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Assessment of Genetic Diversity in Four *Brassica* Species using Randomly Amplified Polymorphic DNA Markers

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Abstract

This investigation was aimed to explore the genetic diversity and relationship among the 30 *Brassica* varieties of four different species such as *Brassica juncea, Brassica rapa, Brassica napus* and *Brassica carinata* using RAPD markers. Forty primers were tested in this study, out of which 33 revealed clear and repeatable RAPD polymorphisms. They produced 530 total loci and the total bands were 6041. Out of the 530 loci produced, 511 were polymorphic and hence the total polymorphism percentage was found to be 95.96 %. Nineteen primers out of 33 analyzed successfully produced 100% polymorphism. The average PIC value for RAPD was 0.92. The highest PIC value obtained was 0.94 for S-70 marker and lowest PIC value was 0.77 for S-40. The number of amplicons produced per primer varied from 9 to 26 with a mean of 16.56 bands per primer. The molecular size of the amplified PCR products ranged from 130 to 3100 bp. On the basis of dendrogram, thirty accessions were categorized mostly according to their species. Cultivar specific RAPD markers for fifteen accessions were also detected. The study revealed that the diversity exhibited by genotypes belonging to various *Brassica* species can be further utilized for crop improvement programme.

Highlights:

- A total of thirty accessions used for diversity analysis in *Brassica* species were obtained from DRMS, Bharatpur, Rajashan.
- 19 primers out of 33 analyzed successfully produced 100% polymorphism.
- The total polymorphism percentage and PIC were found to be 95.96 % and 0.92, respectively.
- Highest genetic similarity was observed between those genotypes which were belong to the same species and least genetic similarity when they belong to different species.
- Cophenetic Correlation between the similarity matrix and dendrogram pattern was found to be 0.94 which is good to fit.

Keywords: *Brassica* species, RAPD, genetic diversity, PIC, PCA, genetic relationships, polymorphism

The Brassicaceae family comprising of approximately 3,700 species in 338 genera with diverse characteristics, includes many economically important edible and industrial oilseed, vegetable, condiments and fodder crop species (Beilstein*et el al.* 2006). *Brassicas* are the third most important oilseed crop in the world after soybean and palm. China, India, Canada, Japan and Germany are the major rapeseed-mustard growing countries. In India, it is mainly grown in Rajasthan, Gujarat, Uttar Pradesh, Madya Pradesh, Punjab, Haryana and Assam. Whereas in Gujarat, it is mostly grown in districts of Mehsana, Kheda, Banaskantha, Sabarkantha, Ahmedabad and Gandhinagar. *Brassica* crops consist of three primary species, namely *Brassica rapa* or chinese

cabbage (n=10), *Brassica oleracea* or Cole (n=9), and *Brassica nigra* Koch (n=8) and three amphidiploids, *Brassica juncea* (n=2x=18), *Brassica carinata* (n=2x17) and *Brassica napus* (n=2x=19) (Ren *el al.* 1995), these exhibit a number of morphological and biochemical characteristics. There is also interest in developing hybrid oilseed rape cultivars because of high levels of heterosis reported (Grant and Beveresdorf, 1985) in these species. Increasing oil content and development of varieties with enhanced oil quality is one of the most important breeding goals for rapeseed. To conserve and use these plant genetic resources effectively, it is essential to develop markers that not only distinguish individuals but also reflect the inherent diversity and relationships among collection holdings (Kresovich and McFerson, 1992). The advent of molecular markers such as RAPD has proved their potential to measure such genetic variations because they are unaffected by environment, detectable at all stages of development, ubiquitous in number and covering the entire genome (Williams *et al*. 1990). In present research, an attempt has been made to correlate the genetic diversity among or within four *Brassica* species and to identify genotype or species specific marker using RAPD markers.

Material and Methods

Plant material

Thirty genotypes belonging to four different species were undertaken in the present study. These genotypes are currently being cultivated in Gujarat and some of these are also cultivated in different agro-climatic zones of India. The plants of representative genotypes was raised in pots during the year 2011-12 and utilized for DNA extraction (Table 1).

