGACAGACAGACGC	79	68	11	86.07
ISSR9	GATAGATAGATAGATAGC	87	84	3	96.55
ISSR10	GACAGACAGACAGACAAT	87	81	6	93.10
ISSR11	ACACACACACACACACYA	91	84	7	92.30
ISSR12	ACACACACACACACACYC	42	33	9	78.57
ISSR13	AGAGAGAGAGAGAGAGYT	50	39	11	78
Total		1064	980	84	
Mean		81.84	75.38	6.46	90.38

Genetic distance and similarities based on ISSR markers

As shown in Table 4 and 5, genetic distance and similarities were calculated the 13 Canola genotypes based on ISSR data analysis. Based on ISSR markers, the maximum genetic similarity was 0.83 between line 8 and line 11, while the lowest genetic similarity of 0.06 was between Line 11 and line75. On the contrary the lowest genetic distance was 4.4 between line 8 and line 11, while the maximum genetic distance of 13.2 was between Line 63 and Line 92.

Knowledge of genetic similarity or genetic distance between genotypes is useful in breeding programs because it provides more efficient sampling of genotypes to hybridize for the

development of populations (Afiah *et al.*, 2007). In this study, the clustering analysis revealed good relationships between some cultivars and their derived mutant lines (Tables 5; Figure 4).

Phylogenetic analysis based on ISSR

The phylogenetic relationships among 13 Canola genotypes (Figure 4) were analyzed by clustering analysis method (Rokach, Lior, and Oded Maimon. 2005). The clustering data indicated that all the genotypes could be distinguished by ISSR markers. A dendrogram based on clustering analysis grouped the 13 Canola genotypes into four clusters, the Egyptian cultivar Serow 4 and its mutant lines formed a separate cluster showing less similarity

with the other genotypes. The cultivar Serow 6 and its mutant formed II. Cluster III included German cultivar Evita and its mutant lines, while French (Pactol) cultivar formed cluster IV. Within all clusters, cultivars showed moderate and high similarity with mutant lines, while showed low similarity with other cultivars and their mutant lines (Figure 4).

In general, the clustering analysis did not show clear pattern of clustering according to the origin of cultivars. It is clear from the cluster analysis that

genotypes from the same origin grouped together, I while in some cases they were placed in different clusters (Figure 4).

Thus, in this study the association between genetic similarity and the origin was not essential. Therefore, it is necessary to use more number of genotypes from each origin to confirm the available pattern. Similar results were reported in castor (Gajera *et al.*, 2010), Melocanna (Lalhruaitluanga and Prasad, 2009), rapeseed (Marjanovic-Jeromela *et al.*, 2009) and in Trigonella (Dangi *et al.*, 2004).

Table 4. Genetic distance, calculated as the total number of ISSR band differences, among the 13 Canola genotypes.

Genotypes	Serow 4	Line 8	Line 11	Serow 6	Line 38	Pactol	Line 63	Line 66	Evita	Line 74	Line 75	Line 78	Line 92
Serow 4	0.0												
Line 8	4.6	0.0											
Line 11	5.8	4.4	0.0										
Serow 6	9.9	10.1	9.9	0.0									
Line 38	10.3	10.0	9.8	9.1	0.0								
Pactol	11.7	11.5	11.3	12.5	11.9	0.0							
Line 63	12.3	12.0	11.8	12.6	12.4	8.6	0.0						
Line 66	11.6	11.4	11.3	12.8	11.8	8.2	7.9	0.0					
Evita	11.5	11.5	11.4	13.0	12.2	12.8	13.0	12.5	0.0				
Line 74	10.8	10.4	10.8	12.0	11.2	12.4	12.7	12.2	7.8	0.0			
Line 75	9.9	9.5	9.6	11.0	10.3	11.2	11.5	11.3	9.0	6.5	0.0		
Line 78	11.4	11.3	11.5	12.8	12.2	12.8	12.9	12.5	7.7	6.7	8.3	0.0	
Line 92	11.7	11.5	11.6	12.9	12.2	12.9	13.2	12.5	9.3	8.2	8.6	7.3	0.0

Table 5. Similarity coefficient (Dice Similarity Measure) of 13 Canola genotypes based on ISSR data analysis.

