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Evaluation of Genetic Diversity of Raya (*Brassica juncea*) through RAPD Markers

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Abstract

This study was designed to explore the genetic relatedness among 30 *Brassica juncea* lines and varieties included in "development of canola varieties in *B. juncea*" program, using Randomly Amplified Polymorphic DNA (RAPD) markers. All used RAPD markers produced a total of 104 loci with an average of 8.6 bands per primer. The range of amplified bands was between 300bp to 3kb. The largest fragment of 3kb was amplified with three primers, A-5, A-19 and B-05. On an average 84% similarity matrix (SM) was observed between all thirty genotypes. The most diverse genotype was RBJ-97001 with average genetic diversity of 29% while RBJ-02017 showed the highest average genetic SM (87%) to all other genotypes. The genotypes RBJ-07017 and RL-18 were observed most similar to each other with genetic SM of 95%, whereas, Raya 49/2 and RBJ-97001 were found the most diverse lines with genetic SM of 71%. Unweighed pair group method of arithmetic means cluster analysis indicated that 30 genotypes were capable of being classified into two major groups, A and B. Both groups were further divided into two sub-groups. The information obtained on genetic distances will be useful in future breeding of Raya cultivars for the development of canola type varieties along with germplasm characterization. © 2013 Friends Science Publishers

Keywords: *Brassica juncea*; RAPD; Canola; Genetic diversity; Pakistan

Introduction

Improved seed oil and meal quality have facilitated oilseeds Brassicas in gaining the second largest oilseed crop position and a major crop for the raw material supply in biodiesel production (Burbulis *et al.,* 2004; Hasan *et al.,* 2006). Brassica is loosely classified into oilseeds, vegetable and condiment crops. Improvement of seed quality is one of the most important objectives in *Brassica* breeding for satisfying future edible oil requirements (Becker *et al.*, 1999; Shengwu *et al.,* 2003). Oilseed breeders have developed "canola" forms of *B. napus* and *B. rapa* to improve oil and meal quality. Due to wider adaptability to the hotter and drier areas and relatively higher oil content of up to 44%, *B. juncea* is being widely grown in Pakistan, India, China and in south-western areas of the former Soviet Union as oil seed crop. However, *B. juncea* types grown in Pakistan are known as mustard quality as they contain higher percentage of erucic acid in the oil and elevated concentrations of glucosinolates in the cake. In Pakistan, *B. juncea* is the first choice for conversion to canola quality as it exhibits better tolerance against heat and drought stresses, and resistance against diseases and pod shatter compared to *B. napus* (Woods *et al.,* 1991; Kjellstorm, 1993; Getinet, 1996). Breeding for quality and yield has led to narrowing the genetic diversity in modern cultivars in almost all major crops. Resultantly, new cultivars are more prone to biotic as well as abiotic stresses, necessitating induction of diverse lines from germplasm in breeding program. Analysis of genetic diversity amongst tailored cultivars or worthy breeding materials may generate useful information to broaden genetic base in modern cultivars (Talebi *et al*., 2008). Genetic relationships could be obtained from pedigree analysis, morphological characters or using molecular markers (Mohammadi and Prasanna, 2003). However, molecular markers are the best choice for calculating genetic diversity as they are not influenced by environmental factors. These molecular assays vary in principle, function and type. Task and time requirements are also considered, while selecting the molecular methods. Polymerase chain reaction (PCR) based methods are being commonly used for the characterization of plant and animal genetic resources. Randomly amplified polymorphic DNA is perhaps the simplest method among numerous available molecular techniques recruited to measure constitution, change and progression of genetic material (Williams *et al*, 1990). Despite controversies on limitations and reproducibility of RAPD markers, they are still significantly used to tackle various objectives in molecular biology, like tagging a simple and dominant gene as well as to identify multiple chromosome intervals controlling a quantitative

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trait (Basha and Sujatha, 2007; Grimmer *et al.*, 2007; Pan and Chen, 2010).

