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Comparison of winter and summer canola (*Brassica napus*) genotypes in Turkey

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ABSTRACT. We examined genetic relationships between canola (*Brassica napus*) genotypes cultivated in winter and spring in Turkey. Genomic DNA was isolated from the seeds by two modified CTAB protocols: EZ1 nucleic acid isolation method and a commercial kit (Dneasy Plant Mini Kit, Qiagen). Diversity and genetic relationships in the genotypes were analyzed with RAPD markers; 156 reliable bands were found for both genotypes, of which 24% were polymorphic. Fifteen primers gave at least one consistent polymorphic band. The dendogram developed by pooling data of RAPD analysis of summer and winter genotypes had similar patterns. This technique allowed us to examine the relationship between canola genotypes.

Key words: *Brassica napus* L.; Canola; Biodiversity; Genomic DNA; RAPD-PCR

INTRODUCTION

Canola (*Brassica napus* L.) is one of the most important members of the Cruciferae. Total production of canola seed was over 46.4 m in 2005 (Food and Agriculture Organization, 2005). *B. napus* is an important source of vegetable oil and is the second largest oilseed crop after soybean (Food and Agriculture Organization, 2007). Evaluation of genetic diversity among wild and crop plant population is necessary for the protection, conservation and useful application of germplasms, identification of suitable parents for high-quality crosses and characterization of the genetic content of important breeding traits (Kresovich et al., 1992; Diers and Osborn, 1994; Cruz et al., 2007). Oilseed *B. napus* includes annual and biennial types, the latter of which require vernalization to flower and are grown as an over-wintering crop in cooler climates. Biennial forms have a higher frequency of winter survival, which was also correlated with acclimated freezing tolerance in a study of annual and biennial cultivars (Teutonico et al., 1995). Winter survival is an important characteristic for over-wintering of oilseed *Brassica*, and depends on the expression of many interacting traits (Kole et al., 2002). One of these traits is freezing tolerance, which can be increased in some genotypes by acclimating plants to cold temperatures (Kasperska-Palacz, 1978; Palta, 1991). On the other hand, low levels of genetic diversity increase vulnerability to diseases and pests (Jordan et al., 1998). Genetic diversity in these plants has been characterized by using various morphological, protein, isozymes, and DNA-based markers (Shengwu et al., 2003). DNA-based markers are a powerful tool for studies of genetic diversity. One DNA-based method for studying genetic diversity is RAPD (random amplified polymorphic DNA). This method facilitates the identification of polymorphisms in all races of plants and animals (Welsh et al., 1991). Previous studies have suggested that the RAPD marker is more precise than other markers such as proteins and isozymes in studies of genetic diversity. Because of the important role of canola in global vegetable oil production daily dietary consumption, and its nutritional and industrial uses, studies on canola breeding are essential. Molecular markers are the best tools for determining genetic relationships. A variety of molecular markers has been used to study the extent of genetic variation in 2 diverse groups of important crops in the genus *Brassica* (Liu et al., 2006).

Our group has studied the biochemical characteristics of 2 *B. napus* (canola) genotypes, in Turkey, cultivated in winter and spring (Maltas and Yildiz, 2011). In this study, we characterized the genetic diversity and determined the pedigree relationships between both genotypes of *B. napus* using RAPD and the OPA1-15 primers.

MATERIAL AND METHODS

Isolation of genomic DNA

Two genotypes of *B. napus*, a spring and winter canola species, were provided as fresh, frozen, and old leaves, and seeds or grains by the Agricultural Faculty of Selcuk University, Konya, Turkey. Different types of plant material (fresh, frozen, young, old, lyophilized leaves, and seed leaves dried at 40°C, and plants grown *in vitro*) were sampled for DNA isolation, and different protocols were tested: 2 modified CTAB protocols (Park et al., 2006), EZ1 nucleic acid isolation method (Maltas et al., 2011a) and a commercial kit (Dneasy Plant Mini Kit, Qiagen).

Fresh, seed, plantlets, and herbarium specimens were sampled for DNA extraction.

