



ASSESSMENT OF GENETIC DIVERSITY OF SOME CANOLA GENOTYPES

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ABSTRACT

Analyzed of genetic diversity for six canola genotypes using 5 inter-simple sequence repeats (ISSR) primer pairs were done. The cluster results of UPGMA method indicated that all the genotypes could be distinguished by ISSR markers. Six genotypes were categorized into two main clusters. Cluster I comprised Egyptian genotypes Serw-6 and Serw-4 and German genotype AD 201/Gi/81. Genotypes within cluster II comprised of French (Pactol), Chinese (Wan-you 25) and German (RG 4514).

Key words: Canola (*Brassica napus* L.), genetic diversity, ISSR, principal coordinate (PCo) analysis, UPGMA.

INTRODUCTION

Canola (*Brassica napus* L.) has become an object of tissue culture studies and breeding because it is one of the most important non-traditional oil crops which is expanding and spreading as an important source of oil plant extraction, protein-rich meal and biofuels in the world (Burbulis *et al.*, 2008). Canola is a member of the family Brassicaceae. Also, it ranks the second largest oilseed crop after soybean in global oil production in the world (Borjian and Arak, 2013). Canola is a very important oil seed crop in the world. Its oil is the best types of edible oils used in human feeding, because it has the lowest saturated fat content compared to all edible oils, it contains 6% of saturated fatty acids and 94% non-saturated fatty acids as well as, very low level of low density lipids (Cholesterol) (Al-Naggar *et al.*, 2008) and also contains compounds of omega beneficial to human health. It is widely used as cooking oil, salad oil and making margarine. In addition, canola oil is used in lubricants

and hydraulic fluids especially when there is a significant risk of oil leaking to waterways or into ground water (Hussain *et al.*, 2014).

Inter simple sequence repeat (ISSR) is segments of DNA found between two simple sequence repeats (usually in dinucleotide or trinucleotide repeats). 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. ISSR techniques is nearly identical to RAPD techniques except that ISSR primers are designed from microsatellite regions and are longer (approximately 14 or more bp) than RAPD primers. So 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 (Pradeep Reddy *et al.*, 2002).

ISSR segregate mostly as dominant markers following simple Mendelian inheritance (Gupta *et al.*, 1994; Ratnaparkhe *et al.*, 1998). However, they have also been

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shown to segregate as co-dominant markers in some cases, thus enabling distinction between homozygotes and heterozygotes (Wu *et al.*, 1994; Sankar and Moore, 2001).

Also, Wolfe (2005) reported that inter-simple sequence repeat (ISSR) markers were originally devised for differentiating among closely related plant cultivars but have become extremely useful for studies of natural populations of plants, fungi, insects, and vertebrates. This marker is easily generated using minimal equipment and are hyper variable, yielding with a large amount of data for a reasonable cost to the researcher. The methods for Miniprep DNA extraction and cleanup, polymerase chain reaction (PCR) amplification, optimization, data gathering and scoring, and data analyses are outlined.

The genetic diversity of 24 Chinese weak-winter, Swedish winter and spring of *Brassica napus* accessions by inter-simple sequence repeats (ISSRs) were compared by Chao-zhi *et al.* (2003). Using cluster analysis (UPGMA) based on 125 polymorphism bands amplified with 20 primers, the 24 accessions were divided into three groups. Six Swedish winter lines and eight of Chinese weak-winter lines were in group I and group II consisted of two Chinese weak-winter lines, Xiangyou15 and Bao81. The third group contained eight Swedish spring lines. Principal coordinates (PCO) analysis showed similar groupings to cluster analysis. Results from cluster analysis and PCO analysis showed very clearly that Chinese weak-winter, Swedish spring and winter accessions were distinguished from each other and Chinese weak-winter accessions were genetically closer to Swedish winter accessions than to Swedish spring accessions. The Chinese weak-winter accessions had larger diversity than the Swedish spring or winter accessions. This study indicated that ISSR was a suitable and effective tool to evaluate genetic diversity among rapeseed germplasm.

