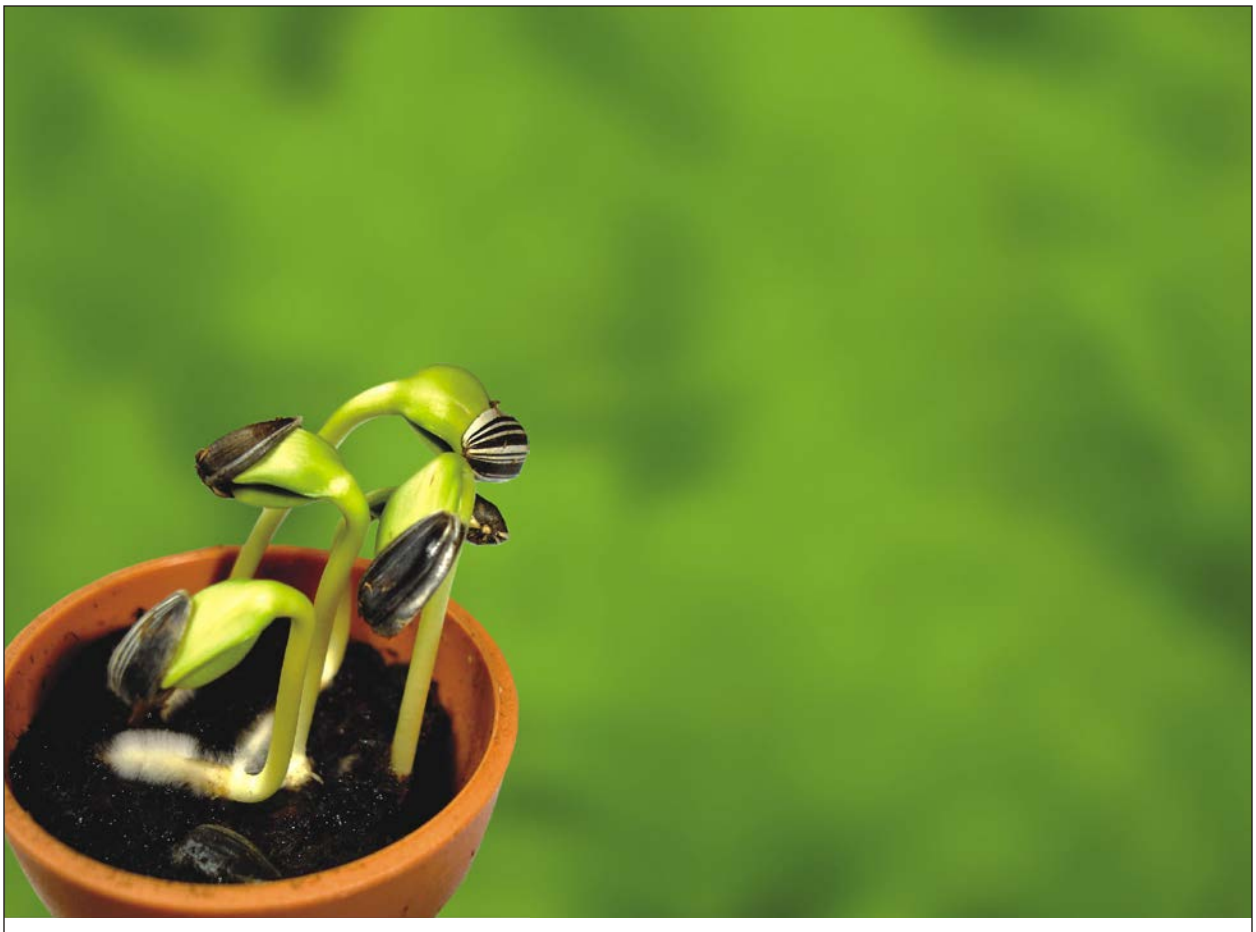

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Genetic Diversity of *Brassica napus* L. Varieties Estimated by Morphological and Molecular Markers