Herbarium samples were collected by the author in the field. Dried canola seeds were used directly for DNA extraction as they yielded DNAs comparable in quality and quantity whether they were isolated by the EZ1 nucleic acid isolation analyzer (QIAGEN, 2007) and the CTAB method, the Plant Genomic DNA Purification Kit method as described by Maltas et al. (2011b) and/or DNA extraction with phenol purification and liquid nitrogen (Sambrook et al., 1989). A bulk sample of canola seed was ground to fine powder. This experiment was repeated twice for all DNA samples. Plant seeds were used directly for DNA extraction as they yielded DNAs comparable in quality and quantity to beans ground in liquid nitrogen. Seeds were placed in 2-mL deep wells of a 96-well plate containing a 4-mm stainless steel grinding ball and soaked for 12 h in distilled water prior to processing. Using a grinding ball dispenser, a grinding ball was placed on top of each seed. The plate was sealed with a fitted Teflon®/silicone mat and placed in the Geno/Grinder. A piece of adsorbent paper was placed on top of the plate and the plate was locked into the grinder. The seeds were disrupted for 2.5 min at 1500 rpm. The deep well plate with lid was centrifuged at 1500 rpm to pellet the lysate and condense the liquid from the rim and walls of the well. Without centrifugation, the probability of well-to-well cross contamination of genetic material is greatly increased. Once centrifuged, the lid was carefully removed. Four different DNA isolation methods were compared in different plant homogenates, namely the CTAB, Plant Genomic DNA Purification Kit, and EZ1 Nucleic acid isolation methods, and DNA extraction with phenol purification and liquid nitrogen. Amplified products were electrophoresed on 2% agarose gel using 0.5X TAE buffer (10 mM Tris-HCl and 1 mM EDTA, pH. 8.0) and visualized by ethidium bromide staining. The gels were photographed and stored as digital pictures in a gel documentation system. Amplification reproducibility was confirmed by repeating each experiment 3 times.

RAPD-PCR optimization

PCR was carried out with optimized DNA and $MgCl_2$ concentrations. Different types of Taq DNA polymerase were also tested. Fifteen arbitrary 10-mer primers (Operon Technologies Inc., Almeda, CA, USA) were applied. A preliminary study of genetic variability using RAPD molecular markers was performed.

To optimize the RAPD reaction using DNA extracted from various accessions, oligonucleotide primers were used to standardize the PCR conditions (Table 1). PCR was carried out with 200 ng template DNA, 67 mM Tris, pH 8.8, 6.7 mM $MgSO_4$, 16.6 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 1 mM of each dNTP, 250 ng primer and 1 U Taq DNA polymerase in a final volume of 50 mL. Amplifications were carried out in a DNA Thermal Cycler (Bio-Rad) under the following conditions: one initial cycle of 3 min at 95°C, 5 min at 35°C, and 5 min at 72°C followed by 43 cycles of 1 min at 95°C, 1 min at 35°C and 2 min at 72°C, with a final extension period of 15 min at 72°C. Tubes containing all reaction components except for template DNA were included as controls. Amplification products were observed on ethidium bromide-stained 2% agarose gels following electrophoresis. Ten primers of different sizes were tested and those producing visible, reproducible, and easily scorable bands were selected. All reactions were repeated 3 times.

The RAPD-PCR results were analyzed. Unequivocally reproducible bands were scored and entered into a binary character matrix (1 for presence and 0 for absence). Genetic similarity between accessions was determined by Nei's genetic distance. A dendrogram was

constructed from the distance matrix, using the unweighted pair group method with arithmetic (UPGMA) averages. Scores entered in the matrix were analyzed using TAXAN version 4.0 based on the degree of band sharing. A similarity matrix was generated using the Dice coefficient as SI = 2Nab / Na + Nb, where *Na* is the total number of bands present in lane a, *Nb* is the total number of bands in lane b, *Nab* is the number of bands common to lanes a and b. The Dice values were then used to perform the UPGMA analysis.

RESULTS AND DISCUSSION

DNA extraction was improved by major modifications of the CTAB method (Doyle and Doyle, 1987). High-quantity good-quality DNA was isolated from canola genotypes from 3 g fresh tissue (Figure 1). The isolated DNA had A_{260}/A_{280} ratios (specific wavelengths of nucleic acids, 260 and 280 nm) were of 1.8. This study presents a novel method that does not require ultracentrifugation for DNA isolation.