Extraction of genomic DNA

Genomic DNA was isolated from freeze-dried young leaves of a single plant of each accession by the CTAB method (Doyle and Doyle 1990) with some minor modification. Fresh and young leaves of 15-20 days old seedlings were used as the sources of genomic DNA.

PCR amplification

PCR was performed in a 25-µl volume containing 40ng/µl of genomic DNA, 250 nM of each primer, 0.25 mMdNTPs, 1X reaction buffer (Thermo scientific, USA) and 5 unit Taq polymerase (Thermo scientific, USA). The reaction mixture was initially denatured at 94°C for 5 min, followed by 35 cycles of amplification at 94°C for 1 min, 36-38°C for 2 min, and 72°C for 1 min, and final extension at 72°C for 10 min in a Veriti Thermal Cycler (Applied Bio-systems).

Agarose gel electrophoresis

The PCR products were fractionated on 2% agarose gel run at 80V for about 1.5h in 1X Tris-borate-EDTA buffer. Molecular size of the amplified product was estimated using a known molecular marker DNA. DNA bands were visualized by staining with ethidium bromide and photographed on a UV transilluminator using gel documentation system.

Data analysis

Differences in the DNA banding patterns were qualitatively scored from gel photographs for presence (1) and absence (0) of bands assuming that each band represents a unique genetic locus. Homology of bands among samples was based on the distance of migration in gel. The software used for the analysis of the scored data was NTSYSpc version 2.02 (Rholf 1994). The molecular weights of the PCR product were estimated by Alpha EaseFC4.0.0 software (Alpha Innotech Corporation, USA) for each primer to analyse allele (band) range. Based on the presence or absence of amplicons, a binary 1-0 data matrix was created and used to calculate Jaccard's similarity coefficient (Jaccard, 1908). Using the Coph module of NTSYSpc version 2.02, r (Cophenetic Correlation) value was calculated and results were also expressed graphically.

PolymorphismInformation content (PIC) was calculated according to formula described by Garcia *et al*. 2004. Clustering pattern in 30 mustard genotypes was constructed using the computer software "XLSTAT" Version 2012.3, based on UPGMA following the numerical taxonomic techniques and methods of Sneath and Sokal (1973).

Results

Forty primerswere initially screened for their ability to produce polymorphic patterns and only 33 of them were selected which gave reproducible and distinct polymorphic amplified products. (Table 2) The data collected from random amplification of polymorphic DNA, with 33 arbitrary primers produced 530 total loci and the total bands were 6041. Out of the 530 loci produced, 511 were polymorphic and hence the total polymorphism percentage was found to be 95.96 %, whereas Fu *el al.* (2006), Ali *el al.* (2007), Khan *el al.* (2011) and Gami *et al*. (2013) obtained only 81.72%, 88%, 59.36 and 84.63 polymorphism, respectively. The reason might be the *Brassica* varieties used in the present study

Table 1. List of thirty mustard genotypes and their species

Sl. No.	Genotypes	Species	Sl. No.	Genotypes	Species
1	PM-67	*Brassica juncea*	16	IC363714	*Brassica rapa*
2	ZEM-1	*Brassica juncea*	17	IC363710	*Brassica rapa*
3	GM-1	*Brassica juncea*	18	IC363713	*Brassica rapa*
4	GM-2	*Brassica juncea*	19	IC346013	*Brassica rapa*
5	GM-3	*Brassica juncea*	20	IC365684	*Brassica rapa*
6	BIO-34192	*Brassica juncea*	21	IC398101	*Brassica rapa*
7	Kranti	*Brassica juncea*	22	GSL-1	*Brassica napus*
8	Vardan	*Brassica juncea*	23	HNS-004	*Brassica napus*
9	Laxmi	*Brassica juncea*	24	HYOLA-401	*Brassica napus*
10	IC560696	*Brassica juncea*	25	Neelam	*Brassica napus*
11	IC491446	*Brassica juncea*	26	IC399790	*Brassica napus*
12	IC3999797	*Brassica juncea*	27	IC399819	*Brassica napus*
13	NRCYS-05-02	*Brassica rapa*	28	IC560699	*Brassica napus*
14	GT-1	*Brassica rapa*	29	PusaSwarnim	*Brassica carinata*
15	IC363714	*Brassica rapa*	30	Kiran	*Brassica carinata*

