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Original Research Article

Assessment of Genetic Variation and Relationship among *Brassica* Species by RAPD Marker

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A b s t r a c t

The present study was aimed at determining genetic diversity and relatedness among four *Brassica* cultivars belonging to three different species using Random Amplified Polymorphic DNA (RAPD) marker. A total of 43 reproducible DNA fragment were generated by six randomly selected primers. Out of the forty three fragments generated 38 were found to be polymorphic. Size of amplified fragments produced ranged from 250 to 2723 bp. Primers OPA11, OPB9, OPB3 showed highest 100% polymorphism, whereas OPA 1 showed minimum of 50% polymorphism. Jaccard's pairwise genetic similarity was calculated, highest value of 0.96 and minimum 0.69 was obtained between two varieties Heera and Varuna of *B. juncea* and between *B. nigra* and variety Varuna, each respectively. A dendrogram was constructed using unweighted pair group method of arithmetic mean (UPGMA). Cluster analysis grouped the four varieties into two groups. One group consists of two cultivars of *B. juncea* (i.e. Heera and Varuna) along with *Brassica rapa* var. pusa kalyani and *Brassica nigra* var. IC257 was present in second group distantly related to the first group.

K e y w o r d s

Brassica cultivars

Genetic similarity UPGMA

Polymorphism

RAPD markers

Introduction

Crops of the genus *Brassica* belongs to the family Brassicaceae. The family comprises of more than 300 genera and 3500 species across the world. Most of the species belonging to genera *Brassica* belong to economically important crop plants and are consumed as oilseed, vegetable or as condiments, cattle feed, fertilizer and for medicinal purposes worldwide. Out of the hugely diversified genus *Brassica* some species like *Brassica nigra*, *Brassica carinata* and *Brassica juncea* are commonly known as mustard while *Brassica campestris* and *Brassica napus* are called rapeseed (Yarnell, 1956). *Brassica oleracea* is known as cole crop which includes vegetables and oil seed crop plants. Thus in broad terms the genus *Brassica*

comprised of two major groups; Vegetable crops and Oil yielding crops. India is one of the top most oilseed producing countries of the world. Out of the several oil seed crops cultivated in India, rapeseed mustard contributes 28.6% of the India's total oilseed production and ranks second after groundnut having an appreciable share of 27.8% of the India's oilseed economy. In India maximum production is accounted from Rajasthan, Uttar Pradesh, Haryana, Madhya Pradesh Gujarat and some south Indian states such as Karnataka, Tamil Nadu, and Andhra Pradesh (Shekhawat et al.,2012).

In lieu of the huge diversity and immense economic importance of *Brassica*, lot of work for understanding its biology status in India and other countries of the World. In order to cope with the high demands, crop improvement using biotechnological tools is essential. Availability of a wider range of genetic resources *vis-a-vis* assessment of genetic diversity or genetic relatedness at species, subspecies, cultivar or varietal level is a pre-requisite for crop improvement (Abbas et al., 2009). Several conventional methods like collection and articulation of germplasm accessions, development of breeding lines and segregating populations based upon pedigree data, morphological data, agronomic performance, cytological and biochemical data are used for analyzing genetic diversity. However these markers are labour intensive and time consuming moreover they are not suitable for analyzing big sample size or populations. With the advent of molecular tools, DNA based molecular markers are being extensively used now a days due to their precision and reproducibility and more importantly they are not influenced by environmental factors (Mohammadi and Prasanna, 2003). Diversity analysis has been studied using various molecular tools where large numbers of highly informative DNA markers have been developed for identification of genetic polymorphism. These molecular marker systems have been broadly classified as Polymerase chain reaction (PCR) technique based or hybridization marker systems (Ali et al., 2007). A lot of studies have been done on unraveling the genetic relationships of *Brassica* species by using different molecular markers such as RAPD (Random amplified polymorphic DNA) (Wang et al.,2002) , RFLP (Restriction fragment length polymorphism) (Ferreira et al.,1994), AFLP (Amplified fragment length polymorphism) (Liu and Meng, 2006), SSR (Simple sequence repeats) (Ramchiary et al.,2011), SNP (Single nucleotide polymorphism) (Hayward et al.,2012), sequence characterized amplified region (SCAR) (Iniguez-Luy et al.,2008), allele specific associated primers (ASAP), single primer amplification reaction (SPARs), SSR - anchored PCR (Charters et al.,1996), cleaved amplified polymorphic Sequences (CAPs) (Park et al.,2008), microsatellitic repeat polymorphisms allele specific PCR (Slankster et al.,2012), allele specific ligation (Tanhuanpaa et al.,1998), single strand conformational polymorphism and DNA amplification fingerprinting (DAF) (Niu et al., 2009) etc. based marker systems (Ren et al., 1995; Yousuf et al., 2013). All these marker systems are based on different principles and correspondingly generate highly variable data. In the last few decades, the random amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction (PCR) has been one of the most commonly used molecular marker. RAPD markers have been reported as a powerful technique for the estimation of genetic relatedness between genotypes and identification of species or strains (Welsh and McClelland, 1990; Williams et al., 1993; Thormann et al., 1994; Yousuf et al., 2013). RAPD markers are amplification products of unknown DNA sequences using short and arbitrary oligonucleotide primers, and hence do not require prior information of a DNA sequence for primer designing. Large number of DNA markers can be developed in a short time span for analyzing large germplasm size, requiring less sophisticated equipment and methodology. All these qualities have made RAPD technique the most extensively utilized molecular marker for germplasm evaluation.