Thus, assessment of genetic diversity among *B. juncea* accessions from present study will be helpful in base building for the development of canola type varieties more acceptable to farmers and ultimately share of good quality oil will be increased in local economy.

Materials and Methods

Plant Material

Thirty *B. juncea* accessions, comprising approved cultivars as well as exotic and local germplasm lines, from Oilseed Research Institute (ORI), AARI, Faisalabad were used in the study (Table 1). Genomic DNA (gDNA) was extracted using CTAB method (Rahman *et al.*, 2002) from fresh and young leaves of two weeks old seedlings of each accession selected at random. Quality and quantity of total gDNA was assessed through NanoDrop-1000 3.3.1 spectrophotometer. A sub-sample of three randomly selected accessions was used to screen 50 arbitrary decamer primers for their suitability to amplify DNA sequences, which could be scored precisely. RAPD primers were evaluated for unambiguous, visible bands, constancy and presence of smearing. A final set of 12 primers showing good quality banding patterns and ample polymorphism were selected for further analysis. RAPD primers producing faint or hard to detect bands were not used for amplification.

PCR reactions were performed in a 20 µl mixture containing 10 mmol/L TrisHCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 µmol/L each dNTP, 0.4 µmol/Lof decamer primer, 1 unit Taq DNA Polymerase and approximately 20 ng of template DNA. Reaction mixtures were overlaid with paraffin to avoid evaporation. DNA amplification was performed in a programmable thermal cycler (Master Cycler Gradient, Eppendorf, Germany). The reaction mixture was preheated at 95°C for 3 min followed by 40 cycles of one min denaturation at 94°C, one min annealing at 37°C and extension at 72°C for 2 min. After the last cycle, a final step of 7 min at 72°C was further added to allow complete extension of all amplified fragments. The PCR products were resolved on 1.2% agarose gel and sizes of the amplified bands were estimated using a known molecular weight marker (1kbp DNA ladder). The gels were post-stained with ethidium bromide and gel imaging was recorded on Nyxtechnik electrophoresis documentation system.

Molecular data were recorded on the basis of presence or absence of DNA fragments. Only clear and score able bands were considered for genetic diversity analysis. The binary data obtained were used to estimate the similarity on the basis of the number of shared amplified products to combine the data of all the accessions. Nei and Li's (1979) coefficients were used to generate genetic similarity matrices. NTSyspc 2.0 software package was used for dendrogram construction using unweighed pair group method of arithmetic means (UPGMA).

Results

During present study, the surveyed RAPD primers produced 104 amplicons with an average of 8.6 bands per primer. It is indicated in Table 2 that a maximum of 15 amplicons were generated by primer A-05; whereas, B-01 amplified only five DNA bands. The amplified products ranged from 300 to 3000 bp in size. Lowest band size (300 bp) was amplified with three primers viz; A-05, A-10 and A-19, while two primers A-05 and B-05 produced 3000 bp fragments. Primer B-07 produced seven polymorphic bands as depicted in Fig. 1.

Data obtained from RAPD analysis were employed to generate similarity matrix (SM) using Nei and Lei coefficient (Nei and Li, 1979). Twelve RAPD primers generated 104 markers, which were utilized for final analysis. The resulting SM revealed a mean genetic diversity (84%) among the genotypes studied. As far as the pair-wise combinations are concerned, similarity between accessions varied with a range of 95 to 71% (Table 3). The accession pairs BARD-1 and RBJ-03050 and RL-18 and RBJ-07017 emerged as the most similar pairs with the highest SM of 95%. RBJ-03050 and RJ-96026 also showed very low dissimilarity of 6%. Three accession pairs including RBJ-03047 and Varuna, Varuna and KJ-127 and Khanpur Raya and RBJ-07010 exhibited 8% diversity. The accessions J-90-43001 and Varuna were found most dissimilar pair with dissimilarity coefficient of 29%. J-90-43001 is a high yielding, low erucic acid and low glucosinolate line introduced from Canada, while Varuna is a high yielding *Brassica juncea* variety form India with high erucic acid and glucosinolate contents. J-90-43001 also showed higher dissimilarity with RBJ-02019, a line selected from the cross of high yielding accessions from Khanpur Research Station (KRS), a sub-station of ORI mainly involved in rapeseed and mustard breeding. While, 27% dissimilarity was observed between KH-38 and KJ-117, J-90-43001 and KJ-127 and J-90-43001 and BARD-1 pairs of accessions.

