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Potential Use of Random Amplified Polymorphic DNA Marker in Assessment of Genetic Diversity and Identification of Rapeseed (*Brassica napus* L.) Cultivars

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Abstract: In this study the genetic diversity among 27 rapeseed (*B. napus*) cultivars and one cultivar of *B. rapa* was investigated using Random Amplified Polymorphic DNA (RAPD) marker. A set of 24 arbitrary primers was used which produced 133 reliable polymorphic DNA bands ranging in size from 440 to 2988 bp. RAPD data were used to compute genetic similarities based on Jaccrad (J) and Simple Matching (SM) indexes. Similarity coefficients values ranged from 0.22 to 0.87 with a mean of 0.48 for J and from 0.13 to 0.61, with a mean of 0.31 for SM coefficient, for all genotypes. Similarity matrices were used to contrast dendrograms using Unweighted Paired Group of Arithmetic Mean (UPMGA) algorithm. In cluster analysis, based on both similarity coefficients, *B. napus* cultivars were grouped together and Hysyn110, which belongs to *B. rapa* was classified as an outlier. Winter and spring *B. napus* cultivars were clustered into two separate groups. Results of Principal Component Analysis (PCA) supported cluster analysis results strongly. RAPD profiling was found efficient to reveal DNA polymorphism among rapeseed cultivars. RAPD data showed relatively low level of genetic diversity among studied cultivars, but also revealed some genetically distinct cultivars. This finding should be useful to breeding programs for selection of the genetically divergent parents in order to obtain the highest level of heterosis.

Key words: *Brassica napus*, genetic diversity, RAPD molecular marker

INTRODUCTION

Oilseed rape (*Brassica napus* L.) is one of the most important oilseed crops worldwide. *B. napus* is an amphidiploid (2n = 38, AACC) that has been arisen by spontaneous interspecific hybridization between two diploid species, *B. rapa* (syn. *campestris*) (2n = 20, AA) and *B. oleracea* (2n = 18, CC) (U, 1935). *B. napus* has evolved through different combinations of the diploid morphotypes (Song *et al*., 1988). RFLP analysis on organelle and nuclear genome strongly support the concept of multiple origins of *B. napus* (Song and Osborn, 1992).

B. napus is predominantly self-pollinated with about 20% out-crossing (Luhs *et al*., 1994). Most oilseed rape cultivars are open-pollinated populations derived from inbred plants (Ripley and Beversdorf, 2003). There is also interest in developing hybrid oilseed rape cultivars because high levels of heterosis have been reported (Grant and Beveresdorf, 1985). Increasing oil content and development of varieties with enhanced oil quality is one of the most important breeding goals for rapeseed (Hu *et al*., 1999). There is intensive attempt in developing rapeseed cultivars for different growing regions.

Characterization of genetic diversity in plant varieties and breeding lines is essential for effective conservation and utilization of plant genetic resources. These information are essential for implementation of plant breeding strategies. Molecular markers, especially DNA markers, have been employed for analysis of genetic diversity, because of their advantages. Their expression is independent of environmental conditions and potential number of polymorphic molecular markers is nearly unlimited. One of the most extensively used molecular markers is Random Amplified Polymorphic DNA (RAPD). The main advantages of RAPD technology are its simplicity and low cost. It does not require any digestion by restriction enzymes, any radioactive probes or previous knowledge about the sequences and require small amount of DNA (Williams *et al*., 1990). RAPD provide a level of resolution equivalent to RFLPs for determination of genetic relationships between genotypes at the intraspecific level (dos Santos *et al*., 1994; Graham *et al*., 1996).