Taking this in consideration, four genotypes of *Brassica* belonging to three species of genus *Brassica* viz. *B. rapa*, *B. nigra* and two cultivars-Varuna and Heera of *B. juncea* are analysed for genetic diversity among them using six primer combinations. *Brassica* crops studied basically belong to three primary species *viz.*, *B. rapa* (Turnip, Chinese cabbage, n = 10), *B. nigra* (Black mustard) (Koch, n = 8) and one amphidiploids, *B. juncea* (Indian mustard) (n = 2x = 18) (Ren et al., 1995). The first two species are closely related distinct species however the later is an amphidiploids arose from crossing and paleopolyploidization among the primary species (*B. rapa* and *B. nigra*) which has led to morphological and botanical variability in the many subspecies and cultivar groups of *B. juncea* (Li, 1981; Lee, 1982; Opena et al., 1988; Turi et al., 2012). An attempt in

analyzing the genetic diversity among these genotypes using genetic molecular markers will be fruitful for future breeding programmes in *Brassica*.

Materials and methods

Plant material

Fresh and young leaf samples from 12-15 days old seedlings for four *Brassica* cultivars (Table 1) were procured from Department of Botany, University of Delhi.

Table 1. List of *Brassica* genotypes analyzed in present study.

S. No.	Taxa
A	*Brassica rapa* var. pusa kalyani
B	*Brassica nigra* var. IC257
C	*Brassica juncea* cultivar Varuna
D	*Brassica juncea* cultivar Heera

Primer selection

Twenty decamer arbitrary primers (Operon Technologies Inc., California, USA) were initially screened for two randomly chosen genotypes to test their suitability for amplification of DNA sequences (Table 2). Primers were selected on the basis of clarity, easily detectable bands, consistency, less background smear, able to generate polymorphic loci. Finally six primers exhibiting a good quality and polymorphic banding patterns were selected for further analysis (Table 3).

Extraction of total genomic DNA

Genomic DNA was isolated using the CTAB extraction procedure of Saghai-Maroof et al. (1984) with minor modifications. Five grams of young leaves frozen in liquid nitrogen were ground to a fine powder using mortar and pestle and transferred to 50 ml centrifuge tube containing 20 ml pre-heated (60°C) DNA extraction buffer. The samples were incubated at 60°C for 1h with intermittent mixing by gentle shaking. Twenty milliliter of chloroform:iso-amyl alcohol (24:1, v/v) was added and all the tubes were gently mixed for 5 min. The mixture was then centrifuged at 17,000 rpm for 20 min at room temperature. The aqueous phase was transferred to a fresh centrifuge tube, mixed with equal volume of isopropanol an tubes were kept at –20°C for 20 min to precipitate the DNA.

Table 2. List of decamer random primers initially screened.

