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PCR-Based Genetic Diversity of Rapeseed Germplasm Using RAPD Markers

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Abstract: The most challenging hurdle facing Pakistan is the production of *Brassica* germplasm with a wider genetic base and using them properly in rapeseed genetic improvement. Genetic diversity was evaluated in 20 rapeseed lines (10 entries each of *B. napus* and *B. campestris)* using RAPD as molecular markers. Four Randomly Amplified Polymorphic DNA primers were used to estimate the genetic distances among the genotypes in all the possible combinations. The genetic diversity study revealed different levels of polymorphism for RAPD primers GLA07, GLB07, GLD18 and GLE07 that resulted in amplification of 3.2, 1.5, 3.0 and 3.5 scorable bands (loci) per genotype of *Brassica napus* and 2.5, 1.3, 2.6 and 3.7 scorable bands (loci) per genotype of *B. campestris*. Among *Brassica napus* genotypes, maximum genetic distance (79%) was observed between Torch+Maluka, Torch+Baro and Torch+Global, while, maximum genetic distance (91%) was observed between T-16 and P1-367601 genotypes of *Brassica campestris*. Individual genetic distance observed among the *B. napus* and *B. campestris* lines ranged from 21.50 to 59.41% and 53.75 to 60.09%, respectively. The dissimilarity coefficient matrix of these lines based on the data of four RAPD markers using UPGMA (Unweighted Pair Group of Arithmetic Means) method was also used to construct a dendrogram. The dendrogram analysis indicated that lines Torch and 366822 of *B. napus*, while 2163 and P1-392029 of *B. campestris* were genetically apart from other lines. These results provide valuable information for fingerprinting that can be used in a synergistic way to create wider genetic base and augment the breeding program of *Brassica* in Pakistan.

Key words: Rapeseed, genetic diversity, RAPD, molecular markers

INTRODUCTION

Genetic diversity within the genus gives us an important source of variation that can be used to modify *Brassica* crop species by various methods. Estimates of genetic relationship are also very important in designing crop improvement programs, management of germplasm and evolution of conservation strategies.

The use of DNA profiling techniques for genotype identification offers several advantages over the use of morphological data. DNA sequences are independent of environmental conditions; furthermore, identification can be determined at any stage of plant growth (Lakshmikumaran, 2000; Smith and Smith, 1992) and very similar genotypes, including clonal variants, can be discriminated using DNA techniques (Demeke *et al*., 1992). Finally, the use of the Polymerase Chain Reaction (PCR) has enabled the development of simple and rapid DNA profiling methods (Oliveira *et al*., 2004; Morell *et al*., 1995).

Molecular markers are the best tools for determining genetic relationships. A variety of molecular markers have been used to study the extent of genetic variation among the diverse group of important crops in the genus *Brassica*. These include Restriction Fragment Length Polymorphism (RFLP), Simple Sequence Repeats (SSR), random amplification of polymorphic DNA (RAPD) (Osborn and Lukens, 2003; Karp *et al*., 1997) and Amplified Fragment Length Polymorphism (AFLP) (Negi *et al*., 2001; Vos *et al*., 1995). These techniques differ in their principles and generate varying amounts of data. RFLP analysis is labour intensive, time consuming and expensive.

With the development of Polymerase Chain Reaction (PCR) based RAPD and SSR, most of the problems associated with RFLP were overcome. Random amplified polymorphic DNA (RAPD) markers offer quick screening of different regions of the genome for genetic polymorphisms. The technique of RAPD gained importance due to its simplicity, efficiency, relative ease to perform and non-requirement of sequence information (Karp *et al*., 1997).

RAPD analysis has also been extensively used to document the genetic variation in *Brassicas* (Zhu *et al*.,

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1998; Demeke *et al*., 1992; Jain *et al*., 1994; Bhatia *et al*., 1995; Thormann *et al*., 1994). PCR-based fingerprinting techniques, is very efficient both in cost and time to identify RAPD markers associated with a trait. The RAPD markers are easier and quicker to use and are preferred in applications where the relationships between closely-related breeding lines are of interest (Halldén *et al*., 1994). However, most of the earlier studies were carried out with *B. juncea* germplasm and not much information is available on the extent of genetic variation present in rapeseed species using DNA based marker systems in Pakistan.

The present study was therefore undertaken to estimate the genetic diversity of different rapeseed lines based on molecular characterization and obtain reliable information that can be utilized for selecting better parents and used in a synergistic way in future *Brassica* breeding program. For this purpose 20 rapeseed (*B. napus* and *B. campestris*) genotypes were analyzed at molecular level using Randomly Amplified Polymorphic DNA primers (RAPDs) and genetic distances among the genotypes were estimated.