In addition, Abdelmigid (2012) determined the genetic diversity evaluations among 10 canola (*Brassica napus* L.) genotypes using ISSR markers. The five ISSR primers produced 94 bands across 10 genotypes, of which 76 were polymorphic with an average of 15.2 polymorphic fragments per primer. The number of amplified bands varied from 4 to 32, with size of amplicons ranging from 127 to 3011 bp. The percentage of polymorphism using ISSR primers ranged from 25 to 100.0 with an average of 78.8%. Dice similarity coefficient was calculated for all pairwise comparisons and was used to construct a UPGMA dendrogram. The similarity coefficient ranged from 0.47 to 0.73 with ISSR and combined dendrogram.

Havličková *et al.* (2014) using microsatellites (SSRs), ISSRs and AFLPs to determine the genetic diversity of 94 accessions of winter oilseed rape (*Brassica napus* L.), representing past and contemporary material utilized in the Czech breeding programmes and found that the greatest genetic distance was found by ISSRs (62.3%).

Abd-El-aziz and Habiba (2016) assessed the genetic diversity among ten homozygous lines of canola using five ISSR primers, were found the high significant positive correlations for molecular distance among all of these molecular marker techniques and combined data, which indicates the reliability of the combined data for molecular distances in accurate assessment for genetic diversity and identifying the genetic relationships between all studied homozygous lines in canola. Accordingly, cluster analysis and principal coordinate (PCo) analysis based on combined data were used for indicating degree of similarity which was high between all the studied homozygous lines. Moreover, PCo analysis was succeeded in assessment of genetic diversity and description of heterogeneity within studied lines.

In this study, molecular characteristics of the selected genotypes using ISSR- technique were investigated.

MATERIALS AND METHODS

Plant Materials

Six canola genotypes from Egypt, China, Germany and France were used in this study. The genotypes included are shown in Table 1.

DNA Extraction

Genomic DNA for each genotype was isolated using the protocol for medicinal and aromatic plants. To remove RNA contamination, RNase A (10 mg/ml, Sigma, USA) was added to the DNA solution and incubated at 37°C for 30 min. Estimation of the DNA concentration in different samples was done by measuring optical density at 260 nm according to the following equation: Concentration ($\mu\text{g/ml}$) = $\text{OD}_{260} \times 50 \times \text{dilution factor}$. The quality of DNA was determined using agarose gel (0.8%) electrophoresis.

ISSR Amplification

Five primers for ISSR were used in the study. The names and sequences of the selected primers are shown in Table 2. PCR analysis was performed in 25 μl reaction volume containing 1x PCR buffer, 4 mM MgCl_2 , 0.2 mM dNTPs, 20 pmol/ μl primer, 2 units Taq DNA polymerase and 25 μg template DNA. PCR amplification was programmed to fulfill 40 cycles after an initial denaturation cycle for 4 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 40°C for 1 min and an extension step at 72°C for 2 mins, followed by extension cycle for 7 mins at 72°C in the final cycle.

Detection of PCR Products

The PCR products were visualized in agarose gel 1.5% in 1X TBE buffer, stained with ethidium bromide then fragments sizes were estimated visually by comparison with a standard 100-bp DNA ladder.

Data Analysis

The ISSR reproducible bands were scored as present (1) or absent (0), each of which was treated as independent locus regardless of its intensity. By comparing the banding patterns of genotypes for a specific primer, genotypes -specific bands were identified. Faint or unclear bands 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 10000 bps using FAMD (Version 1.31) program. Genetic similarity among accessions was calculated according to Dice similarity coefficient and used to construct a dendrogram using un-weighted pair group method with arithmetic average (UPGMA) (Smith *et al.*, 1997). Polymorphism information content (PIC) values were calculated according to Smith *et al.* (1997), using the following formula as follows: $\text{PIC} = 1 - \sum f_i^2$, where f_i is the frequency of the allele. PIC provides an estimate of the discriminatory power of a locus by taking into account, not only the number of alleles but also the relative frequencies of those alleles. PIC values vary from 0 (monomorphic) to 1 (very highly discriminative, with many alleles in equal frequencies).