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ABSTRACT

Six canola (*Brassica napus*) varieties were used in this study which was chosen as a representative sample of commercial canola varieties used for oil production. The objectives of this study were; 1) cluster the varieties understudy using the morphological characters oil and protein percentages, 2) estimation the genetic diversity among varieties understudy using RAPD markers, and 3) find the correlation between RAPD and morphological diversity. Data showed that the averages of all varieties for oil and protein percentages were 39.38 and 16.77, respectively. Euclidean distances based on oil and protein percentages were calculated. Their values ranged from 14.14 to 92.05 and the average of the distances was 42.9. This data indicated that the amount of phenotypic variation among the varieties was relatively high. The cluster diagram based on Euclidean distance separated varieties into two groups. Also, data of the 10 random primers used indicated a total of 401 scorable bands. Total amplified DNA fragments were 93 and number of DNA fragments per primer varied from 6-13. Total polymorphic fragments were 55 with an average of 57.76% polymorphic fragment per primer. The similarity coefficient values based on RAPD markers ranged from 0.114 to 0.261 with an average of 0.183. The conducted dendogram showed that five varieties formed one main cluster. RAPD results (81.7% divergence) indicated higher diversity than indicated by oil and protein morphological markers (42.9%). Finally, Mantel test was calculated to find out the correlation between RAPD markers and morphological characters matrices. A lack of correlation was obtained (r = -0.08992) and many possible reasons were discussed.

Key words: *Brassica napus*, oil and protein content, euclidean distance, RAPD, similarity coefficient, mantel test

INTRODUCTION

Family Brassicaceae contains many genus one of them is *Brassica* L. which contains 19 species one of them is canola (*Brassica napus* L.). The first oilseed crop in the world is soybean and the second is canola, therefore, it considered as an important source of vegetable oil (FAO, 2009). In the seasons 2010-2011, the worldwide production was about 58.4 million tons (http://www.fas.usda.gov/oilseeds/circular/2011/March/oilseeds).

In the national breeding programs, the study of genetic relationships (diversity) is important for the selection of suitable diverse parents to obtain heterotic hybrids, predict progeny performance, conserve and characterize used germplasms. In addition, knowledge about relatedness between parents in canola breeding programs could be used to avoid the possibility of elite germplasm to become genetically uniform, thereby endangering long-term selection gains and conserved genetic resources.

The main objective of canola breeding programs is enhancement of protein and oil concentrations of seeds. Therefore, evaluation of genetic diversity related to these two characters is important for sustainable production of canola since losses of genetic diversity among any collection for these characters may limit chances of canola improvement (Kresovich *et al*., 1992; Diers and Osborn, 1994; Hallden *et al*., 1994; Cruz *et al*., 2007). Many techniques had been used to estimate genetic diversity in plants such as morphological, protein, isozyme and DNA-based markers (Shengwu *et al*., 2003).

Genetic diversity has been conventionally estimated on the basis of phenotypic traits (Goodman, 1972). These traits had many advantages for canola which includes; (i) germplasm collections has its own databases (Spagnoletti-Zeuli and Qualset, 1987), (ii) most of canola varieties have well studied phenotypic traits with high heritability values, (iii) biometrical techniques such as phenotypic index, principal components analysis and coefficient of parentage for phenotypic traits are available, (iv) phenotypic traits are important for our understanding of ideotype-performance and (v) estimated genetic distance on the basis of phenotypic traits in many cases showed a good correlation with heterosis (Cox and Murphy, 1990).

However, molecular markers reveal differences of natural sites at the DNA level. These variations are not seen in the phenotype and each might be a single nucleotide difference in a gene or a piece of repetitive DNA (Chen *et al*., 2010). Thus, they have unlimited number and do not affect the organism.