MATERIALS AND METHODS

Plant material: Twenty genotypes/lines of rapeseed (10 entries each of *B. napus* and *B. campestris*) were characterized molecularly during the present study. The germplasm, kindly provided by the Institute of Biotechnology and Genetic Engineering, NWFP Agricultural University Peshawar was planted on March 15, 2005 in the IBGE green houses. Standard growth conditions were used during the experiment. The list of rapeseed genotype used in the present study is given in the Table 1.

DNA extraction: For genomic DNA isolation, about 10 cm of fresh leaf material from 3-4 weeks old seedling was ground to fine powder in a 2 mL eppendorf tube under liquid nitrogen, following the method of Weining and Langridge (1991). The powder was homogenized with 500 μL of DNA extraction buffer (4% SDS, 0.1M Tris-HCl, 10 mM EDTA, pH 8.0) and an equal volume of phenol:chloroform:isoamyl alcohol in the ratio of 25:24:1 respectively, was added to it. The whole mixture was vigorously shaken for 20-30 sec and aqueous phase was recovered by centrifugation at 5000 rpm for 5 min. The supernatant was transferred to a fresh tube and the DNA was precipitated from it by adding 1/10th volume of 3M sodium acetate (pH 5.0) with an equal volume of isopropanol. The DNA was pelleted by centrifugation for 7 min. The pellet was washed twice with ice cold 70% ethanol, dried at 37°C and dissolved in 40-45 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing

Table 1: Description of all 20 rapeseed genotypes used in the current study

B. napus	*B. campestris*
Torch	T-16
Altex	2163
Maluka	2065
ww-15171	1203
Baro	366
Global	399596
Puma	459976
8a111-1	TP-57-1
Narindra	P1-367601
366822	P1-392029

Table 2: Detailed description and sequence information of the primers used

Name of primer	Sequence	Size (bp)	TM (°C)	Mol. Wt (Da)
A07 primer	(GAAACGGGTG)	10	29.5	3117.04
B07 primer	(GGTGACGCAG)	10	33.6	3093.03
D18 primer	GAGAGCCAAC)	10	29.5	3046.00
E07 primer	(AGATGCAGCC)	10	29.5	3037.00

TM = Melting temperature of primer, Mol. Wt = Molecular weight of the primer

40 μg mL^{-1} RNase A. The concentration of DNA was estimated by comparing the Ethidium Bromide staining intensity with that of the λ DNA (standards) of known concentration on a 2% Agarose, Tris Borate EDTA (TBE) gel. The DNA was diluted with double distilled, autoclaved and deionized water at the ratio of 1:5 concentrations for use in PCR.

PCR (Polymerase Chain Reaction): The polymerase chain reaction was carried out using protocols of Prasad *et al.* (2000) with modifications in thermal profile given below: Denaturation at 94°C and primer annealing at 34°C for 1 min each, followed by extension at 72°C for 2 min and total cycles (40) with final extension at 72°C for 10 min.

The Randomly Amplified Polymorphic DNA markers (RAPDs) synthesized from the Gene Link Technology, USA were employed for genetic diversity analyses. Detailed description and sequence information of the primers is given in the Table 2.

DNA amplification was carried out in PCR tubes containing 25 μL reaction mixture, having 1 μL template DNA, 1 μL RAPD primer, 15 μL of dd H_2O and 7 μL of PCR mix (composed of 460 μL H_2O, 500 μL buffer, 10 μL each of dNTPs (100 mM) and 300 μL $MgCl_2$ (25 mM)). The PCR reaction mixture, except DNA and primer in the above mentioned volume was pooled to sterilized PCR tubes and was mixed thoroughly by gentle pipetting. DNA and primer were added to the reaction mixture and centrifuged mildly to collect all constituents and were subjected to the thermal profile given above. The reaction was carried out in the Thermocycler (Gene Amp 2700).

Statistical analysis: For statistical analysis of Randomly Amplified Polymorphic DNA (RAPD), all the scorable bands were considered as single locus/allele. The loci were scored as present or absent. Bivariate 1-0 data matrix

was generated. Genetic distances were calculated using Unweighted Pair Group of Arithmetic Means (UPGMA) procedure as follows (Nei and Li, 1979).

$$GD = 1\text{-}dxy/dx+dy\text{-}dxy$$

Where:

GD = Genetic distance among two genotypes,
dxy = Total number of common loci (bands) in two genotypes,
dx = Total number of loci (bands) in genotype 1 and
dy = Total number of loci (bands) in genotype 2.

The 1-0 bivariate data matrix for each set of wheat lines based on the data of four RAPD primers was used to construct a Dendrogram using compute program Popgene32 version 1.31 (http://www.ualberta.ca./~fyeh/fyeh).