Genotypes	Serow 4	Line 8	Line 11	Serow 6	Line 38	Pactol	Line 63	Line 66	Evita	Line 74	Line 75	Line 78	Line 92
Serow 4	1.00												
Line 8	0.82	1.00											
Line 11	0.72	0.83	1.00										
Serow 6	0.37	0.32	0.34	1.00									
Line 38	0.26	0.26	0.29	0.51	1.00								
Pactol	0.17	0.16	0.19	0.19	0.21	1.00							
Line 63	0.14	0.14	0.17	0.21	0.19	0.65	1.00						
Line 66	0.17	0.17	0.18	0.14	0.21	0.66	0.70	1.00					
Evita	0.18	0.15	0.15	0.12	0.16	0.17	0.18	0.20	1.00				
Line 74	0.16	0.17	0.11	0.12	0.17	0.10	0.12	0.13	0.64	1.00			
Line 75	0.08	0.08	0.06	0.09	0.12	0.11	0.12	0.09	0.42	0.63	1.00		
Line 78	0.19	0.17	0.13	0.13	0.15	0.16	0.19	0.20	0.69	0.73	0.50	1.00	
Line 92	0.16	0.14	0.13	0.12	0.16	0.15	0.16	0.19	0.55	0.60	0.46	0.73	1.00

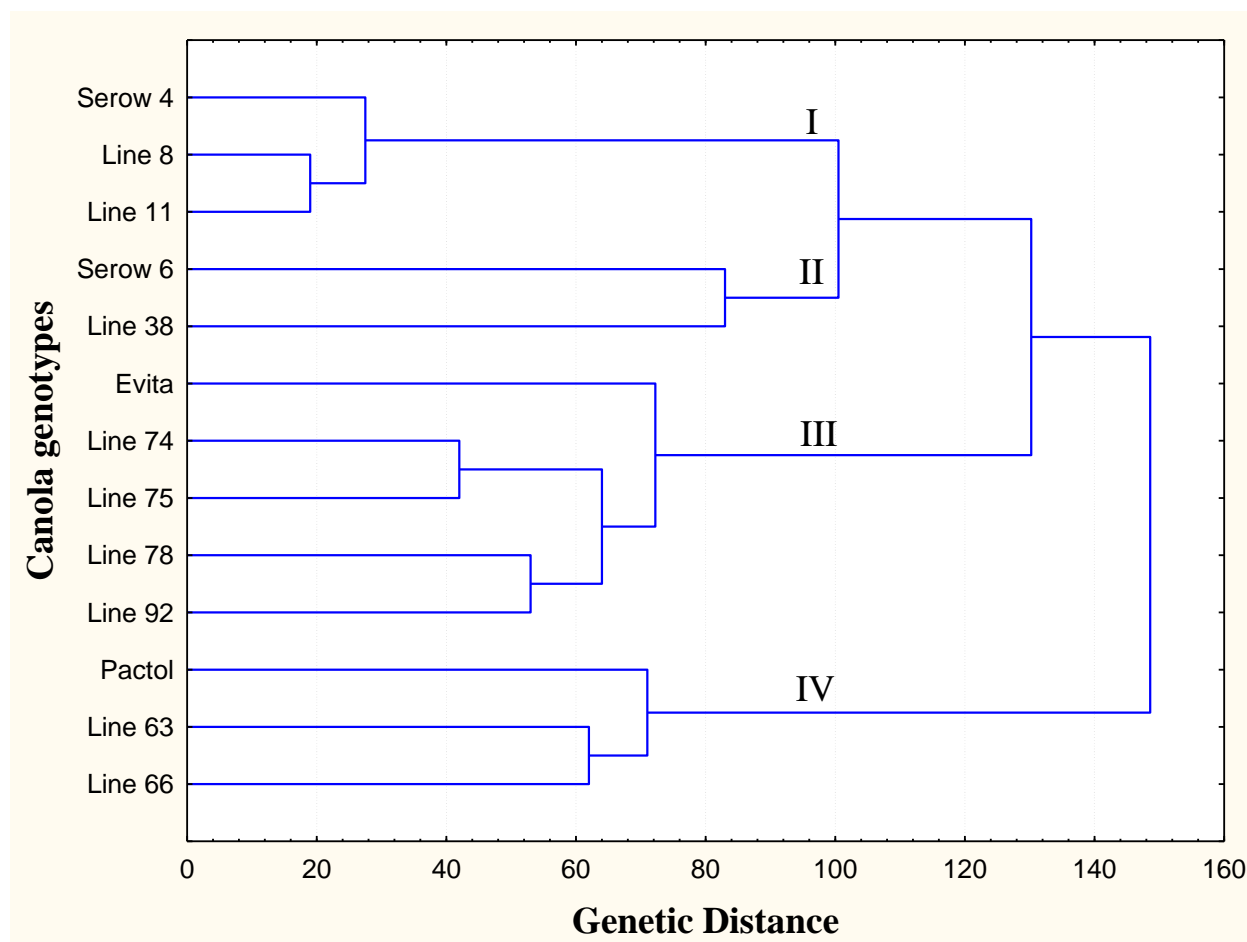


Figure 4. Phylogenetic tree (Linkage dendrogram) of studied Canola genotypes based on their ISSR-PCR banding patterns.

