Research Article

Assessment of Genetic Diversity among Various Genotypes of *Brassisa Napus* L. using Molecular Markers

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Abstract

Nine reproducible RAPD's were used to amplify 22 genotypes of *Brassica* species. Out of which 18 genotypes belong to *Brassica napus* L. and four genotypes of different *Brassica* species were used to check the authenticity of work. It was observed that the PCR amplification profiles of other *Brassica* species were totally different from *Brassica napus* L. genotypes. Polymorphic information content of each primer was calculated and ranged from 0.17(primer OPA-09) to 0.27 (primer OPD-18) with an average of 0.23.The percentage polymorphism was evaluated and ranged from 72.7 to 100%. With an average of 88.60% per primer. Out of 9 primers used we recognized primer OPD-18 as the best primer in studying genetic diversity. Molecular similarity matrix showed that CNH-11-2, CNH-11-13, HNS1001 and NUDB-26 had the highest similarity. To exploit heterosis, the hybridization programme postulated on the basis of genetic divergence may be successful between the genotypes viz, AKGS-3 and CNH-11-7 or EC55208 and CNH-11-7.

Keywords: *Brassica napus*, RAPD markers, Polymorphism, Genetic diversity

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Introduction

Brassica napus is a young species that originated by mean of interspecific hybridization between *B. rapa* L. and *B. oleracea* L. Now a day due to its intensive cultivation for seed and oil quality traits, it represents one of the most important sources of vegetable oil world wide. Because of intensive breeding processes Brassica napus L. has a relatively narrow genetic diversity in current germplasm (Rubby Sandhu *et al.* 2017). Research on *Brassica* germplasm and evaluation of its genetic diversity could accelerate the efficient use of genetic variation through establishing a breeding programme (Harper *et al*. 2012). For developing promising varieties through hybridization a careful choice of parents and breeding methodology are matters of great concern to a plant breeder (S.K.Rai *et al.* 2017). Heterosis in hybrids is based on genetic completion between divergent parents, so the information on genetic diversity could help breeders better understand the genetic structure of germplasm and to predict which cross combinations would produce good F1 hybrids (Yu *et al.* 2007).

There are various techniques available for evaluation of crop genetic variability, such as morphological, biochemical and molecular markers. Molecular (DNA) markers have many advantages over other techniques (independent of environment and plant growth stage, unlimited number, etc.) and they have been increasingly employed for analysis of genetic diversity (Nyende, 2008). A variety of molecular markers including restriction fragment length polymorphism (RFLP), simple sequence repeats (SSR), amplified fragment length polymorphism (AFLP) and random amplified polymorphic RAPD analysis has been widely used on important crop species in the genus Brassica (Shengwu *et al.*, 2003). The use of RAPD technique for the study of genetic variation has been demonstrated as for determining the genetic relationships between different related species (Thormann *et al.*, 1994), In this study, an initiative was taken to assess the genetic diversity and the relationship among *Brassica napus* L. genotypes cultivated in India using RAPD markers.

Materials and Methods

Plant material

The seed material of 18 genotypes of *Brassica napus* and 4 different *Brassica* species was procured from different institutes of India including SKUAST-Jammu. Genotypes with their pedigree used for diversity analysis are shown in **Table 1**.