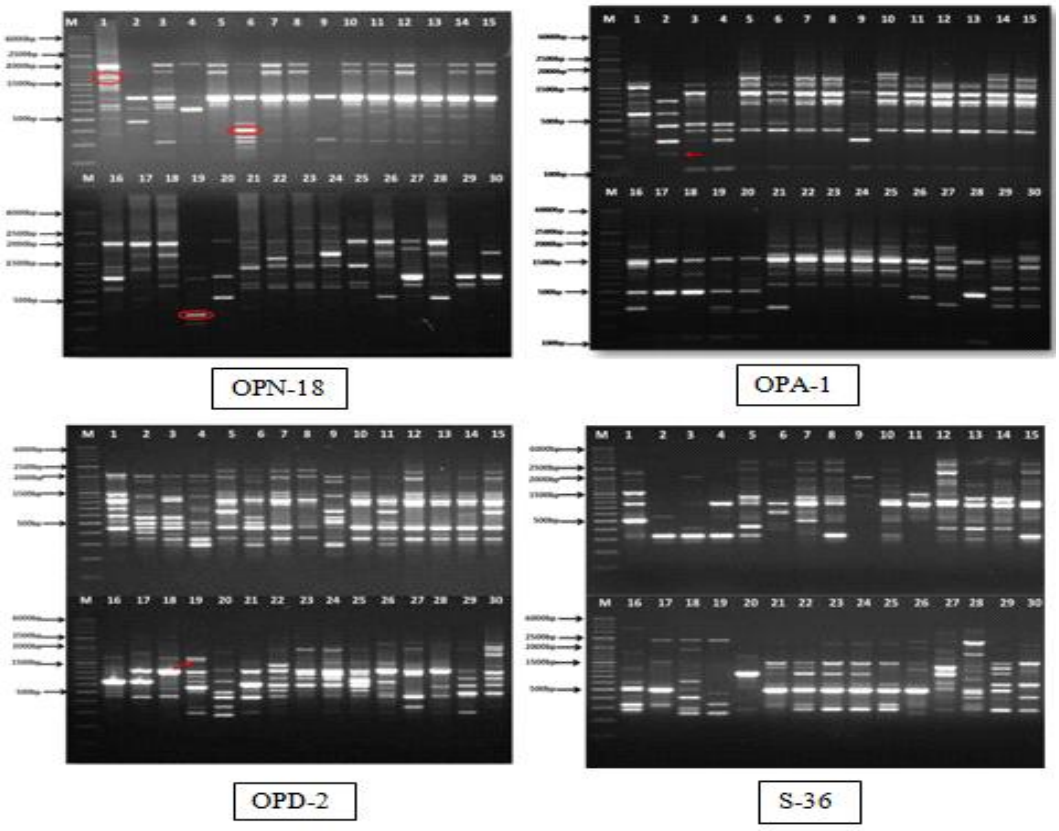

Fig. 1. PCR profile of 30 genotypes generated by RAPD primers

Lane M is 100+500bp DNA ladder and Lanes 1-30 represent genotypes, viz. HNS-004, HYLO-401, PM-67, ZEM-1, GM-1, GM-2, GM-3, Varuna, BIO-34192, Kranti, Vardan, Laxmi, IC560696, IC 491446, IC 3999797, NRCYS-05-02, GT-1, IC363714, IC363710, IC363713, GSL-1, Neelam, IC399790, IC399819, IC560699, IC346013, IC365684, IC398101, Pusa Swarnim and Kiran.