Random Amplified Polymorphic DNA (RAPD) marker is based on PCR method which uses one and sometimes two short arbitrary primers (usually 8-10 bases) to amplify random genomic DNA fragments (Williams *et al*., 1990). It has been used worldwide for evaluation of genetic diversity because of its simplicity, speed and relatively low-cost (Rafalski and Tingey, 1998). Also, compared to other types of molecular markers *B. napus* RAPD data showed proportional distribution of RAPD markers within genome. Because of the high rate of RAPD-markers production, numerous numbers of RAPD-markers have been included in canola genetic map (Quiros *et al*., 1991). Also, it had been proved to be suitable for classification of *Brassica* species at population, species and genus levels (Demeke *et al*., 1992). For example, RAPD markers had been used successfully to estimate genetic diversity among 25 Australian canola varieties (Mailer *et al*., 1997). Also, Shengwu *et al*. (2003) had used RAPD-markers for estimation of genetic diversity among germplasms collected from several countries. Therefore, the objectives of this study were; (i) to cluster canola varieties as a representative sample of Egyptian commercial varieties into groups using the morphological characters oil and protein content percentages, (ii) to apply RAPD markers for estimation of genetic diversity among these varieties and (iii) to find the correlation between RAPD and morphological diversity.

MATERIALS AND METHODS

This research has been conducted during the years of 2009- 2010. Plant material used in these experiments consisted of six canola (*Brassica napus* L.) varieties namely; Sedo, Dupluo, Canola103,

Canola101, Serw4 and Drakkar. These varieties were a representative sample of Egyptian commercial varieties.

Oil and protein content percentages determination: This experiment was carried out at Faculty of Agriculture, Menofiya University. The oil content was determined in triplicate using Soxhlet extraction method with petroleum ether for 4 h at 60-80°C according to the Crude protein content was determined in triplicate according to the method of Cottenie *et al.* (1982). Finally, oil and protein content percentages were calculated.

RAPD experiments: RAPD technique was conducted in collaboration with the laboratory of Biotechnology Department, Faculty of Science, Taif University. DNA was extracted from two-week old plants. Leaves were collected and subjected to liquid nitrogen extraction using Dellaporta *et al.* (1983) method with some modifications. Ten arbitrary 10-mer primers were used (Table 3). 2X PCR Master Mix from (Fermentas®, Lithuania) was used for PCR reaction. The thermocycler was programmed by an initial standard denaturation cycle at 94°C for seven minutes. The following 40 cycles were composed of: denaturation step at 94°C for 1 min, annealing step at 35°C for 1 min and elongation step at 72°C for 2 min. The final cycle was a polymerization cycle performed at 72°C for 7 min. The PCR products of each reaction were analyzed by electrophoretic separation in 1.2% agarose gel.

Data analysis: In order to detect patterns of genetic relationship in the varieties using oil and protein content percentages, data analysis was initially performed based on the Euclidean distance matrix. The output was analyzed using an agglomerative hierarchical clustering method with complete linkage strategy. Firstly, the data was subjected to analysis to produce a matrix of dissimilarity values and the phenotypic distance between each pair of varieties was estimated as Euclidean distance. Secondly, cluster analysis was conducted on the Euclidean distance matrix with un-weighted pair-group method based on arithmetic averages (UPGMA) to develop a dendogram using computer program NTSYS-pc ver 2.1 (Rohlf, 2000). Also the PCA analysis were obtained from standardized oil and protein content data in order to grouping varieties based on principal components of studied traits using computer program NTSYS-pc ver 2.1 (Rohlf, 2000).

RAPD data was scored as (+) and (-) which stands for the presence and the absence of PCR products in all primers used in the study. The output was analyzed using an agglomerative hierarchical clustering method with complete linkage strategy. Data was subjected to analysis to produce a matrix of similarity values according to Nei and Li (1979). Cluster analysis was conducted on the genetics similarity matrix with un-weighted pair-group method based on arithmetic averages (UPGMA) to develop a phenogram using computer program NTSYS-pc ver 2.1 (Rohlf, 2000).

To compare Euclidean distance and RAPD distance the similarity matrix of RAPD data was converted to dissimilarity matrix. Mantel test (1967) of matrix comparisons was applied on every pair of matrices to calculate the correlation between each distance pair using computer program NTSYS-pc ver 2.1 (Rohlf, 2000).