Comparison of each line with the rest of the accessions showed a SM ranging from 81 to 95% (Table 4). RBJ-96026 came out as the most diverse accession when compared with all other 29 cultivars with a mean dissimilarity of 19%. RBJ-07017 depicted the highest mean SM of 95% with other accessions. Sultan Raya, Varuna and Khanpur Raya showed mean SM of 86, 83 and 93%, with the remaining accessions. Zem-II and J-90-43001, showed 82% SM with all other genotypes.

Based on the analysis carried out on Nei and Li, SM using the unweighted pair group method with arithmetic mean (UPGMA), 30 *B. juncea* accessions were grouped into two main clusters and a number of independent accessions (Fig. 2). Group A (the largest cluster) comprised

Table 1: *B. juncea* accessions used in the study

Genotypes	Origin
Sultan Raya	Arid Zone Research Institute, Bahawalpur. (AZRI)
Khanpur Raya, RL-18	ORI, AARI, Faisalabad.
BARD-1	National Agriculture Research Centre, Islamabad. (NARC)
Varuna	India
Zem II	Australia
RBJ-96024, RBJ-99026, Raya 49/2, RBJ-03047, KJ-127, KJ-117, RBJ-02019, RBJ-03050, RBJ-96026, Raya 63/2, RBJ-02018, RBJ-2K024, RBJ-02017, KS-74, KH-33, KH-34, KH-38, KJ-119, RBJ-2K022, RBJ-97001, RBJ-2K027, J-90-43001, RBJ-07010, RBJ-07017	*Brassica juncea* lines selected from Breeding Program at ORI, AARI, Faisalabad.

Table 2: RAPD primers with corresponding bands scored polymorphic bands observed in 30 *B. juncea* accessions

Oligo Name	Sequences (5'-3')	Total No. of Bands Scored	Size Range (bp)	No. of Polymorphic Bands
GL DecamerA-04	AATCGGGCTG	8	400-1800	8
GL DecamerA-05	AGGGGTCTTG	15	300-3000	8
GL DecamerA-08	GTGACGTAGG	9	500-2100	4
GL DecamerA-09	GGGTAACGCC	7	400-1500	3
GL DecamerA-10	GTGATCGCAG	9	300-1500	2
GL DecamerA-17	GACCGCTTGT	8	500-2000	5
GL DecamerA-18	AGGTGACCGT	6	600-1700	1
GL DecamerA-19	CAAACGTCGG	13	300-3000	10
GL DecamerB-01	GTTTCGCTCC	5	900-2700	2
GL DecamerB-03	CATCCCCCTG	7	500-2200	2
GL DecamerB-05	TGCGCCCTTC	7	700-3000	7
GL DecamerB-07	GGTGACGCAG	10	400-1500	7
Total		104		59