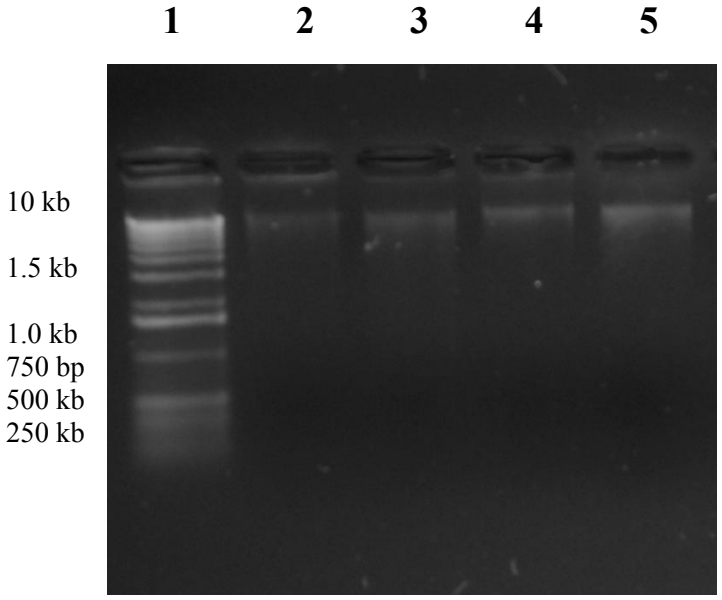


Figure 1. Genomic DNAs were loaded on a 1% agarose gel and separated by electrophoresis for 90 min at 100 V, then visualized by ethidium bromide staining with transillumination. *Lane 1* = 1-kb ladder; *lane 2* = genomic DNAs isolated from spring canola (*Brassica napus*) with Bio Robot EZ-1; *lane 3* = genomic DNAs isolated from winter canola (*B. napus*) with Bio Robot EZ-1; *lane 4* = genomic DNAs isolated from spring canola with Bio Robot EZ-1; *lane 5* = genomic DNAs isolated from winter canola with Bio Robot EZ-1.

The RAPD procedures proved to be useful tools for assessing genetic variability, since the banding profiles were reproducible and their patterns of inheritance proved to be Mendelian for a dominant marker. Despite the high proportion of excluded bands, a substantial number of polymorphic markers were detected. Reproducibility was determined by replicating all RAPD reactions at least 3 times. Variations among canola genotypes

across the primers were evaluated from pairwise comparisons of the proportion of shared amplified bands (Nei and Li, 1979). The presence of polyphenols, which are powerful oxidizing agents, can reduce DNA yield and purity by binding covalently with the extracted DNA, making it inaccessible to most enzymes (Katterman and Shattuck, 1983; Peterson and Stack, 1997), including restriction enzymes (Katterman and Shattuck, 1983), causing it to interact irreversibly with proteins and nucleic acids (Loomis, 1974). This phenomenon is mainly due to the oxidation of polyphenols to quinines and quinones by polyphenol oxidase followed by covalent coupling or oxidation of the proteins by the quinines. During homogenization, polyphenols are released from vacuoles and react rapidly with cytoplasmic enzymes.

RAPD assays were as described in previous surveys. PCRs were performed in a Thermal-Cycler Engine (BioRad). Negative controls were included in each run to check for contamination. To ensure reproducibility, 2 replicates were performed for each reaction. PCR amplification products were separated on 2% agarose gel in 1X TAE buffer, visualized by staining with ethidium bromide, and photographed over UV-Transilluminator systems. Molecular weight markers were used to estimate the sizes of amplification products and to compare duplicate reactions. A set of 15 random 10-mer primers was purchased from Invitrogen. After screening, 15 primers that amplified clear, reproducible banding patterns were chosen for further studies. RAPD assays were repeated twice for each primer; only the reproducible bands were scored, with specific attention to the repeatability of polymorphic bands. The presence or absence of bands was scored by eye and Quantity One; only unequivocal bands were scored, with weak and spurious bands excluded. PCR amplification was successful for all genotypes. A similarity matrix was constructed using the Dice coefficient as in Nei and Li (1979), and was converted into distances using the formula $GD_{ab} = -In (S_{ab})$, where S_{ab} is the measure of genetic similarity between accessions a and b and is defined as $S_{ab} = 2N_{ab} / N_a + N_b$, where N_{ab} is the number of common bands in accesssions a and b; N_a and N_b are the sum of the scored bands in accessions a and b.