RESULTS

In order to estimate the relationship among the six canola varieties understudy, two types of experiments were conducted. The first one included measurements of oil and protein content

Table 1: Oil and protein content percentages of canola varieties

Varieties	Oil%	Protein%
Sedo	39.55	16.82
Duplou	42.19	17.18
Canola103	40.50	15.65
Canola101	37.73	15.32
Serw4	38.35	17.53
Drakkar	37.97	18.16
L.S.D. 0.05	02.33	02.57
0.01	03.12	02.44

Table 2: Euclidean distance among canola varieties based on oil and protein percentages

Varieties	Sedo	Duplou	Canola103	Canola101	Serw4	Drakkar
Sedo	00.00					
Duplou	36.12	00.00				
Canola103	50.01	14.14	00.00			
Canola101	18.11	54.23	68.07	00.00		
Serw4	20.03	16.49	30.07	38.01	00.00	
Drakkar	42.05	78.16	92.05	24.00	62.01	0.00

Table 3: Primer used in RAPD analysis with six Canola varieties and total number of fragments detected by each primer and number of polymorphic fragments

Primer	Sequence (5'-3')	Total scorable bands	Total fragments	Polymorphic fragments	Polymorphic fragments (%)
OP-O03	CTGTTGCTAC	040	13	10	76.92
OP-A09	GGGTAACGCC	054	13	07	53.85
OP-O10	TCAGAGCGCC	025	06	04	66.67
OP-O14	AGCATGGCTC	051	10	06	00.60
OP-Z05	TCCCATGCTG	037	07	03	42.86
OP-Z08	GGGTGGGTAA	041	08	03	37.05
OP-Z12	TCAACGGGAC	042	09	05	55.56
OP-Z10	CCGACAAACC	037	07	03	42.86
OP-Z13	GACTAAGCCC	027	09	07	77.78
OP-Z17	CCTTCCCACT	047	11	07	63.64
Total		401	93	55	
Mean		040.1	09.3	05.5	57.76

percentages (Table 1) which indicated that the amount of variation among the varieties was relatively high. Euclidean distances among varieties based on the standardized values of oil and protein content percentages were calculated (Table 2). Euclidean distances ranged from 14.14 between Dupluo and Canola103 to 92.05 between Canola103 and Drakkar (the highest) and the average of the distances among varieties was 42.9. Also, a dendogram based on Euclidean distance was generated for the six varieties (Fig. 2). Generated dendogram divided varieties into two groups separated at Euclidean distance of 55.14. The first group included Sedo, Canola103 and Drakkar and the second group included Dupluo, Canola103 and Serw4.

Secondly, RAPD experiments were conducted using 10 random primers (Table 3). The total number of loci determined using these primers was 93 with an average of 9.3 loci per primer. A total of 55 polymorphic loci with an average of 5.5 polymorphic loci per primer were detected. The percentage of polymorphic loci ranged from 42.86 to 77.78% with an average of 57.76%. The high percentage of polymorphic primers indicates that they have been well selected for this study