Table 3: Pair-wise similarity matrix (%) for thirty *B. juncea* accessions

	J2	J 3	J 4	J 5	J 6	J 7	J 8	J 9	J 10	J 11	J 12	J 13	J 14	J 15	J 16	J 17	J 18	J 19	J 20	J 21	J 22	J 23	J 24	J 25	J 26	J 27	J 28	J 29	J 30
J 1	0.87																												
J 2	0.90	0.81																											
J 3	0.84	0.82	0.89																										
J 4	0.81	0.78	0.82	0.87																									
J 5	0.82	0.80	0.85	0.87	0.92																								
J 6	0.87	0.79	0.88	0.87	0.88	0.92																							
J 7	0.84	0.81	0.83	0.87	0.85	0.89	0.91																						
J 8	0.84	0.86	0.84	0.86	0.84	0.86	0.90	0.94																					
J 9	0.88	0.81	0.89	0.90	0.85	0.86	0.92	0.89	0.91																				
J 10	0.88	0.80	0.87	0.90	0.85	0.87	0.91	0.90	0.89	0.95																			
J 11	0.83	0.79	0.86	0.88	0.83	0.86	0.87	0.89	0.87	0.93	0.94																		
J 12	0.80	0.81	0.82	0.84	0.85	0.82	0.85	0.85	0.86	0.84	0.88	0.88																	
J 13	0.82	0.83	0.84	0.83	0.82	0.84	0.85	0.87	0.89	0.86	0.84	0.85	0.84																
J 14	0.84	0.80	0.84	0.83	0.83	0.83	0.89	0.89	0.90	0.88	0.90	0.87	0.87	0.84															
J 15	0.87	0.78	0.85	0.82	0.82	0.82	0.85	0.83	0.84	0.86	0.86	0.86	0.85	0.87	0.87														
J 16	0.92	0.87	0.87	0.83	0.82	0.82	0.85	0.88	0.88	0.86	0.88	0.86	0.85	0.87	0.86	0.88													
J 17	0.91	0.82	0.86	0.85	0.79	0.81	0.84	0.82	0.83	0.87	0.90	0.85	0.84	0.84	0.86	0.90	0.92												
J 18	0.88	0.84	0.86	0.83	0.86	0.86	0.84	0.83	0.88	0.85	0.84	0.82	0.83	0.88	0.85	0.90	0.86	0.90											
J 19	0.90	0.85	0.83	0.82	0.80	0.82	0.81	0.81	0.87	0.87	0.86	0.82	0.80	0.85	0.84	0.85	0.85	0.89	0.89										
J 20	0.83	0.84	0.81	0.77	0.75	0.75	0.73	0.75	0.78	0.78	0.79	0.76	0.75	0.83	0.81	0.84	0.85	0.87	0.87	0.86									
J 21	0.84	0.80	0.82	0.81	0.82	0.78	0.80	0.87	0.84	0.83	0.84	0.83	0.84	0.81	0.83	0.78	0.87	0.79	0.78	0.83	0.79								
J 22	0.88	0.81	0.86	0.83	0.83	0.81	0.82	0.81	0.82	0.82	0.84	0.84	0.84	0.85	0.83	0.87	0.89	0.88	0.85	0.84	0.85	0.85							
J 23	0.83	0.77	0.81	0.81	0.71	0.73	0.75	0.72	0.73	0.80	0.79	0.79	0.76	0.76	0.78	0.79	0.80	0.80	0.77	0.79	0.83	0.77	0.85						
J 24	0.88	0.86	0.83	0.81	0.77	0.78	0.78	0.83	0.84	0.83	0.82	0.84	0.78	0.84	0.81	0.85	0.87	0.84	0.84	0.86	0.84	0.84	0.84	0.84					
J 25	0.89	0.84	0.89	0.83	0.76	0.78	0.80	0.82	0.85	0.85	0.81	0.83	0.81	0.87	0.81	0.85	0.87	0.83	0.86	0.84	0.85	0.81	0.83	0.82	0.89				
J 26	0.90	0.87	0.89	0.83	0.79	0.81	0.80	0.84	0.85	0.86	0.84	0.85	0.79	0.87	0.85	0.84	0.92	0.86	0.85	0.88	0.87	0.86	0.85	0.85	0.90	0.92			
J 27	0.86	0.84	0.83	0.80	0.78	0.81	0.79	0.86	0.87	0.84	0.83	0.80	0.78	0.85	0.85	0.86	0.91	0.87	0.85	0.86	0.82	0.81	0.80	0.77	0.84	0.86	0.94		
J 28	0.85	0.83	0.83	0.84	0.78	0.78	0.76	0.81	0.82	0.82	0.83	0.84	0.80	0.82	0.82	0.83	0.88	0.84	0.81	0.86	0.84	0.80	0.82	0.81	0.88	0.88	0.92	0.90	
J 29	0.85	0.83	0.88	0.83	0.79	0.81	0.80	0.85	0.86	0.86	0.84	0.86	0.82	0.88	0.86	0.88	0.90	0.86	0.84	0.88	0.86	0.84	0.87	0.83	0.90	0.91	0.94	0.92	0.95