Table 1 Genotypes with their pedigree used for diversity analysis

S. No	Genotypes	Species	Pedigree	Source
1	CNH-11-7	*Brassica napus*	OCN8NA X China 1006BCR	PAU Ludhiana
2	HNS0901	*Brassica napus*	Selection from exotic selection	CCS HAU, Hisar
3	CNH-11-1	*Brassica napus*	Ag Outback NA X China 6 1006 NAR	PAU Ludhiana
4	CNH-11-13	*Brassica napus*	RT108NA X China 1006BCR	PAU Ludhiana
5	HNS1001	*Brassica napus*	HNS0004 X EC552585	CCS HAU, Hisar
6	GSL-1	*Brassica napus*	Selection from farmer's field	PAU Ludhiana
7	GSC-101	*Brassica napus*	Rivette X RR001	PAU Ludhiana
8	CNH-11-2	*Brassica napus*	ECN 3 NA X China 6- 1006 NAR	PAU Ludhiana
9	GSC-6	*Brassica napus*	-	PAU Ludhiana
10	NUDB2611QC	*Brassica napus*	-	Faizabad.
11	EC552608	*Brassica napus*	An exotic line of Gobhisarson	CSKHPKV
12	RSPN-29	*Brassica napus*	DGS-1 X GSL 1	SKUAST-J
13	RSPN-25	*Brassica napus*	B. napus x B. hirta	SKUAST-J
14	AKGS-3	*Brassica napus*	HPN-1-36-16-9	CSKHPKV
15	DGS-1	*Brassica napus*	Selection from exotic collection	SKUAST-J
16	RSPN-28	*Brassica napus*	DGS-1 X RSPN 25	SKUAST-J
17	CNH-55	*Brassica napus*	BCN61 X China 6A.	PAU Ludhiana
18	CNH-13-1	*Brassica napus*	BCN3575NA X China 6-1006-2	PAU Ludhiana.
19	PusaTarak	*Brassica juncea*	SEJ-8 X PusaJagannath	IARI New Delhi
20	PTC-2009-3	*Brassica campestris*	Composite(IGT-1+TS-29+TS-36+TS-38+TS-46+TS-50+Bhawani)	GB PUA & T, Pantnagar.
21	RSPT-2	*Brassica campestris*	Mass selection from local germplasm	SKUAST-J
22	Varuna	*Brassica juncea*	Selection from Varanasi Local 786,02.021976	Kanpur

Genomic DNA Isolation

The genomic DNA isolation for each genotype was carried out by Doyle and Doyle, (1990) method, with slight modifications. About 7-8cm young and actively growing fresh leaves were harvested from the field for genomic DNA extraction.

DNA Quantification

Quality and quantity of genomic DNA was estimated by using Agarose gel electrophoresis and UV Spectrophotometric method.

Agarose gel electrophoresis

The concentration of DNA was determined by comparing its intensity of bands with that of 100 bp molecular ladder quality was indicated by having intact band.

UV Spectrophotometric method

Optical density of DNA samples was measured at 260nm and 280nm using spectrophotometer (Peq-Lab Nanodrop).

(a) Estimation of DNA concentration in different samples was done as follows:

$$\text{Concentration of DNA } (\mu g/ml) = OD_{260} \times 50 \times \text{Dilution factor}$$

(b) Quality analysis was done by calculating the ratio of absorption maxima at 260nm and 280nm.

Ratio between 1.8 and 2.0 shows the presence of fairly pure DNA. The value less than 1.8 indicates the presence of protein contaminants and greater value than 1.8 indicates the presence of RNA. DNA samples were diluted using sterilized Milli Q water, to have final concentration of 25ng/µl.

A set of 9 arbitrary random 10-mer primers were selected for use in amplification of genomic DNA shown in the **Table 2**. These were got synthesized from IDT (Integrated DNA Technologies, USA).

Table 2 Arbitrary 10 mer primers with their sequence selected for DNA amplification

primer	sequence	Amount oligo n moles)	Water added (µl)	Primer (µl)	Water (µl)
OPA-02	5'-TGC CGA GCT G-3'	97.1	971	50	950
OPA-03	5'-AGT CAG CCA C-3'	60.7	607	50	950
OPA-07	5'-GAA ACG GGT G-3'	85.8	858	50	950
OPA-09	5'-GGG TAA CGC C-3'	75.4	754	50	950
OPA-11	5'-CAA TCG CCG T-3'	82.3	823	50	950
OPD-18	5'-GAG AGC CAA C-3'	70.5	705	50	950
OPE-01	5'-CCC AAG GTC C-3'	81.1	811	50	950
OPE-02	5'-GGT GCG GGA A-3'	84.5	845	50	950
OPE-03	5'-CCA GAT GCA C-3'	77.2	772	50	950

Components used for PCR Reaction

Reaction mixture contained 2.5 µl of DNA(25ng/µl), 2.5 µl of 10X PCR Buffer,$Mgcl_2$ (2mM), 0.2 mM of each dNTPs (dTTPs, dGTPs, dCTPs, dATPs), primer (1.67Mm) concentration, 5 units Taq DNA polymerase.