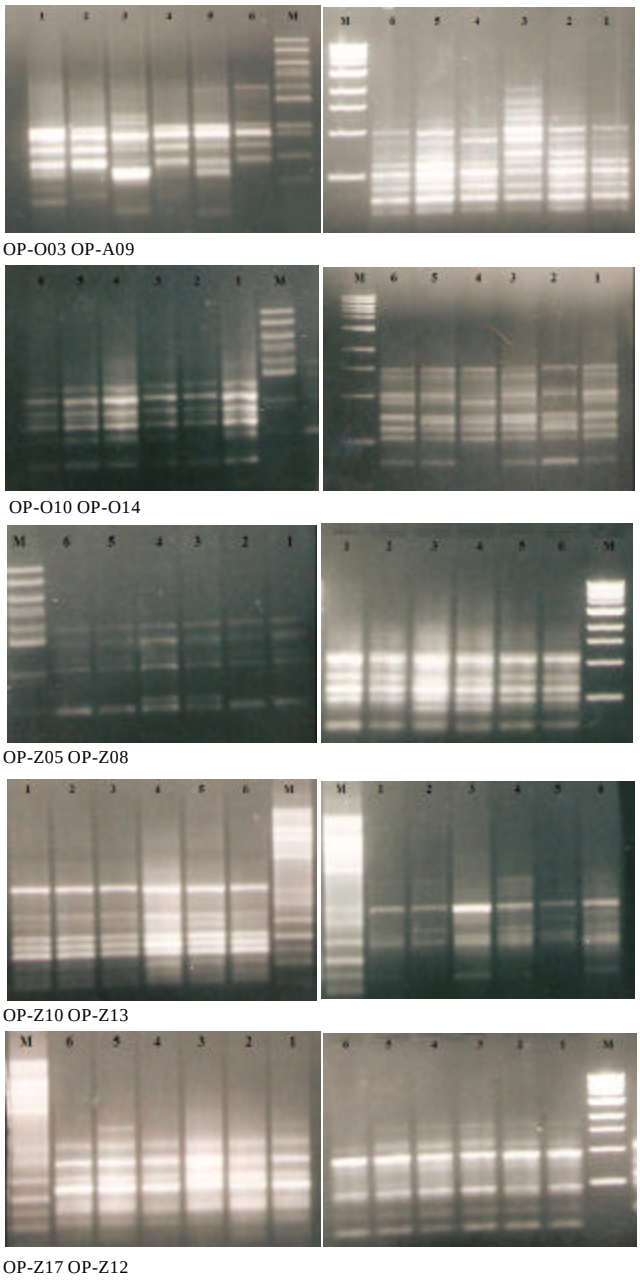

Fig. 1: DNA fragments detected by RAPD primers with Canola varieties. (1) Sedo, (2) Duplou, (3) Canola103, (4) Canola101, (5) Serw4 and (6) Drakkar (M) DNA marker

(Fig. 1). Genetic similarity coefficient of Nei and Li (1979) between all pairs of varieties were illustrated (Table 4). Data showed that the similarity coefficient values ranged from 0.114 between Duplou and Drakkar to 0.261 between Canola 101 and Drakkar. The average of similarity coefficient values among verities was 0.183. This RAPD data indicated low genetic similarity and high genetic variation among varieties. Finally, dendogram of the six varieties using genetic

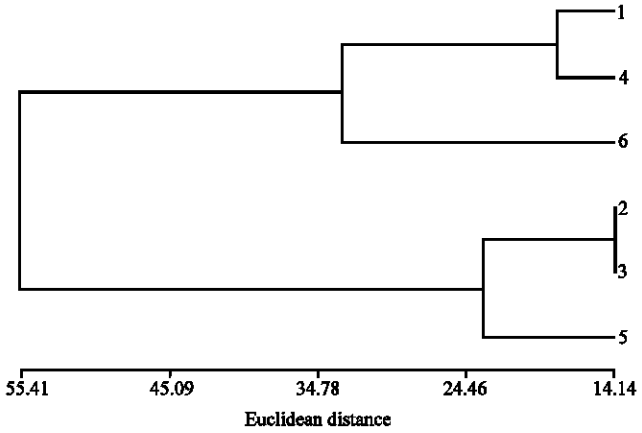

Fig. 2: Dendogram based on Euclidean distance of protein and oil content. (1) Sedo, (2) Duplou, (3) Canola103, (4) Canola101, (5) Serw4 and (6) Drakkar

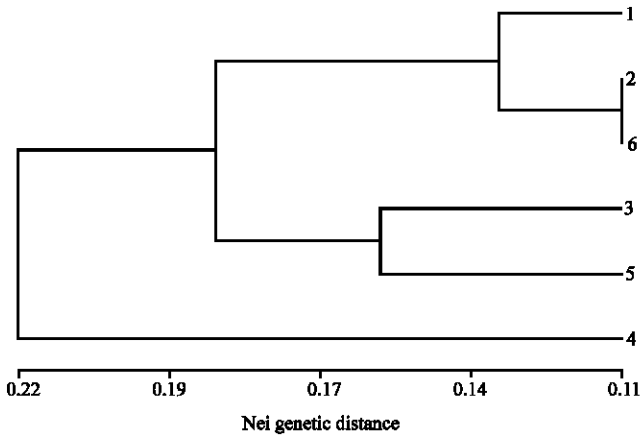

Fig. 3: Dendogram based on Nei and Li (1979) similarities of 55 RAPD loci. (1) Sedo, (2) Duplou, (3) Canola103 (4) Canola101, (5) Serw4 and (6) Drakkar