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Several molecular markers such as RFLPs, AFLPs, microsatellites and isozymes have been used for genetic analysis in *B. napus* germplasm (Diers and Osborn, 1994; Lombard *et al*., 2000; Pliesk and Struss, 2001; Zhao-Jian *et al*., 1998). Several studies have confirmed the significance of RAPD marker as a powerful tool in evaluation of genetic diversity in *B. napus* cultivars and its wild relatives (Dulson *et al*., 1998; Lanner *et al*., 1996; Mailer *et al*., 1997). RAPD markers are useful for investigation of genetic relationships among cultivars in other plant species (Shiran *et al*., 2004; Diaby and Casler, 2003; Fu *et al*., 2003; Shiran and Raina, 2001; Stallen *et al*., 2000). RAPD also has been used for constructing genetic linkage map in *B. napus* (Foisset *et al*., 1996; Lombard and Delourme, 2001). RAPD and SCAR markers associated with low linolenic acid and other important traits in rapeseed have been identified (Hansen *et al*., 1997; Rajcan *et al*., 1999; Zhang *et al*., 2003).

The objectives of the present study are to study the extent of genetic diversity among *B. napus* cultivars and to identify the genetic relationships among them.

MATERIALS AND METHODS

Plant material: Twenty-seven *B. napus* cultivars including both spring and winter types with canola quality were studied (Table 1). Most of cultivars were chosen to represent the genetic diversity in rapeseed germplasm. Also, these double-Zero cultivars represented breeding lines currently used in conventional breeding systems in order to introduce the most proper cultivars for commercial cultivation in Iran. This collection included open pollinated and hybrid rapeseed cultivars from different origins. Finally, one cultivar of *B. rapa* (Hysyn110) were included for comparison to *B. napus* cultivars. Seeds were supplied with Seed and Plant Improvement Institute-Oilseed Crop Research Department, Karaj, Iran.

Total DNA isolation and RAPD fingerprinting: Total cellular DNA was isolated from freshly germinated young leaves following a modification of the CTAB method of Murray and Thompson (1980): about 1-1.5 g of fresh leaf tissue was crushed to powder in liquid nitrogen and transferred to pre-warmed (60°C) 2×CTAB buffer. The DNA was purified by phenol-chloroform extraction and ethanol precipitated. The final DNA pellet was dissolved in TE buffer, were stored in -20°C until use.

An initial screening of 40 random primers (Genset, France) was performed in order to evaluate their capacity to produce polymorphic, reproducible and reliable RAPD patterns. Twenty-four primers selected (Table 2). Polymerase chain reaction was performed based on the protocol of Williams *et al*. (1990), with minor modification. Amplification were carried out in 25 μL of reaction mixture containing 25 ng of genomic DNA, 0.5 unit of *Taq* DNA polymerase (Amersham biosciences), 2.5 μL of PCR buffer, 240 μM each of dNTPs (Amersham biosciences), 15 ng of the primer. DNA amplification was

Table 1: Characteristics of accessions used in this study

No.	Cultivar	Origin	Type
1	Okapi	France	Winter
2	Fornax	Germany	Winter
3	Fusia	Mexico	Spring
4	Cyclon	Canada	Spring
5	Cobra	Germany	Winter
6	SLM-046	Germany	Winter
7	Chinese	China	Spring
8	Licord	Germany	Winter
9	Zeus	Germany	Winter
10	Hyola308	Canada	Spring
11	Option501	Canada	Spring
12	Pronto	Germany	Winter
13	Garisson	Canada	Spring
14	Hyola420	Canada	Spring
15	Colvert	France	Winter
16	Sponsor	Sweden	Spring
17	Option500	Canada	Spring
18	PF7045/91	Germany	Spring
19	Hylight201	Canada	Spring
20	Orient	Germany	Winter
21	Kristina	Canada	Spring
22	Foseto	Mexico	Spring
23	Hysyn110	Canada	Spring
24	Hyola401	Canada	Spring
25	Regent× Cobra	Iran	Winter
26	LG3310	Canada	Spring
27	Profit	Canada	Spring
28	Eurol	France	Winter