J1= KH-33, J2 = KJ-119, J3 = KH-34, J4 = Raya-49-2, J5 = RBJ-03047, J6 = Varuna, J7 = KJ-127, J8 = KJ-117, J9 = RBJ-02019, J10 = BARD-1, J11 = RBJ-03050, J12 = RBJ-96026, J13 = RBJ-02018, J14 = Raya-63/2, J15 = RBJ-2K024, J16 = RBJ-02017, J17 = KS-74, J18 = RBJ-97001, J19 = RBJ-99026, J20 = KH-38, J21 = Zem-II, J22 = RBJ-2K022, J23 = J-90-43001, J24 = RBJ-2K027, J25 = RBJ-96024, J26 = Khanpur raya, J27 = RBJ-07010, J28 = RBJ-07017, J29 = RL-18, J30 = RBJ-07017

of 13 accessions, which were subdivided into two subgroups A1 and A2. The A1 subgroup contained seven lines *i.e*., Sultan Raya, RBJ-02017, KS-74, RBJ-2K022, RBJ-2K024, RBJ-97001 and RBJ-99026, whereas A2 included six accessions (RBJ-2K027, RBJ-96024, Khanpur Raya, RBJ-07010, RBJ-07017 and RL-18). Group B consisted of 11 constituents including two approved varieties Varuna and BARD-1, and nine advanced breeding lines KJ-119, KH-34, KJ-127, KJ-117, RBJ-02019, RBJ-03050, Raya63/2, Raya 49/2,

Table 4: Mean similarity of individual genotypes with rest of the accessions

Genotypes	Mean Similarity	Genotypes	Mean Similarity	Genotypes	Mean Similarity
Sultan Raya	0.86	RBJ-03050	0.84	RBJ-2K022	0.84
KH-33	0.82	RBJ-96026	0.81	J-90-43001	0.82
KH-119	0.85	RBJ-02018	0.85	RBJ-2K027	0.88
KH-34	0.84	Raya 63/2	0.84	RBJ-96024	0.89
Raya 49/2	0.82	RBJ-2K024	0.85	Khanpur Raya	0.93
RBJ-03047	0.82	RBJ-02017	0.88	RBJ-07010	0.91
Varuna	0.83	KS-74	0.85	RBJ-07017	0.95
KJ-127	0.84	RBJ-97001	0.84	RL-18	0.86
KJ-117	0.85	RBJ-99026	0.85		
RBJ-02019	0.85	KH-38	0.84		
BARD-1	0.85	Zem-II	0.82		