PCR Amplification Program

PCR tubes containing master mix and DNA template were thoroughly mixed and subjected to the thermal profile. The amplification reaction was carried out in a gradient mastercycler. An initial denaturation step of 4 minutes was programmed in the thermocycler, followed by a loop of 35 cycles each consisting of denaturation (at 94^0C for 1 minute), annealing (at 36^0C for 1 minute) and extention (at 72^0C for 2 minutes). The final extension was performed at 72^0C for 10 minutes. The PCR products were then stored at 4^0C.

RAPD-PCR banding Profile

The amplification products were then subjected to electrophoretic separation using horizontal agarose gel electrophoresis. 1.5 % agarose gel was prepared in 1X TBE buffer stained with ethidium bromide. In each PCR tube, 7 µl of loading dye was added and then loaded to separate wells. 100bp DNA ladder was used as a molecular weight marker for determining the molecular weights of RAPD based PCR bands. Electrophoresis was carried out at 60 V for 3 hours. The gel was visually examined under UV and documented using gel documentation system.

Scoring of Bands

RAPD-PCR bands were detected in the gel using gel documentation system. Bands were seen for their presence (1) or absence (0). All the scorable bands were considered as single locus/allele. Their sizes were estimated using 100bp standard marker. The presence and absence of bands in all genotypes for 9 primers were used to generate Bi-nomial data using excel sheet. Bands were marked as present only if the DNA amplification produced the fragment of a particular sequence and absent if the DNA amplification lacked that fragment. The banding patterns of all genotypes against each primer were compared. Bands present in one genotype and absent in another genotype, were regarded as variable and used to score for polymorphism. In order to check the informativeness and discriminatory power of RAPD primers utilized in this study, certain parameters like polymorphism percentage, polymorphic information content, were calculated.

(a) Polymorphism (%)

It was calculated by dividing the polymorphic bands by the total number of scored bands:

$$\frac{\text{Number of Polymorphic Bands}}{\text{Total Number of Bands}} \times 100$$

(b) Polymorphism Information Content (PIC)

Bi-nomial data matrix of all genotypes generated from 9 primers was subjected to the UPGMA analysis and a dendogram was constructed using NT SYS software. Genotypes were divided in various clusters, sub-cluster and sub-sub clusters based on genetic diversity among them and linkage distance was calculated.

Results and Discussion

Genomic DNA isolation and quantification of all genotypes of *Brassica napus* and other species was done and subjected to PCR amplification using 9 reproducible RAPD markers. Other *Brassica* species were exploited to check the reliability of the markers being used. The amplified bands were scored and dendogram was constructed to evaluate genetic diversity.

Data Analysis

A total of 104 fragments were produced with an average of 11.6 per primer. Out of these only 94 polymorphic bands were obtained with an average of 10.22 bands per primer. One unique band each from OPA-09, OPD-18 and OPE-02 was obtained. The PIC values for each primer was calculated and ranged from 0.17(primer OPA-09) to 0.27 (primer OPD-18) with an average of 0.23.The percentage polymorphism was evaluated by the formula as discussed and ranged from 72.7 to 100% with an average of 88.60% per primer as shown in the **Table 3**.