RESULTS AND DISCUSSION

ISSR Analysis

High level of polymorphism was observed among the studied canola genotypes (Table 3). ISSR primers produced different numbers of DNA fragments, depending on their simple sequence repeat motifs (Fig. 1). The ISSR primers produced 41 bands across the 6 genotypes, of which 16 were polymorphic.

The number of bands varied from six (17899B) to 9 (17899A, HB11 and HB12). Average number of bands and polymorphic bands per primer were 8.2 and 3.2, respectively. The percentage of polymorphism

Table (1): List of used canola genotypes and their origin.

No.	Genotype	Origin	Type
S ₁	Serw-6	Egyptian	spring
S ₂	Pactol	French	spring
S ₃	Wan-you 25	Chinese	winter
S ₄	RG 4514	German	winter
S ₅	Serw-4	Egyptian	spring
S ₆	AD201/Gi/81	German	winter

Table (2): List of ISSR primers.

No.	Primer	Primer sequence (5' 3')
1	17898A	5' [CA] ₆ AC 3'
2	17899A	5' [CA] ₆ AG 3'
3	17899B	5' [CA] ₆ GC 3'
4	HB11	5' [GT] ₆ CC 3'
5	HB12	5' [CAC] ₃ GC 3'

Table (3): List of ISSR primers, the number of amplified products, the number of polymorphic and monomorphic bands, and percentage of polymorphism obtained by analyzing 6 genotypes of canola.

No.	Primer	Primer Sequence (5'-3')	Total No. of bands	No. of Polymorphic band	No. of Monomorphic band	Polymorphism (%)
1	17898A	(CA) ₆ AC	8	1	7	12.50
2	17899A	(CA) ₆ AG	9	4	5	44.44
3	17899B	(CA) ₆ GG	6	3	3	50.00
4	HB11	(GT) ₆ CC	9	4	5	44.44
5	HB12	(CAC) ₃ GC	9	4	5	44.44