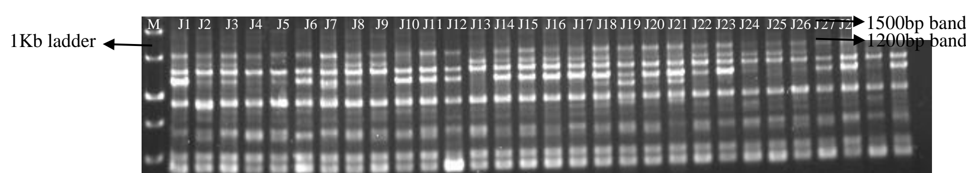

Fig. 1: PCR amplification profile of 30 *Brassica juncea* accessions using GL Decamer B-07 RAPD primer
M = marker (1Kb), J1= KH-33, J2 = KJ-119, J3 = KH-34, J4 = Raya-49-2, J5 = RBJ-03047, J6 = Varuna, J7 = KJ-127, J8 = KJ-117, J9 = RBJ-02019, J10 = BARD-1, J11 = RBJ-03050, J12 = RBJ-96026, J13 = RBJ-02018, J14 = Raya-63/2, J15 = RBJ-2K024, J16 = RBJ-02017, J17 = KS-74, J18 = RBJ-97001, J19 = RBJ-99026, J20 = KH-38, J21 = Zem-II, J22 = RBJ-2K022, J23 = J-90-43001, J24 = RBJ-2K027, J25 = RBJ-96024, J26 = Khanpur raya, J27 = RBJ-07010, J28 = RBJ-07017, J29 = RL-18, J30 = RBJ-07017

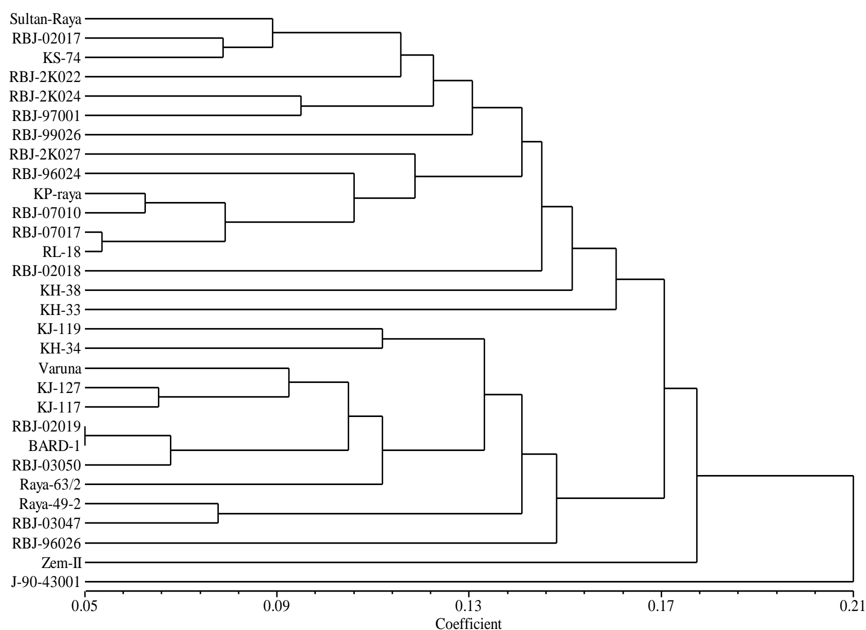

Fig. 2: Dendrogram generated by UPGMA cluster analysis of RAPD fragments based on similarity matrix representing genetic relationship among 30 *B. juncea* accessions

RBJ-03047 and RBJ-96026. Advanced breeding lines Raya 49/2 and RBJ-03047 in group B formed a separate sub cluster. As expected from the SM, most of the accessions were positioned close to each other by cluster analysis, depicting a higher level of relatedness. However, lines emerged from the crosses of varieties/lines of ORI, AARI, Faisalabad and allied research stations grouped together, while the accessions descendent from exotic and other varieties introduced from NARC were crowded together. KH-38 and KH-33 are heat tolerant lines from Khanpur research station, showed a distinct pattern in grouping. ZemII and J-90-43001 were distant from all the

accessions. Both these lines are low in erucic acid and glucosinolate concentration introduced from Australia and Canada, respectively did not crowd with any other cluster having accessions with higher erucic acid and glucosinolate contents.