Table 2: Primers included in the study

No.	Primer name	Sequence
1	OPG1	TGC CGA GCT G
2	OPG2	AGT CAG CCA C
3	OPG3	GAA ACG GGT G
4	OPG4	GGG TAA CGC C
5	OPG5	GTG ATC GCA G
6	OPG6	CAA TCG CCG T
7	OPG7	CAG CAC CCA C
8	OPG8	CCG CCC AAA C
9	OPG9	AGC GAG CAA G
10	OPG10	GAA CAC TGG G
11	OPG11	CCC TAC CGA C
12	OPG12	AAT GGC CCA G
13	OPG13	CTC CTG CCA A
14	OPG14	CCC AGC TGT G
15	OPG15	GTG TCG CGA G
16	OPG16	CTG CTG GGA C
17	OPG17	GTA GAC CCG T
18	OPG18	CCT TGA CGC A
19	OPG19	AGG GAA CGA G
20	OPG20	CCA CAG CAG T
21	OPG21	CCT GGG CCT A
22	OPG22	GAG GGC GTG A
23	OPG23	GAG CAC CAG G
24	OPG24	GGG CCC CAG G

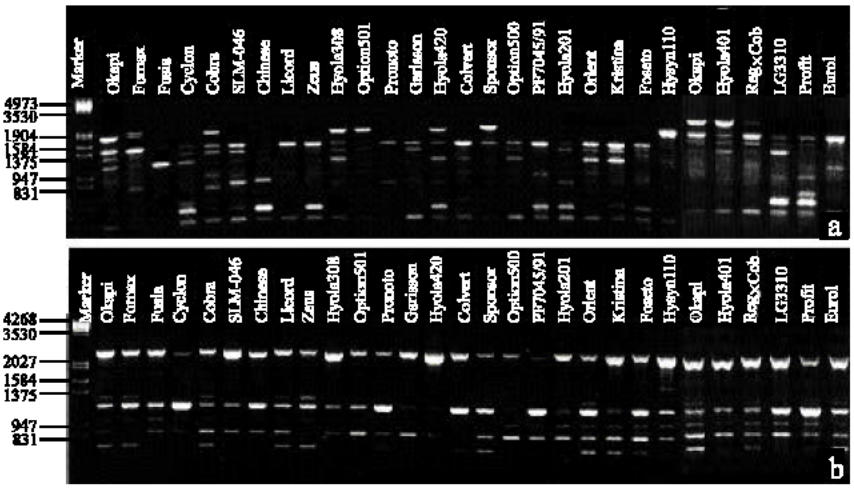

Fig. 1: Random amplified polymorphic DNA banding patterns produced using OPG10 (a) and OPG19 (b) on a subset of 28 rapeseed cultivars

performed in an Eppendorf Mastercycler Gradient programmed as follows: pre-denaturation 3 min at 92°C followed by 45 cycles, each of 1 min at 92°C, 1 min at 35°C and 2 min at 72°C, followed by one final extension cycle of 10 min at 72°C. The amplification products were size-separated by gel electrophoresis in 1.2% agarose gels with 0.5×TBE and stained with ethidium bromide. *Eco*R1/*Hin*dIII digested lambda DNA served as molecular size marker. PCR reactions were repeated at least twice to establish reproducibility of results. After AGE, the gel was photographed in UV light (Fig. 1). The molecular size of each fragment was calculated by using UVI bandmap software 10.02. Special care was taken to eliminate variation in DNA and any other reaction reagent concentration and to ensure consistent reaction conditions.

Data analysis: All polymorphic bands were scored as (1) for present and (0) for absence. Only reproducible and reliable bands were scored. Bands of identified size amplified with the same primer were considered to be the same DNA marker. Two similarity coefficients were computed, the Jaccard's coefficient (Jaccard, 1908) and Simple matching coefficient (Sokal and Michener, 1958). Cluster analysis was done and dendrogram were constructed using unweighted paired group of arithmetic mean (UPGMA) algorithm.

The structure of the genetic diversity was further analyzed by Principal Component Analysis (PCA) from the correlation matrix among the markers. Both cluster analysis and principal component analysis were performed using NTSYS 2.02 statistical software.