Primer name	Primer sequence	GC content %	Reference
OPA 01	CAGGCCCTTC	70	Jain et al. (1994) and Cartea et al. (2005)
OPA 02	AGGGGTCTTG	60	Jain et al. (1994) and Cartea et al. (2005)
OPA 11	CAATCGCCGT	60	Jain et al. (1994) and Cartea et al. (2005)
OPB 03	CATCCCCCTG	70	Cartea et al. (2005)
OPB 05	TGCGCCCTTC	70	Cartea et al. (2005)
OPB 09	TGGGGGACTC	70	Cartea et al. (2005)
OPA 03	AGTCAGCCAC	60	Jain et al. (1994) and Cartea et al. (2005)
OPA 04	AATCGGGCTG	60	Jain et al. (1994) and Cartea et al. (2005)
OPA 15	TTCCGAACCC	60	Jain et al. (1994) and Cartea et al. (2005)
OPA 18	AGGTGACCGT	60	Jain et al. (1994) and Cartea et al. (2005)
OPL 14	GTGACAGGCT	60	Jain et al. (1994) and Cartea et al. (2005)
OPL 07	AGGCGGGAAC	70	Cartea et al. (2005)
OPK 10	GTGCAACGTG	60	Cartea et al. (2005)
OPD 02	GGACCCAACC	70	Cartea et al. (2005)
OPC 09	GATGACCGCC	70	Jain et al. (1994) and Cartea et al. (2005)
OPC 05	GATGACCGCC	70	Cartea et al. (2005)
OPB 10	CTGCTGGGAC	70	Cartea et al. (2005)
OPB 04	GGACTGGAGT	60	Cartea et al. (2005)
OPA 07	GAAACGGGTG	60	Jain et al. (1994) and Cartea et al. (2005)
OPB 09	TGGGGGACTC	70	Cartea et al. (2005)

The DNA was spooled out, washed with 70% ethanol and dried under vacuum. The DNA was finally suspended in 2 ml of TE (10 mM Tris-HCl and 1 mM EDTA) buffer. The isolated DNA was further purified to remove the RNA, protein, polysaccharides and phenols. Two micro-liter RNase (10mg/ml) was added to each tube and incubated for 1h at 37°C. Further, 4µl proteinase K (10mg/ml) was added and incubated for 1h at 37°C. An equal volume of phenol: chloroform: iso-amyl alcohol (25:24:1) was added and gently mixed end-to-end for 10 min. The mixture was centrifuged for 5 min and supernatant was transferred to fresh tubes. The above phenol: chloroform extraction was repeated three times. The supernatant was added with 1/10 volume of 3M sodium acetate and 2.5 times of the total volume of chilled ethanol to precipitate the DNA. The precipitated DNA was spooled out, washed twice with 70% ethanol and dried under vacuum. The purified DNA was dissolved in minimum volume of TE buffer at room temperature and stored at -20°C for further use.

PCR amplification

The RAPD reaction conditions were optimized by varying the concentrations of the template DNA initially (5, 15, 25, 35, 45, 55, 60 ng), Taq DNA polymerase (0.5U, 1U, 1.5U) and Mg++ salt (0, 0.5, 1, 1.5, 2, 2.5 mM). The final 25 µl reaction mix contained 200 µM each of dATP, dCTP, dGTP and dTTP; 3.0 mM of MgCl2; 1 Unit of Taq DNA polymerase (Bangalore Genei, Bangalore); 10 ng of genomic DNA; 10 mM of PCR buffer and 0.4 µM of 10mer random primer (Table 2). Amplification was carried out in a thermocycler using the following PCR conditions. Initial denaturation at 94°C for 4min, Denaturation at 94°C for 1min, Annealing at 35°C for 1min, Extension at 72°C for 2min, followed by final extension at 72°C for 5min. The cycle was repeated for 40 cycles.

Agarose gel electrophoresis

PCR products, thus obtained were mixed with 2.5 µl of 10X loading dye. The amplification products were resolved on 1.5 % agarose gel electrophoresed at 120 V for about 1 h in 1X TAE buffer. Molecular sizes of amplified products were estimated using a known molecular weight marker DNA (2 Log DNA ladder) (Fig. 1). DNA fragments were visualized by staining with ethidium bromide and photographed using UV gel documentation system.

Fig.1: RAPD profiles of four *Brassica* varieties (Lane 1-4; *Brassica rapa* var. pusa kalyani, *Brassica nigra* var. IC257, *Brassica juncea* cultivar Varuna, *Brassica juncea* cultivar Heera) using primer I(OPA1), primer II (OPA2) and primerIII (OPA 11), M: Molecular weight marker (2 log DNA ladder).