A marker index for RAPD markers was calculated in order to characterize the capacity of each primer to identify polymorphic loci among the genotypes. The index is the sum of the polymorphism information content (PIC) of all markers produced by a particular primer. The PIC value was calculated using the formula $PIC = 1 - \Sigma pi\ 2$, where *pi* is the frequency of the i^{th} allele (Smith et al., 1997). The RAPD profiles were scored individually and collectively, and then used to construct a similarity matrix using the Jaccard coefficients (Jaccard, 1908). The similarity values were used for cluster analysis. Sequential agglomerative hierarchical non-overlapping clustering was performed using the UPGMA method. Data analysis was performed in NTSYSpc v. 2.02 (Rohlf, 1998). The product-moment correlation (r) based on Mantel Z-value (Mantel, 1967) was computed to measure the relationship between similarity index matrices produced by any 2-marker systems.

DNA from canola genotypes was amplified by RAPD analysis with 15 primers. The number of scorable markers per primer ranged from 1 to 15 and product size ranged from 10 to 250 kb. RAPD marker profiles produced by primers OPA1-15 for spring and winter canola are shown in Table 1. The PIC values, a reflection of allele diversity and frequency among varieties, were not uniformly higher for all RAPD loci tested. Cluster

analysis was performed based on Jaccard similarity coefficient matrices, calculated from RAPD markers to generate a dendrogram of canola genotypes. RAPD markers revealed their ability to reveal polymorphisms between accessions. These results suggest that it is possible to distinguish accessions and evaluate genetic diversity by this technique. Further studies are in progress with the aim to characterize all the accessions. In this study, 156 reliable bands were found, and scored for both genotypes (*B. napus*-spring and *B. napus*-winter), among which 37 (23.8%) produced polymorphic bands These markers could be used for moleculer linkage mapping, gene location and the markers could be used for selection.

Table 1. Primers used for generating randomly amplified polymorphism DNA in *Brassica napus* genotypes grown in Turkey.

Primer No.	Primer sequence (5'-3')
OPA-1	5'-CAGGCCCTTC-3'
OPA-2	5'-TGCCGAGCTG-3'
OPA-3	5'-AGTCAGCCAC-3'
OPA-4	5'-AATCGGGCTG-3'
OPA-5	5'-AGGGGTCTTG-3'
OPA-6	5'-GGTCCCTGAC-3'
OPA-7	5'-GAAACGGGTG-3'
OPA-8	5'-GTGACGTAGG-3'
OPA-9	5'-GGGTAACGCC-3'
OPA-10	5'-GTGATCGCAG-3'
OPA-11	5'-CAATCGCCGT-3'
OPA-12	5'-TCGGCGATAG-3'
OPA-13	5'-CAGCACCCAC-3'
OPA-14	5'-TCTGTGCTGG-3'
OPA-15	5'-TTCCGAACCC-3'

The findings of this preliminary study indicate that such research should be continued. Future studies should include a greater number of genotypes and markers in order to cover a large portion of the rapeseed genome and to obtain valuable information on the genetic variability of this important crop. Fifteen primers were tested in 2 genotypes (*B. napus*-spring and *B. napus*-winter). The result indicated that 15 primers (60%) vielded at least one consistent polymorphic band. The 15 informative primers were selected and used to evaluate the degree of polymorphism and genetic relationships within and between genotypes. One hundred and fifty-six amplified fragments were distinguished across the selected primers and statistical analysis showed 37 polymorphic bands between genotypes with an average of 2.4 polymorphic bands per primer. The results showed that OPA3, OPA6 reproducible bands and OPA7, OPA8 reproducible bands OPA11, and OPA12, OPA13, OPA14, and OPA15 RAPD markers correlated with the performance of canola genotypes. Reproducible, common bands were observed in both genotypes with these RAPD primers (Table 2). A dendogram generated from the RAPD data is shown for *B. napus*-winter and *B. napus*-spring in Table 3. Varying cultivation conditions have produced changes in canola genotypes. Our results provide valuable fingerprinting information that can be used in a synergistic way to create a wider genetic base and augment the *B. napus* breeding program in Turkey. More biochemical and molecular data are required to improve our understanding of genetic variability in the *Brassica* germplasm and allow more efficient utilization of existing variability to improve the *Brassica* crop.