RESULTS

Twenty four primers were selected to amplify total DNA from samples of all genotypes among which amplification products of 22 primers (91.67%) showed polymorphism. A total of 173 reproducible bands amplified against 28 genotypes among which 133 products (76.88%) were polymorphic. Among total scored bands 164 fragments observed only in *B. napus* cultivars.

The number of amplicons generated varied from 5 (OPG1, OPG2, OPG7, OPG15, OPG16) to 18 (OPG8) and presented molecular weight between 440 to 2988 bp. The ability of primers made a wide range of polymorphism varied from 20% (OPG15) to 100% (OPG7, OPG11). OPG21 and OPG24 failed to reveal any polymorphism. RAPD profile produced using OPG11 against Pronto was clearly different from other ones. OPG1 and OPG16 produced uniform patterns for all *B. napus* cultivars. One fragment (OPG5-1419 bp) was produced only in Option500 and Option501. OPG5 also produced a band (1788 bp) in the profiles of two cultivars (PF7045/91 and Foseto). OPG11 produced a fragment of 2863 bp in the RAPD pattern of Hysyn110 and Fusia and another one (OPG11-663 bp) at Eurol. These polymorphic bands were not presented into the patterns of other cultivars.

The distance matrices based on the two similarity coefficients, Jaccard and SM, were highly correlated. Genetic distances ranged from 0.22 to 0.87 with a mean of 0.48 for J and from 0.13 to 0.61, with a mean of 0.31 for SM. If we ignore Hysyn110, the average of GDs will reduced to 0.45 for J and 0.29 for SM. Whatever the estimator considered, Kristina and SLM-046 were the

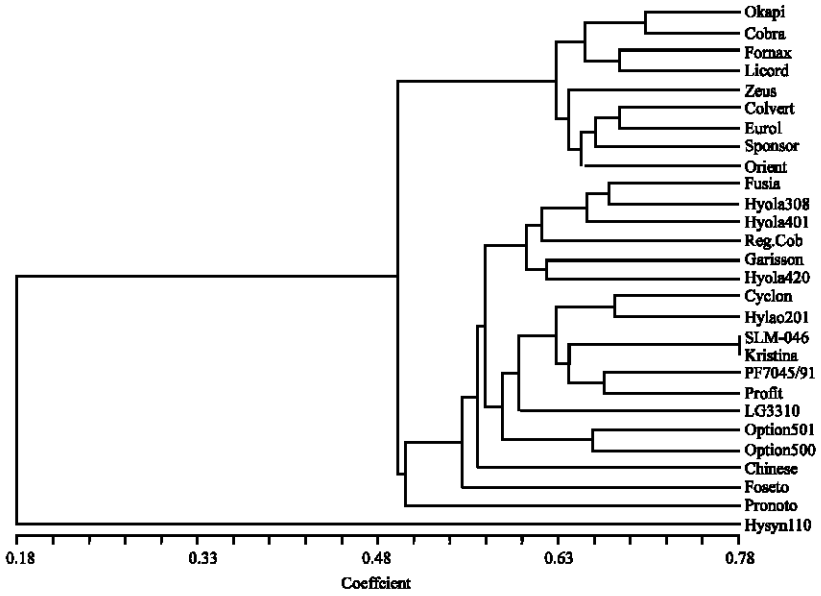

Fig. 2: Dendrogram illustrating genetic relationship among 27 cultivars of *Brassica napus* and one cultivar belongs to *Brassica rapa*, generated by unweighted paired group of arithmetic mean (UPGMA) cluster analysis based on Jaccard coefficient

closet *B. napus* cultivars and LG3310 (a Canadian spring cultivar) and Licord (a German winter cultivar) had the largest GD. The Canadian spring cultivar, Hylight201, had the largest genetic distance with Hysyn110, 0.87 for J and 0.61 for SM.