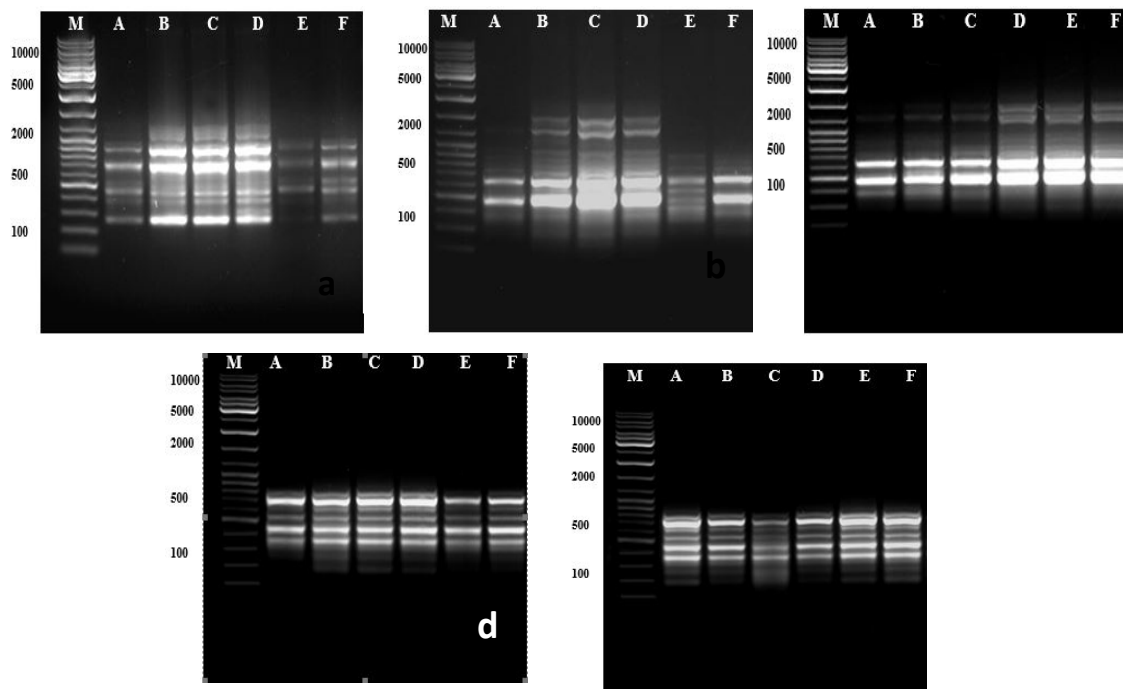


Fig. (1): ISSR-PCR products of 6 genotypes of canola produced with (a) primer 17898A; (b) primer 17899A; (c) primer 17899B; (d) HB11; (e) HB11. Lane M is 1 kb ladder and lanes 1 to 6 represent different canola accessions as listed in Table 1.

ranged between 12.50% (17898A) and 50% (17899B) with an average of 39.2%. ISSR bands were scored for presence (1) or absence (0) among the genotypes and used for UPGMA analysis.

Molecular Distances

Based on ISSRs results presented in Table 4 show that the highest molecular distance (MD) was among Wan-you 25 with Serw-4 (0.30), on the other hand the lowest MD was between Pactol and Wan-you 25 (0.07). According to ISSR results the MD ranged from 0.07 to 0.30.

Cluster Analysis and Principal Coordinate Based on ISSRs Data

Accordingly, cluster analysis and principal coordinate (PCo) analysis for 6 homozygous lines of canola were performed based on the relative genetic distances from ISSRs results (Fig. 2).

Adhikari et al. (2015) and Abd-El-Aziz and Habiba (2016) determined the

consistency of the differentiation among the genotypes defined by the cluster analysis using the PCo analysis. Although the high similarity degree between all the canola homozygous lines, the PCo analysis was succeeded in assessment of genetic diversity and description of heterogeneity within studied lines efficiently (**Sonja et al., 2008**).

Phylogenetic Analysis Based on ISSR

The phylogenetic relationships among 6 accessions of *B. napus* were analyzed by UPGMA method (Fig. 3). The cluster result indicated that all the genotypes could be distinguished by ISSR markers. A dendrogram based on UPGMA analysis with ISSR results is shown in Fig. 3 Using MEGA program version 5.0 (**Tamura et al., 2011**). The 6 genotypes were grouped into two main clusters. Cluster I comprised Egyptian genotypes Serw-6 and Serw-4 and German genotype AD 201/Gi/81. Genotypes within cluster II comprised of French (Pactol), Chinese (Wan-you 25) and German (RG 4514).

Table (4): Simple conelations of molecular distances (MD) between the six canola homozygous lines based on Dice dissimilarity index for ISSR technique.

	Serw-6	Pactol	Wan-you 25	RG 4514	Serw-4
Pactol	0.20				
Wan-you 25	0.28	0.07			
RG 4514	0.19	0.13	0.11		
Serw-4	0.08	0.22	0.30	0.21	
AD 201/ Gi/81	0.18	0.21	0.29	0.25	0.16

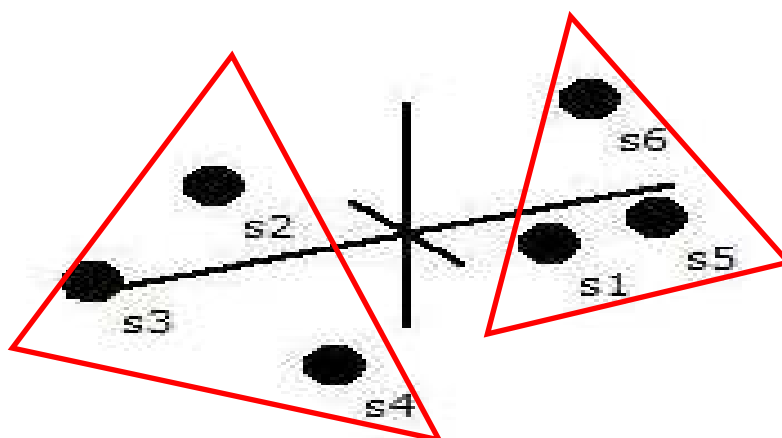


Fig. (2): Principal coordinate (PCo) analysis for 6 homozygous lines based on dice dissimilarity index for ISSRs data.