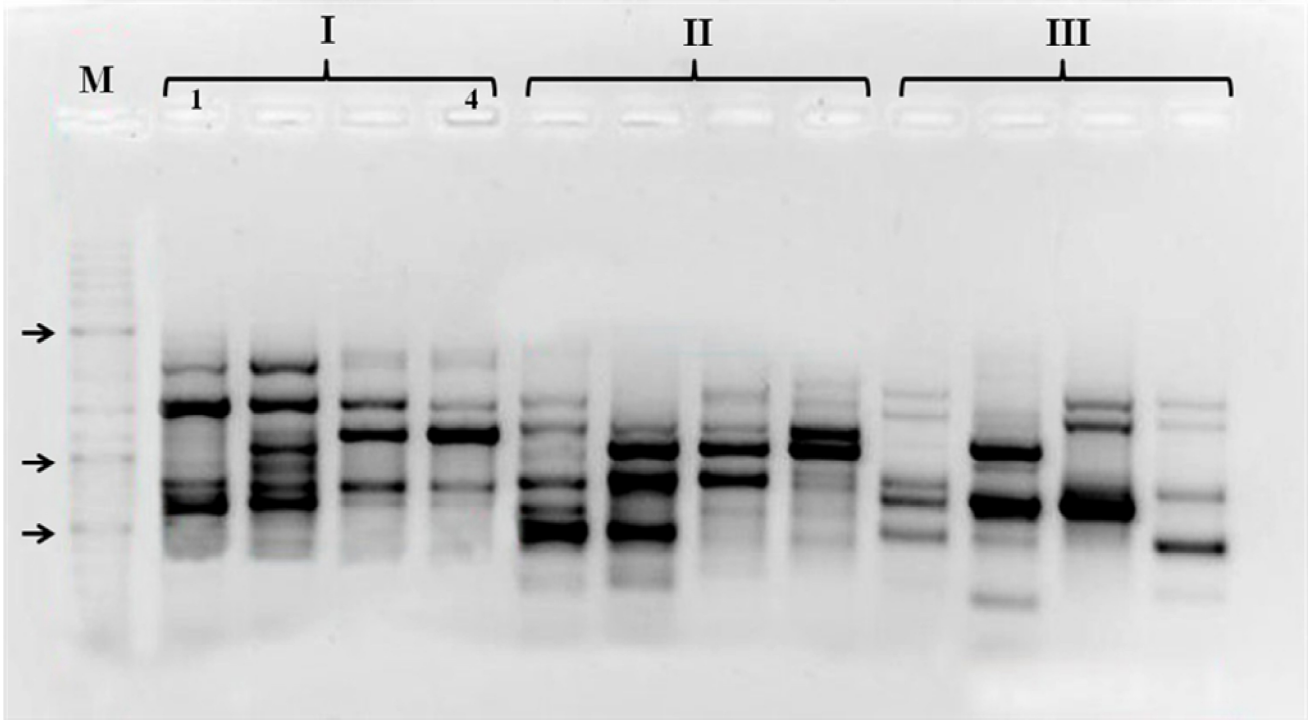


Table 3. Polymorphism obtained with six random primers for *Brassica* cultivars.

Primer	Sequencce (5'-3')	Size range (bp)	Total no. of bands scored	No. of polymorphic bands	% polymorphism
OPA 01	CAGGCCCTTC	2720-400 bp	6	3	50
OPA 02	AGGGGTCTTG	1240-450 bp	8	7	87.5
OPA 11	CAATCGCCGT	1257-250 bp	9	9	100
OPB 03	CATCCCCCTG	2100-550 bp	5	5	100
OPB 05	TGCGCCCTTC	2500-500 bp	7	6	85.7
OPB 09	TGGGGGACTC	1500-750 bp	8	8	100

Data analysis

The bands were scored from the gel profile. For each accession, a binary matrix reflecting presence (1) or absence (0) for all RAPD band which were generated. Using Jaccards similarity coefficient, genetic similarities were calculated for all accession and a similarity matrix was constructed (Table 4). Based on genetic similarity values, cultivars were clustered using UPGMA (Unweighted Pair Group Method of Arithmetic Mean) and dendrogram was constructed using NTSYS-PC- Version 2.0 software package (Rholf, 1993).

Results and discussion

Twenty primers were initially screened for their ability to produce reproducible polymorphic patterns and only six were finally selected (Table 2). A total of 43 DNA fragments were generated by selected primers out of which 37 were found to be polymorphic giving 86% polymorphism which is much closer to polymorphism reported in Chinese mustard (Fu et al., 2006) and *B. napus* germplasm (Cartea et al., 2005). Out of six primers used in present study, three (OPA 11, OPB 3 and OPB 9) generated 100% polymorphism discriminating all four genotypes (Table 3) (Fig. 1). In RAPD profiles, absence of bands at a particular locus may be a result of failure of primer to anneal at that site due to variation in nucleotide sequence or because of insertion / deletions in the primer binding site (Clark and Lanigan, 1993). A appreciably high polymorphism obtained in present study can be attributed to high (60-70%) GC content. Increase in GC content of the primer is directly correlated with increase in number of bands (Fukuoka et al. 1992) because G pairs with C with triple bonds thereby increasing stability of base complementation.