Table 4: Genetic distance among canola varieties based on 55 RAPD markers

	Sedo	Duplou	Canola103	Canola101	Serw4	Drakkar
Sedo	0.000					
Duplou	0.119	0.000				
Canola103	0.172	0.233	0.000			
Canola101	0.220	0.147	0.260	0.000		
Serw4	0.152	0.147	0.156	0.208	0.000	
Drakkar	0.152	0.114	0.192	0.261	0.212	0.00

similarities coefficient values of Nei and Li (1979) was developed and presented in Fig. 3. It showed that the variety Canola101 separated from the rest of the varieties at 0.22 genetic similarities of Nei and Li (1979). While, varieties namely Sedo, Duplou and Drakkar formed a distinct group that were separated from other varieties at genetic similarity of 0.185. The other two varieties formed a cluster at genetic similarity of 0.16.

At the same time, some of the primers had unique fragments with the varieties understudy. Primer OP-O03 had four unique fragments with molecular weights 2647, 617, 344 and 298 bp for varieties Canola101. The first two fragments, Serw4 and Drakkar; respectively. Primer OP-A09 has three fragments with molecular weights 3054, 2036 and 1900 bp for the variety Canola101. Primer OP-O10 has a unique fragment 1218 bp for Canola103. Primer OP-Z12 had a unique fragment with 1118 bp for Serw4. Primer OP-Z13 had two fragments 3453 and 1701 bp unique for Canola101 and Drakkar, respectively.

DISCUSSION

In this investigation a fundamental question concerning genetic relationship among six canola (*Brassica napus*) varieties was addressed. These varieties were chosen as a representative sample of commercial canola varieties used for oil production. Variety genetic diversity information is very significant for any breeding program.

In this report, cluster analysis method was used to divide varieties under study into phenotypic groups. Data indicated that the amount of phenotypic variation among the varieties was relatively high. These values, which are assumed to reflect the genetic diversity of the loci controlling these characters, make it possible to obtain broad segregated characters when diverse parents are used. Also, dendogram of eulidean distance was a good illustration of how interrelationships among genotypes within a dendrogram are influenced not only by extreme values but also by the other characteristics. These results showed that the use of cluster analysis of quantitative traits graphically depicts similarities and differences among genotypes. These findings of diversity data agree with the findings of other authors as well (Crossa and Cornelius, 1997; Hristov, 1999; Marjanovic-Jeromela *et al.*, 2003; Mahasi and Kamundia, 2007).

RAPD results (81.7% divergence) indicated higher diversity than indicated by oil and protein morphological markers (42.9%). This finding is obvious because molecular markers are more powerful in indicating variations. This RAPD data agreed with percentage of 65% as reported by McGrath and Quiros (1992) and the value cited by Geraci *et al.* (2001) and Ahmad *et al.* (2007). Also, RAPD results were used for further calculations and the construction of a dendrogram. This dendogram made it possible to visualize and more easily interpret the findings and compare them with those obtained using other techniques. A number of authors have successfully used the RAPD technique for determining genetic diversity and inferred that PCR based assays can be effectively used to analyze the genetic diversity in *B. napus* (Ali *et al.*, 2011; Fazeli *et al.*, 2008; Geraci *et al.*, 2001; Xu and Gai, 2003; Pankovic *et al.*, 2000; Prasad *et al.*, 2009). This confirmed that RAPD was a simple, fast and cheap technique for evaluation genetic diversity of *B. napus*.

On the other hand, the unique fragments which were identified in some of the varieties were valuable. They may be used to distinguish and indentify these varieties at the molecular level without using field data. Five RAPD markers were unique for Canola101. While, other unique markers were identified in varieties Canola103, Serw4 and Drakkar. This result showed the power of RAPD marker to differentiate among these varieties. Also, using these primers and other RAPD primers it can be establish an appropriate multilocus genotype to identify each individual variety of canola.