Table 3 Monomorphic, polymorphic and unique bands and calculated parameters for the nine primers used

S.No.	Primer	NB	NPB	NMB	NUB	PPB	PIC
1	OPA-02	8	7	1	0	87.5	0.18
2	OPA-03	10	9	1	0	90	0.26
3	OPA-07	14	12	2	0	85.7	0.24
4	OPA-09	11	8	3	1	72.7	0.17
5	OPA-11	13	12	1	0	92.3	0.24
6	OPD-18	17	17	0	1	100	0.27
7	OPE-03	12	9	3	0	75.0	0.25
8	OPE-02	9	9	0	1	100	0.21
9	OPE-01	10	9	1	0	90.0	0.25
Avg.		11.6	10.22	1.33	0.33	88.60	0.23

Where, NB = Total no. of Bands ; NPB = No. of Polymorphic Bands
NMB = No. of Monomorphic Bands; NUB = No. of Unique Bands
PPB = %age of Polymorphic bands; PIC= Polymorphic Information Content

Dendogram Analysis

Dendogram was constructed based on UPGMA using NT SYS software and all the genotypes were clustered in two major groups based on the genetic variation present in them. The cluster I as represented in **Figure 2** includes four genotypes which are grouped in two sub-clusters. The second major cluster is grouped in 2 sub-clusters which are further divided in many sub-sub clusters. The second major cluster contains 18 genotypes which are grouped on the basis Jaccard's similarity coefficients. The genotypes present in the second sub-sub clusters 2a viz. AKGS-3 and EC55208 are more diverse from the genotypes present in the second sub-sub clusters 2b and 2c. So, these genotypes can be used in making hybrids with the genotypes present in the sub-sub clusters 2b and 2c.To exploit heterosis, the hybridization programme postulated on the basis of genetic divergence may be successful between the genotypes viz, AKGS-3 and CNH-11-7 or EC55208 and CNH-11-7.

Polymorphism among rapeseed genotypes using RAPD markers

Nine reproducible 10-mer arbitrary oligonucleotide primers were employed to generate polymorphic products of 22 samples. The selected primers varied greatly in their ability to resolve variability among the genotypes. Primers with higher polymorphic bands OPD-18, OPA-03 were more efficient to discriminate the varieties. Percentage of polymorphism ranged from 72.7% to 100% with an average of 88.60% per primer. Higher level of genetic polymorphism points towards the power of selected primers for the identification of individual genotypes. Abdelmigid *et al*., (2011) reported a percentage of polymorphism to be 87% with 13.4 polymorphic fragments in *Brassica napus* comparable to that of 88.60 polymorphism percentage and 10.22 bands per primer reported in this study while 97.66% polymorphism was reported for 16 primers by Ahmad *et al*. (2009). In other studies, percentage of polymorphic primers in mustard reported by Ali *et al*. (2007) was in the range of 21.54 to 59.36%; exactly similar polymorphism percentage i.e. 21.54 to 59.36% was found by Khan *et al*., (2011). Low level of polymorphism could be attributed to the utilization of only four primers in both cases. Asghari *et al*., (2011) reported among the fifty

RAPD primers used, nine primers produced a 102 polymorph and high resolution bands, in total. Banding average for each primer was 11.3 bands. Comparable to that of 94 polymorph and 10.22 bands per primer reported in this study.

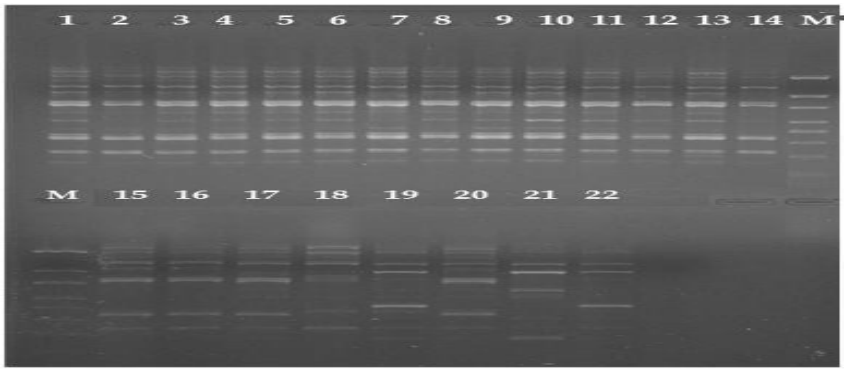

Figure 1 DNA bands amplified from leaves of some understudied genotypes using RAPD marker and electrophoreses in a 1.5% agarose gel