Relationship between species and varieties were determined by Jacarard's similarity coefficient. The value for intra- variety similarity indices between Heera and Varuna was higher (0.96) than intervarietal indices (0.70) between *B. nigra* and *B. juncea* (Table 4).

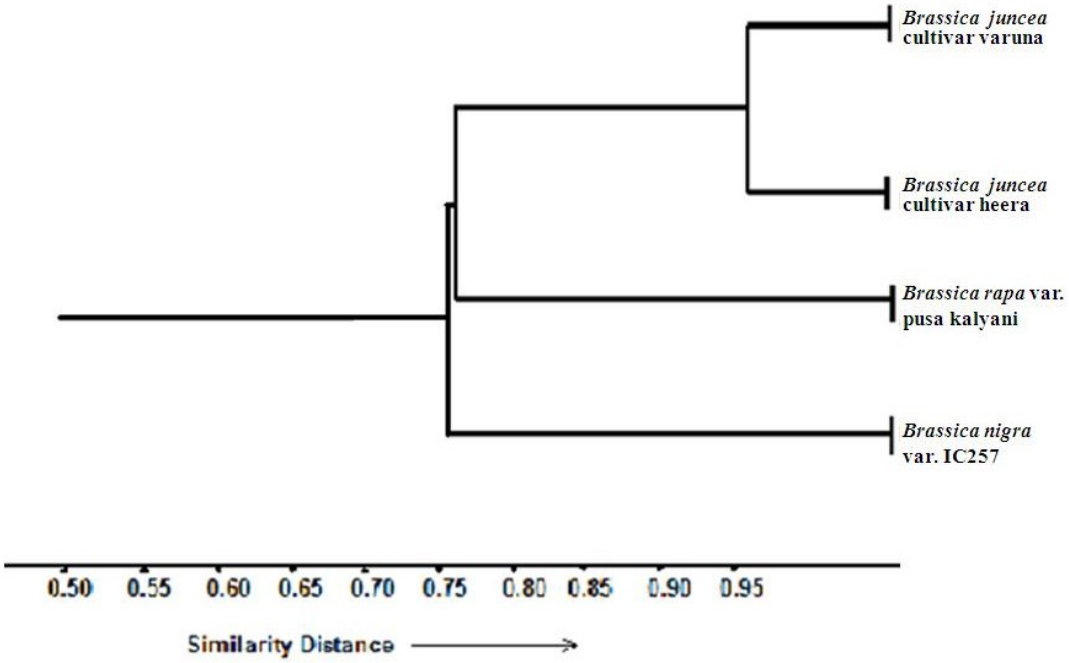
Table 4. Jaccards pairwise genetic similarity matrix.

	Brassica rapa var. pusa kalyani (A)	*Brassica nigra* var. IC257 (B)	*Brassica juncea* cultivar Varuna (C)	*Brassica juncea* cultivar Heera (D)
Brassica rapa var. pusa kalyani (A)	1	0.93	0.76	0.81
Brassica nigra var. IC257 (B)		1	0.69	0.71
Brassica juncea cultivar Varuna (C)			1	0.96
Brassica juncea cultivar Heera (D)				1
Calculation for similarity/ linkage: A & B = 25/14+6+7 = 25/27 = 0.93 A & D = 25/14+11+6 = 25/31 = 0.81 B & D = 25/10+15+10 = 25/35 = 0.71			A & C = 25/13+11+9 = 25/33 = 0.76 B & C = 25/13+13+10 = 25/36 = 0.69 C & D = 25/16+6+4 = 25/26 = 0.96	

Based on Jaccard's genetic similarity distance a dendrogram was prepared using UPGMA where four genotypes were segregated into two groups (Fig.2). The major group could be divided into two subgroups in which first subgroup included two cultivar Varuna and Heera of *B. juncea* and second subgroup included

B. rapa. The other group included *Brassica nigra* which was distantly related to first group. Grouping of *B. rapa* along with *B.juncea* in one group is also supported by Saha et al. (2008). This clustering of different species in one group may be because of extensive introgressive hybridization between *B. juncea* and *B. rapa*. Both the varieties of *B. juncea* showed very high genetic similarity of 0.96 and clustered together in one group since varieties have close proximity in their origin, breeding stategy and morphological traits so they tend to show less genetic distance from each other. Hence, these varieties could be exploited for selecting parents in future breeding programmes for improvement of *Brassica* varieties. Further intensive study involving more newly released varieties or lines and RAPD primers is needed to support present findings.

Fig. 2: UPGMA dendrogram based on Jaccard similarity coefficient, summarizing the data obtained for four *Brassica* varieties based on RAPD analysis.



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