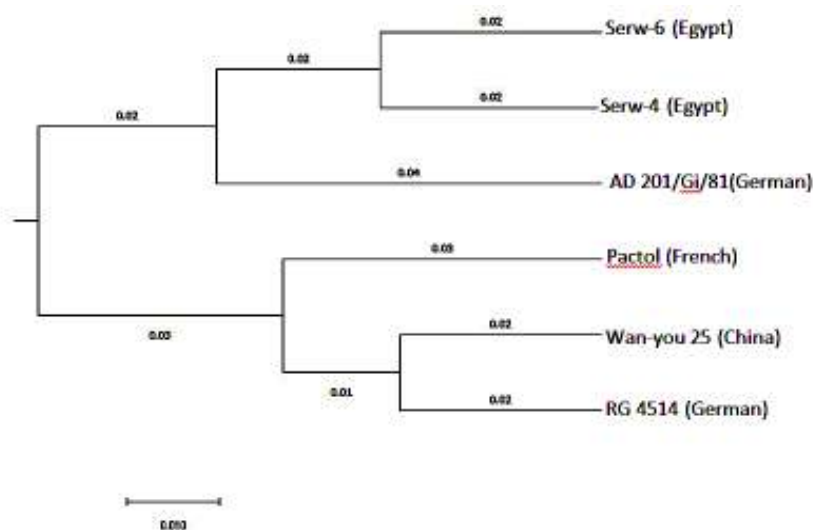


Fig. (3): ISSR data-based dendrograms obtained from 6 accessions of canola with UPGMA based on Dice coefficient using MEGA sof.

Conclusion

1. ISSR primers produced different numbers of DNA fragments, depending on their simple sequence repeat motifs. The ISSR primers produced 41 bands across the 6 genotypes, of which 16 were polymorphic. The number of bands ranged from six (17899B) to 9 (17899A, HB11 and HB12). Average number of bands and polymorphic bands per primer were 8.2 and 3.2, respectively. The percentage of polymorphism ranged between 12.50% (17898A) and 50% (17899B) with an average of 39.2%.
2. The highest molecular distance (MD) was among Wan-you 25 with Serw-4 (0.30), while the lowest MD according to the same results was between pactol and Wan-you 25 (0.07). According to ISSR data the MD ranged from 0.07 to 0.30.
3. The cluster result of UPGMA method and principal coordinate (PCo) analysis indicated that all the genotypes could be distinguished by ISSR markers. Six genotypes were categorized into two main clusters. Cluster I comprised Egyptian genotypes Serw-6 and Serw-4 and German genotype AD 201/Gi/81. Genotypes within Cluster II comprised of French (Pactol), Chinese (Wan-you 25) and German (RG 4514).

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الملخص العربي

تقييم التنوع الوراثي لبعض التراكيب الوراثية من الكانولا

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تم تحليل التنوع الوراثي لـ ٦ تراكيب وراثية من الكانولا باستخدام ٥ بريميرات من تتابعات معلمات ISSR، وأظهرت نتائج التحليل باستخدام طريقه UPGMA أن جميع التراكيب الوراثية يمكن تمييزها باستخدام معلمات الـ ISSR، حيث تم تقسيم الستة تراكيب وراثية إلى مجموعتين رئيسيتين: تتألف المجموعة الأولى من التراكيب الوراثية المصرية سر-٦، سر-٤ والألماني ادا ٢٠١١/جي اي/٨١، بينما تتألف المجموعة الثانية من التراكيب الوراثية الفرنسي (باكتول)، الصيني (وان-يو ٢٥) والألماني (ار جي ٤٥١٤).

الكلمات الإسترشادية: الكانولا، ISSR، تحليل (PCo) Principal Coordinate، UPGMA، تحليل النسق المميز.

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