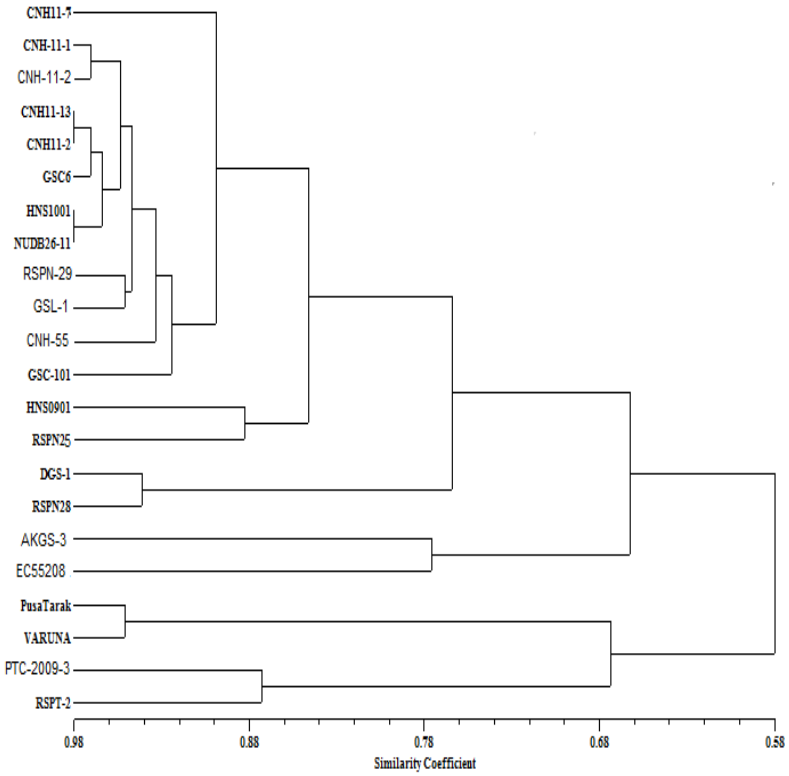

Figure 2 Dendogram illustrating genetic relationship among 22 genotypes generated by UPGMA analysis of RAPD bands based on genetic distance obtained with 9 random primers

Genetic Divergence Analysis

Genetic diversity is known by observing polymorphism among the genotypes revealed by RAPD profiles. More the polymorphic bands among genotypes more will be the expected diversity. In the present study, UPGMA dendogram were constructed based on RAPD analysis. This dendogram made it possible to visualize the diversity among genotypes. It was observed that RAPD markers were able to discriminate all the genotypes effectively. All the genotypes of *Brassica napus* were grouped in one major cluster and genotypes of other *Brassica* species were grouped in different cluster. Cluster I is comprised of four genotypes belonging to other species chosen for authenticity of work. Two *Brassica juncea* varieties i.e., Varuna and Pusa tarak are closely related in the sub cluster, while PTC-2009-3 and RSPT-2 are similar to each other and are relatively different from other two genotypes. The second major cluster is grouped in 2 sub-clusters which are further divided in many sub-sub clusters. The second major cluster contains 18 genotypes. Thus, RAPD markers proved to be very informative in the assessment of genetic variation on the basis of higher polymorphism percentage (88.60%). The analysis with RAPD markers disclosed wide

variation within rapeseed that reflected a high level of diversity within these species. It was retrieved suitable for use with *Brassica* species because of its ability to generate reproducible polymorphic bands. Some studies have indicated the availability of random amplified polymorphic DNA markers throughout the genome and its association with functionally important loci (Penner, 1996). These findings further strengthened previous reports that the RAPD markers can be used effectively to estimate genetic differences among genotypes.

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