Discussion

DNA markers are widely used to reveal DNA polymorphisms (Gupta *et al.*, 1999; Gupta and Varshney, 2000). Different molecular markers have been used to study the extent of genetic relatedness in different crops (Al-Qurainy, 2007; Cruz *et al*., 2007; Lopez *et al*., 2008; Ana *et al.*, 2009; Pezhmanmehr *et al.,* 2009) and differ mainly in their principles and amounts of data generated. Currently, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single nucleotide polymorphism (SNPs), cleaved amplified polymorphic sequences (CAPs) and simple sequence repeats (SSRs) are in use to determine the variability and diversity at molecular level (Palmobi and Damiano, 2002; Agarwal and Shrivastava, 2008; El-Mouei *et al*., 2011).

When compared to present results, more or less similar ranges of genetic dissimilarities in different *Brassica* lines have also been reported in previous studies (Ali *et al.*, 2007; Abbas *et al*., 2009). Qun *et al.* (2009) reported amplification of even shorter DNA fragments ranging from 150-1800bp in *B. campestris*, which might be due to difference in crop specie and primers used.

The results of the study under report indicated a low to moderate level of genetic diversity in the *B. juncea* germplasm. A number of studies have revealed lower levels of genetic diversity in major crop species including Brassica (Rabbani *et al.,* 1998), alfalfa (Flajoulot *et al.,* 2005), white clover (George *et al.,* 2006), rice (Thomson *et al.*, 2003), wheat (Wang *et al.,* 2007), groundnut (Tang *et al.,* 2007) and maize (Leegesse *et al.,* 2007; Yao *et al.,* 2007). A narrower range of genetic diversity has also been formerly documented (Yuan *et al.,* 2004; Astarini *et al.*, 2004) in *B. napus* and *B. oleracea.* Oilseed and vegetable genotypes belonging to these groups are under extensive selection for economic and quality traits. However, An *et al.* (1999) reported the existence of extensive genetic diversity in *B. juncea* germplasm collection. This difference might be due to the inclusion of landraces in the study, while approved varieties and better yielding selected lines were subjected to diversity analysis during present study.

In the present study, lower level of genetic diversity shows the narrow genetic background of the breeding material, which is mainly due to their continuous participation in canola breeding program. Selection for higher yielding lines with better quality traits along with adaptation to local conditions is a major input to diffident diversity. A close association between oilseed cultivars from Pakistan and Spain had been previously reported (Tekelwold and Becker, 2006) through RAPD markers. BARD-1 and Varuna varieties were intermingled with other lines belonging to different research stations in the cluster, demonstrating no relationship between the RAPD banding pattern and the geographic origin. Tekelwold and Becker (2006) reported the amalgamation of *B. carinata* accessions collected from different areas indicating a frail ecological differentiation and non-significant changes in RAPD bands incidence along the accessions grown in different areas. This could be ascribed to absence of gene flow barriers and presence of similar evolutionary factors in different mustard growing regions. Differences in RAPD banding pattern may not reveal the diversity in genes controlling the agronomically important traits (Williams *et al.*, 1993). Hence, RAPD markers not always differentiate accessions on the basis of area of adaptation. Similar results have been reported by various researchers in *B. juncea* (Rabbani *et al.*, 1998), Chinese vegetable Brassicas (Ren *et al.,* 1995), *B. napus* (Mailer *et al.*, 1994), *Ensete ventricosum* (Birmeta *et al.*, 2002), Sorghum bicolor (Ayana *et al.*, 2002). However, Shengwu *et al.* (2003) reported substantial genetic variation between Chinese and European accessions of rapeseed due to difference in gene pool.

It is concluded that an understanding of the presence of genetic variability in Raya (*B. juncea*) germplasm will be useful for efficient utilization of the existing genetic resources for the development of canola type varieties. Genetic diversity among the breeding germplasms of *B. juncea* could be enhanced by broadening the gene pool through international sources. RAPD being a simpler and faster technique could be used to compare the genetic relatedness and the patterns of variation among Raya (*B. juncea*) accessions.

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