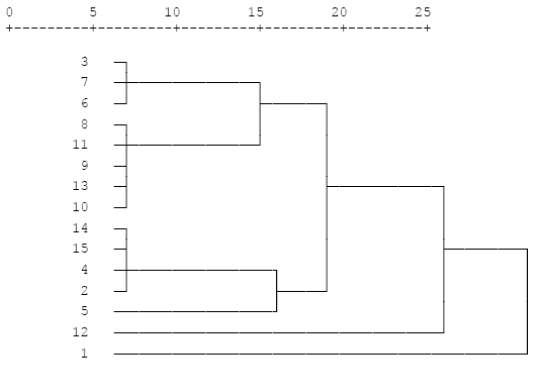
Table 2. Average genetic similarity indices based on 15 RAPD markers related to two canola genotypes.

Case processing summary (single linkage)						
Valid			Missing		Total	
N = 15	100%		N = 0	0%	N = 15	100%

Proximity matrix (similarity)															
	Cosine of vectors														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	1.000	0.970	0.844	0.970	0.924	0.844	0.844	0.759	0.688	0.722	0.744	0.514	0.701	0.970	0.970
2	0.970	1.000	0.949	1.000	0.989	0.949	0.949	0.894	0.844	0.868	0.884	0.707	0.853	1.000	1.000
3	0.844	0.949	1.000	0.949	0.985	1.000	1.000	0.990	0.970	0.981	0.986	0.894	0.974	0.949	0.949
4	0.970	1.000	0.949	1.000	0.989	0.949	0.949	0.894	0.844	0.868	0.884	0.707	0.853	1.000	1.000
5	0.924	0.989	0.985	0.989	1.000	0.985	0.985	0.950	0.913	0.932	0.943	0.803	0.920	0.989	0.989
6	0.844	0.949	1.000	0.949	0.985	1.000	1.000	0.990	0.970	0.981	0.986	0.894	0.974	0.949	0.949
7	0.844	0.949	1.000	0.949	0.985	1.000	1.000	0.990	0.970	0.981	0.986	0.894	0.974	0.949	0.949
8	0.759	0.894	0.990	0.894	0.950	0.990	0.990	1.000	0.995	0.998	1.000	0.949	0.996	0.894	0.894
9	0.688	0.844	0.970	0.844	0.913	0.970	0.970	0.995	1.000	0.999	0.997	0.976	1.000	0.844	0.844
10	0.722	0.868	0.981	0.868	0.932	0.981	0.981	0.998	0.999	1.000	0.999	0.965	1.000	0.868	0.868
11	0.744	0.884	0.986	0.884	0.943	0.986	0.986	1.000	0.997	0.999	1.000	0.956	0.998	0.884	0.884
12	0.514	0.707	0.894	0.707	0.803	0.894	0.894	0.949	0.976	0.965	0.956	1.000	0.972	0.707	0.707
13	0.701	0.853	0.974	0.853	0.920	0.974	0.974	0.996	1.000	1.000	0.998	0.972	1.000	0.853	0.853
14	0.970	1.000	0.949	1.000	0.989	0.949	0.949	0.894	0.844	0.868	0.884	0.707	0.853	1.000	1.000
15	0.970	1.000	0.949	1.000	0.989	0.949	0.949	0.894	0.844	0.868	0.884	0.707	0.853	1.000	1.000

Table 3. Dendogram generated from RAPD markers for *Brassica napus*-winter and *Brassica napus*-spring.

Agglomeration schedule						
Stage	Cluster combined		Cofficients	Stage cluster first appears		Next stage
	Cluster 1	Cluster 2		Cluster 1	Cluster 2	
1	3	7	1.000	0	0	2
2	3	6	1.000	1	0	10
3	14	15	1.000	0	0	4
4	4	14	1.000	0	3	5
5	2	4	1.000	0	4	11
6	9	13	1.000	0	0	8
7	8	11	1.000	0	0	9
8	9	10	1.000	6	0	9
9	8	9	0.999	7	8	10
10	3	8	0.990	2	9	12
11	2	5	0.989	5	0	12
12	2	3	0.985	11	10	13
13	2	12	0.976	12	0	14
14	1	2	0.970	0	13	0



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