Cluster analysis was done using UPGMA method and dendrograms were constructed (Fig. 2). Dendrogram based on the J distance matrix defined one major group, consisted of all *B. napus* cultivars and Hysyn110, that belongs to *B. rapa*, classified as one outlier. The *B. napus* cultivars were distinctly separated into two subgroups and Pronto clustered as an outlier. The first subgroup included winter rapeseed cultivars with the exception of Sponsor, one spring type cultivar and the second one included spring cultivars with the exception of SLM-046 and Regent × Cobra, two winter type cultivars. At lower hierarchy, Foseto (a Mexican spring cultivar) and then Chinese (a Chinese spring cultivar) were separated from all other cultivars in their subgroups as outliers. Dendrogram constructed based on SM coefficient was the same; with this exception that Regent × Cobra clustered with winter type *B. napus* cultivars.

RAPD data also analyzed using PCA. Associations among the cultivars revealed by PCA were represented in Fig. 3. The first principal component explained about 54.4% of total variation among cultivars, this PC separated *B. napus* cultivars from Hysyn110, an cultivar belongs to *B. rapa*. The second PC explained about 6.7% of total variation among cultivars and separated winter and spring *B. napus* cultivars into two major groups.

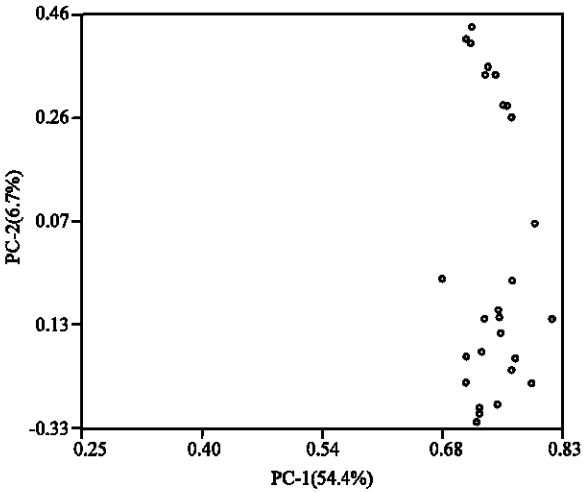

Fig. 3: Scatter plot based on the first and the second principal components from a principal component analysis of RAPD data, based on Jaccard coefficient

DISCUSSION

The present study demonstrated that RAPD profiling is efficient enough to reveal usable level of DNA polymorphism among rapeseed cultivars. This supports the earlier observations with regard to the RAPD profiling in *B. napus* (Hallden *et al*., 1994; Mailer *et al*., 1994, 1997). In this study we used 24 decamer random primers in RAPD analysis of 28 genotypes among which 91.67%

produced polymorphic fragments. OPG1, OPG16, OPG21 and OPG24 failed to reveal any polymorphism among *B. napus* cultivars. Therefore, they would not be recommended to use in *B. napus* germplasm studies. OPG5, OPG11 and OPG19 had the most efficiency in rapeseed genotype discrimination.

Several variety specific RAPD markers were produced for Option500- Option501 (OPG5-1419 bp), PF7045/91- Foseto (OPG5-1788 bp), Hysyn110- Fusia (OPG11- 2863 bp) and Eurol (OPG11-663 bp). These two primers can be employed in varietal registration studies. Specially, about unique band which produced in Eurol, this band can be used for identification of this cultivar from other cultivars if this locus can be proven to be homozygous by examining several plants of this cultivar. Also, if association between these RAPD markers and important traits or special genes can be demonstrated through bulk segregant analysis (Michelmore *et al*., 1991), it will be possible to convert them into codominant SCAR markers (Roy, 2000).

The distance matrices based on two similarity coefficients were highly correlated. Lombard *et al.* (2000) demonstrated that J and SM similarity coefficients were highly correlated among *B. napus* cultivars.