Conclusion

The present findings further strengthened previous reports that the ISSR markers can be used effectively to estimate genetic distances among genotypes. However, it is suggested that a greater number of genotypes and molecular markers is required to have better understanding of the presence of genetic variability in *B. napus* germplasm and consequently, more efficient utilization of existing variability for improvement of this important crop. Results indicated that line 92 and 63 showed the highest seed yield/ plant in Inshas and Ras Sudr respectively. Also those lines showed the highest number of unique bands which must be used as molecular markers for high seed yield. Data showed that line 92 and 63 are excellent performing new genotypes which could be introduced in breeding program to obtain new Egyptian canola varieties. The four primers ISSR2, ISSR4, ISSR6 and ISSR9 could be considered a specific primer for canola genotypes for the discovery of molecular markers associated high seed yield/ plant and consequently oil percentage.

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تحليل التنوع الوراثي بين مجموعة من التراكيب الوراثية لعشيرة الكانولا باستخدام ISSR-PCR وإرتباطها بمحصول البذور ومحتوى الزيت

مني السيد فراج¹ - سعيد سعد سليمان² - إبراهيم محمد عامر¹ - رانية محمد يحيى هيكل² - عبدالله عبدالعظيم حسنين²

1- قسم البحوث النباتية - مركز البحوث النووية - هيئة الطاقة الذرية - مصر.

2- قسم الوراثة - كلية الزراعة - جامعة الزقازيق - مصر.

تم استخدام مجموعة من أربعة أصناف من الكانولا والسلالات الطفرية المستحدثة منهم باستخدام أشعة جاما للتعرف على التنوع الوراثي فيما بين الأصناف بناءً على تحليل ISSR-PCR. إمتدت الدراسة لمعرفة ما إذا كانت أي من متغيرات الـ ISSR تتعلق بإنتاجية البذور ونسبة الزيت. أظهر تقييم السلالات الطفرية الناتجة لمدة ثلاث سنوات متتالية في موقعين مختلفين هما إنشاص ورأس سدر لإنتاجية البذور وسماتها أن السلالتين 92 و 63 تتمتع بأداء ملحوظ عن السلالات الأخرى والآباء من حيث إنتاجية البذور/ نبات في كلا الموقعين. تم استخدام ثلاثة عشر بادئ للتمييز بين المواد قيد الدراسة. أنتجت جميع البودائ 1064 حزمة من الحمض النووي، بمتوسط 81.84 حزمة لكل بادئ، 980 حزمة منها كانت متعددة المظاهر. جميع البودائ حددت حزم فريدة (84 حزمة)؛ لم يلاحظ أي حزم أحادية المظهر في هذه الدراسة. احتوت نتائج البادئ ISSR2 على أعلى نسبة من الحزم المتعددة الأشكال (97.16%). كما أظهرت النتائج أن السلالتين 92 و 63 الناتجة من أصناف Evita و Pactol تمتلك أعلى إنتاجية لمحصول البذور / نبات في مواقع إنشاص ورأس سدر على الترتيب، كما أظهرت تلك السلالات أكبر عدد من الحزم الفريدة التي يمكن أن تعتبر معلمات جزيئية للإنتاجية العالية من محصول البذور. خلصت النتائج إلى أن السلالتين 92 و 63 هما من التراكيب الوراثية الجديدة الممتازة التي يمكن إدخالها في برامج التربية للحصول على أنواع جديدة من الكانولا المصرية. تعتبر البودائ الأربعة ISSR2 أو ISSR4 و ISSR6 و ISSR9 بمثابة بودائ متخصصة لأصناف الكانولا والسلالات الناتجة منها باستخدام أشعة جاما لإكتشاف المعلمات الجزيئية لمحصول البذور / نبات وبالتالي نسبة الزيت.