RESULTS AND DISCUSSION

Level of genetic polymorphism (estimated as percent genetic distance) observed during present study among the *B. napus* and *B. campestris* lines were in the range of 21.50 to 59.41% and 53.75 to 60.09%, respectively. Maximum genetic distances among the *B. napus* and *B. campestris* lines were observed between Torch+Maluka, Torch+Baro, Torch+Global and T-16+P1-367601 and TP-57-1+P1-392029, respectively. These findings were further strengthened by dendrogram analyses (Fig. 3), where *Brassica napus* lines Torch, 366822 and *Brassica campestris* lines 2163, P1-392029 were found to be genetically more distant from other lines. Overall genetic distant among *B. napus* and *B. campestris* was observed between Torch and P1-392029. Similar results were reported by Das *et al.* (1999) and Cansian and Echeverrigaray (2000) who observed more or less similar range of genetic dissimilarities in *Brassica* lines.

For individual RAPD primers, higher level of genetic polymorphism among the *B. napus* and *B. campestris* lines was found in case of RAPD primer GLD18 (not shown) and GLB07 (Fig. 1 and 2), respectively where higher levels of genetic variability were observed among different comparisons, indicating its power for the identification of individual genotypes. The Dendrogram constructed by Unweighted Pair Group of Arithamatic Means (UPGMA) was generally in agreement with the genetic distances calculated, indicating that the RAPD technique can be used reliably for estimation of genetic variability in rapeseed. In *Brassica* and its related genera, RAPD markers have been used successfully for identification and phylogenetic relationship among and within the species (Ren *et al.*, 1995).

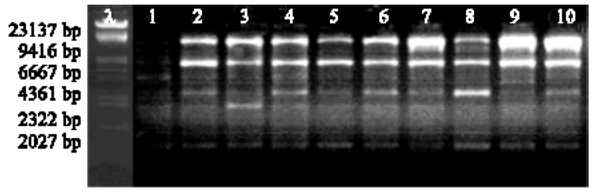

Fig. 1: PCR amplification products of 10 *B. napus* lines using RAPD primer GLE07. (λ. Marker,1. Torch, 2. Altex, 3. Maluka, 4. ww-15171, 5. Baro, 6. Global, 7. Puma, 8. 8a111-1, 9. Narindra, 10. 366822)

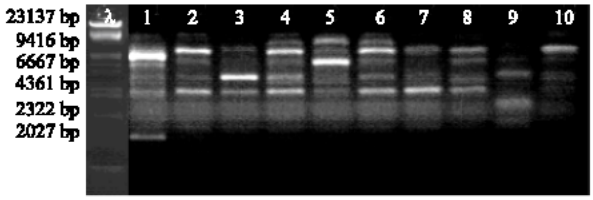

Fig. 2: PCR amplification products of 10 *B. campestris* lines using RAPD primer GLE07. (λ. Marker, 1. T-16, 2. 2163, 3. 2065, 4. 1203, 5. 366, 6. 399596, 7. 459976, 8. TP-57-1, 9. P1-367601, 10. P1-392029)

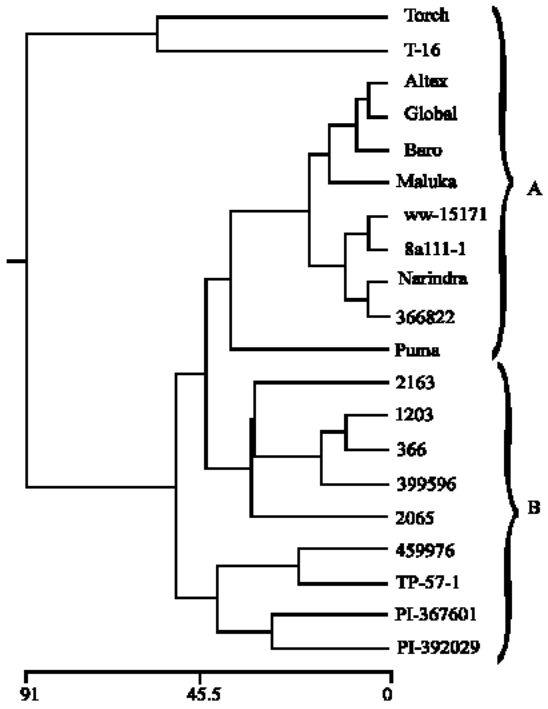

Fig. 3: Dendrogram constructed for 20 rapeseed (*B. napus* and *B. campestris*) lines based on genetic distances using four RAPD primers

Table 3: Average genetic distances among 20 rapeseed (*Brassica napus* and *Brassica campestris)* lines using four RAPD primers