The choice of a genetic distance is a crucial issue for estimating the level of relatedness between cultivars. GD_J takes into consideration only matches between bands-alleles that are presented and ignores pairs in which a band-allele is absent in both individuals. For dominant markers such as RAPD, the expected GD_J of related pairs of lines is a linear function of their coancestry coefficient (Link *et al*., 1995). GD_{SM}, a Euclidian measure of distances, take into account mismatches and matches; it has metric properties that allow its usage in hierarchical strategies such as the minimum variance method within a group (Ward, 1963). However, many researchers do not prefer using GD_{SM} as it gives equal weight to both 0-0 and 1-1 matches in case of binary data (Mohammadi and Parsanna, 2003).

The average GDs among investigated rapeseed cultivars, 0.48 for J coefficient and 0.31 for SM, demonstrated that the level of genetic diversity among them is relatively low. However, according to its autogamous nature, it was predictable. The relatively low level of genetic diversity among rapeseed cultivar is unavoidable since the breeding system is an influential factor of variability within plants and populations of self pollinated species are less polymorphic than out breeding species (Campos-de-Quiroz and Ortega-Klose, 2001). Correlation between the level of intraspecific variation and the reproductive system has been observed for RAPD markers in other species (De Bustos *et al*., 1998; Kazan *et al*., 1993). Also, we should consider that low level of polymorphism in cultivars could be attributed to a narrow genetic base and the frequent inbreeding involved in breeding programs (Pujar *et al*., 1999).

In this study, *B. napus* cultivars and Hysyn110 which belong to *B. rapa* were separately clustered. Cluster analysis also separated winter and spring type *B. napus* cultivars into two separated clusters. PCA revealed the same structure as the cluster analysis. On the basis of the results of PCA, *B. napus* cultivars and Hysysn110 were distinctly separated. PCA also separated winter type *B. napus* cultivars from spring ones. Diers and Osborn (1994) reported similar result using RFLP markers. In their study most of winter and spring *B. napus* cultivars were separated into two different groups. Other similar results have been demonstrated by Plieske and Struss (2001) using microsatellite markers and by Lombard *et al.* (2000) using Amplified Fragment Length Polymorphism (AFLP) markers. Winter and spring *B. napus* cultivars are fairly distinct groups and there has been little intercrossing between these groups in breeding programs (Sernyk and Steffansson, 1983).

Regent×Cobra, an Iranian winter cultivar grouped with spring types in the phenetic analysis based on J coefficient. In principal component analysis, based on both coefficients Regent×Cobra, positioned between spring and winter cultivars. This is evidence that although this cultivar grouped with spring cultivars, its association is not strong. Therefore, further studies must be done in order to identify cultivation type of this cultivar, exactually. Sponsor (a spring type cultivar) clustered with winter types. These results demonstrated that Sponsor has more genetic similarity with winter type cultivars. Unexpected, SLM-046 one winter type cultivar, clustered with spring type cultivars. It should be considered that accuracy of genetic similarity estimates base on molecular markers depends on several variable factors such as the number of markers analyzed, their distribution over the genome and the accuracy in scoring of markers (Schut *et al*., 1997). Moreover, any seed contamination, both mechanical and genetically contamination, might be accrued.

In conclusion, this study demonstrated that RAPD markers provide an effective tool in assessment of genetic variation among rapeseed cultivars and in cultivar identification. This information could be used for selection of most divergent pairs of parents, which could be used for genetic linkage map construction in this species. Information on the genetic diversity in *B. napus* and germplasm also can help breeders to select the best combinations, which produce high level of heterosis. The observed genetic variation among investigated rapeseed

cultivars found in analyzed loci should alert the breeders for introducing new genetic sources in hybridization programs. Investigated collection includes rapeseed cultivars with different cultivation type and different origins. However, direct effort to diversify the genetic base of breeding material and to seek new sources of favorable alleles would be still necessary. Accessions belong to *B. rapa* species and other wild relatives of *B. napus* could be useful to broad the genetic basis of rapeseed germplasm.

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