Correlations among the two matrices generated by morphological traits and RAPD were calculated. The Correlation was [r = -0.08992 (p = 0.3726)]. Qi *et al.* (2008) attribute deviations in the clustering due to the fact that the pressures of artificial selection may have limited mutation effects on the DNA sequence; in other words, numerous changes have occurred in the process of

breeding as a result of artificial selection relative to natural mutations, resulting in a loss of distinctive traits that should characterize genotypes from the same morphological group. A lack of correlation between morrphlogical and genetic distance has been established in other species as well (Johnson *et al*., 2007; Khan *et al*., 2009). Other authors have obtained similar results when comparing the findings of morphological, biochemical and molecular analyses in different species (Vollmann *et al*., 2005; Johnson *et al*., 2007; Khan *et al*., 2009). Also, Schut *et al*. (1997) reported a correlation of -0.1 for AFLP and agronomic data in European barley varieties; Roldan-Ruiz *et al*. (2001), obtained a correlation of -0.06 in perennial ryegrass varieties; and Beer *et al*. (1993), found low correlations (from -0.35 to -0.17) for RFLP and morphological data in wild oats. However, Autrique *et al*. (1996) reported a moderate correlation (0.47) between RFLP and agronomic relatedness in durum wheat.

The accuracy of determining genetic similarity/distance based on molecular markers depends on many factors, such as the number of markers used, their distribution along the genome, and the degree of precision with which the results are analyzed (Schut *et al*., 1997). In addition to that, molecular markers cannot be used for the purpose of drawing conclusions about inter allelic and intra allelic interactions that lead to the expression of particular traits within the genome. Given that a relatively small number of RAPD primers were used in the present study, it is reasonable to expect that a significant portion of the canola genome was not covered by the analysis, hence no major similarity was found between the dendrograms. There are other possible reasons for the lack of correlation between RAPD analysis and morphological data. One of them is that RAPD markers detect polymorphism in coding as well as non-coding gene regions, so it is possible that the bulk of the detected polymorphism was from the non-coding regions. Also, plants may be morphologically similar, but this does not necessarily imply genetic similarity, since different genetic bases can result in similar phenotypic expression (Khan *et al*., 2009). This is especially the case with traits that are significantly influenced by environmental factors. The link between molecular markers and phenotypic traits can be significant if the markers are selected based on their linkage to particular known loci (Persson and Gustavsson, 2001).

The conflict between diversity measurements based on RAPDs and agronomic traits call for new strategies in germplasm collection and breeding. Traditionally, germplasm has been classified based on morphological and agronomic traits, together with geographical origin, and breeders determine which crosses to perform based on these data. However, in the last decade, as molecular markers have become more time and cost efficient, there has been a rapid increase in studies of genetic diversity based on these methods, and it can be expected that in time these techniques will be used routinely in breeding programs and germplasm collections. Nevertheless, the lack of significant correlations between agronomic and molecular diversity measurements in this and other studies showed that germplasm classification and utilization should not be based on one diversity measurement alone. Koebner and Sammer (2002) stated that the reason for the general lack of correlation between molecular and agronomic diversity in cultivated crops is that the former is invisible and, therefore, unselected by breeders, while the latter is subject to selection. Furthermore, as argued by Donini *et al*. (2000), agronomic traits are selected based on their usefulness for distinctness testing in canola, whereas molecular markers are not. In this sense, the molecular approach is more likely to generate an unbiased picture of diversity than an agronomic approach. However, as most desirable traits in plant breeding are the result of interaction among expressed genes, agronomic studies are still vital in germplasm description, and determination of molecular diversity should not be seen as replacing traditional characterization but rather as complementing it.

CONCLUSION

In conclusion, the present study indicated that the genetic diversity information is very important for any breeding program in *Brassica napus*. Genetic diversity study in *Brassica napus* cannot depend only on agronomical traits data but also on molecular markers data such as RAPD. RAPD marker has confirmed to be a good, fast and reliable method for studying genetic diversity in *Brassica napus*.

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