	1	2	3	4	5	6	7	8	9	10	11	12	14	15	16	17	18	19	20
2	0.68																		
3	0.71	0.15																	
4	0.64	0.17	0.17																
5	0.73	0.13	0.15	0.17															
6	0.71	0.6	0.8	0.10	0.6														
7	0.54	0.40	0.42	0.44	0.38	0.33													
8	0.68	0.13	0.15	0.4	0.13	0.0	0.40												
9	0.60	0.25	0.25	0.8	0.22	0.19	0.52	0.12											
10	0.54	0.37	0.38	0.21	0.35	0.31	0.46	0.25	0.13										
11	0.61	0.71	0.76	0.67	0.72	0.71	0.70	0.71	0.65	0.60									
12	0.63	0.36	0.47	0.45	0.44	0.42	0.54	0.48	0.40	0.28	0.53								
13	0.72	0.17	0.28	0.25	0.29	0.23	0.56	0.29	0.33	0.46	0.78	0.38							
14	0.68	0.36	0.44	0.41	0.41	0.39	0.62	0.44	0.39	0.51	0.70	0.34	0.38						
15	0.74	0.23	0.32	0.28	0.31	0.27	0.58	0.44	0.35	0.47	0.68	0.32	0.25	0.13					
16	0.74	0.23	0.32	0.28	0.31	0.27	0.58	0.44	0.35	0.47	0.74	0.41	0.25	0.20	0.10				
17	0.65	0.39	0.53	0.50	0.50	0.48	0.81	0.46	0.46	0.58	0.78	0.54	0.39	0.60	0.54	0.54			
18	0.60	0.69	0.77	0.64	0.72	0.71	0.69	0.69	0.62	0.62	0.71	0.54	0.70	0.64	0.60	0.68	0.43		
19	0.40	0.76	0.73	0.72	0.80	0.78	0.74	0.76	0.68	0.68	0.88	0.72	0.73	0.71	0.77	0.77	0.67	0.63	
20	0.50	0.42	0.35	0.46	0.38	0.35	0.62	0.47	0.42	0.54	0.78	0.50	0.44	0.44	0.50	0.50	0.52	0.72	0.27

B. napus (1. Torch, 2. Altex, 3. Maluka, 4. ww-15171, 5. Baro, 6. Global, 7. Puma, 8. 8a111-1, 9. Narindra, 10. 366822) *B. campestris* (11. T-16, 12. 2163, 13. 2065, 14. 1203, 15. 366, 16. 399596, 17. 459976, 18. TP-57-1, 19. P1-367601, 20. P1-392029)

The level of polymorphism found in the present study in *B. napus* and *B. campestris* lines was less than 59 and 60%, respectively. Similar findings were reported by Uzunova *et al.* (1995) who observed less than 45% level of polymorphism in *B. napus*. In *B. oleracea*, a diploid specie, higher level (>80%) of polymorphism and in *B. juncea* approximately 60% polymorphism was reported by Cheung *et al.* (1997b). Lower levels of polymorphism in amphidiploids may be attributed to the lower level of out-crossing due to a weak and often non-existing self-incompatibility system (Rakow and Woods, 1987).

The 1-0 bivariate data matrix for 20 rapeseed (*Brassica napus* and *Brassica campestris*) lines based on the data of four RAPD primers using UPGMA method was used to construct a dendrogram (Fig. 3). In general the dendrogram agreed with the average dissimilarity matrix presented in Table 3. Based on the dendrogram analysis, the 20 rapeseed (*Brassica napus* and *Brassica campestris*) lines could be categorized in 2 major groups i.e., A and B. In group A all the lines are *B. napus* except T-16, which belongs to *B. campestris* while in group B all the lines are *B. campestris* except Puma which belongs to *B. napus*. Most diverse *B. napus* genotypes were Torch and 366822 while in case of *B. campestris* most diverse genotypes 2163 and P1-392029 were observed among the rapeseed used in the study. The overall genetic distance among *B. napus* and *B. campestris* was observed between Torch and P1-392029. These results were in close agreement with the results of genetic distances where similar relationships between the varieties were found. The lines that showed maximum genetic distances in dendrogram analyses also showed higher Genetic Distances (GD) in average dissimilarity matrix values (Table 3).

It is concluded that a significant genetic variation exists among various combinations of rapeseed germplasm investigated in the present study which can further be utilized in strengthening *Brassica* breeding program. Further, It is evident from present data that PCR based assays like RAPDs can be used effectively to estimate genetic variability in rapeseed (*B. napus* and *B. campestris*). Easy handling of the technique makes it especially suitable for breeding programs where large numbers of lines have to be analyzed. It is also suggested that more rapeseed genotypes and molecular markers should be used to find out the better picture of genetic variability present in these rapeseed genotypes.

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