Table 2. Results of RAPD analysis

Sl. No.	Locus Name	Molecular size range (bp)	Total no. of bands	No. of Amplicons	No. of Polymorphic bands	Percent Polymorphism (%)	PIC value
1	OPA-01	340-2700	212	20	20	100	0.92
2	OPA-03	180-3000	317	20	19	95	0.93
3	OPA-04	170-2250	210	19	19	100	0.92
4	OPA-05	150-2700	167	19	19	100	0.91
5	OPA-07	200-2130	153	13	12	92.3	0.87
6	OPA-10	350-2000	194	14	14	100	0.90
7	OPA-15	250-1900	96	10	10	100	0.83
8	OPA-20	130-1300	183	17	13	76.4	0.87
9	OPC-05	250-1900	115	12	11	91.6	0.84
10	OPD-02	280-1700	238	18	18	100	0.92
11	OPK-10	230-2100	225	17	17	100	0.92
12	OPL-04	350-1300	120	9	8	88.8	0.83
13	OPL-18	250-1400	150	12	11	91.6	0.87
14	OPM15	200-2500	158	12	12	100	0.88
15	OPN-02	280-2200	254	21	21	100	0.92
16	OPN-03	250-1700	203	17	17	100	0.91
17	OPN-05	210-2700	183	19	19	100	0.92
18	OPN-12	200-1600	147	12	12	100	0.86
19	OPN-18	130-1800	259	26	26	100	0.93
20	OPO-20	185-1900	190	16	15	93.7	0.90
21	S-22	150-1800	194	17	16	94.1	0.92
22	S-31	150-1700	229	17	16	94.1	0.92
23	S-34	130-1700	206	15	15	100	0.91
24	S-36	280-2850	204	17	17	100	0.92
25	S-40	225-1400	104	12	10	83.3	0.80
26	S-66	200-2300	171	15	14	93.3	0.93
27	S-67	250-1300	126	11	10	90.9	0.84
28	S-68	175-1750	251	18	17	94.4	0.91
29	S-70	150-3100	213	25	25	100	0.94
30	WR1a	280-1700	142	15	15	100	0.87
31	WR2	150-1600	207	16	14	87.5	0.90
32	WR3	280-2800	105	14	14	100	0.88
33	WR4	150-2000	115	15	15	100	0.88
Total		—	6041	530	511	—	—
Average		216bp-2023bp	183	16.06	15.48	95.96%	0.92

are different in morphology, ploidy level and genome constituents.

The average PIC (Polymorphism Information Content) value for RAPD was 0.92. The highest PIC value obtained was 0.94 for S-70 marker and lowest PIC value was 0.77 for S-40.

The number of amplicons produced per primer varied from 9 to 26 with a mean of 16.56 bands per primer. The molecular size of the amplified PCR products ranged from 130 (OPA-20, OPN-18) to 3100 bp (S-70). The RAPD marker OPA-03 produced maximum number of 317 bands, while OPA-15 produced the minimum number of 96 bands. 19 primers out of 33 analyzed successfully produced 100% polymorphism. The RAPD marker OPA-20 gave lowest of polymorphism *i.e.* 76%. Varieties could be distinguished from each other by means of a specific fragment that always present in one variety and always absent in the other. Cultivar specificRAPD markers for fifteen accessions were also detected. (Fig. 1)

The highest similarity index value of 0.76 was found between Varuna and GM-3 andLaxmi and IC399797 (0.75), while the least similarity index value of 0.25 was found between GM-1 and IC363710 and GM-3 and IC363710. Highest genetic similarity was observed between those genotypes which were foundto belongto the same species and least genetic similarity when they belong to different species (*Brassica juncea* and *Brassica rapa*.) The average similarity coefficient among genotypes was 0.41.

Correlation study was carried out to compare the correlation of original similarity matrix of RAPD results with the dendrogram clustering pattern. High correlation between the similarity matrix and dendrogram pattern was justified by the r value which was found to be 0.94 which is good to fit. (Fig. 2)

Clustering Pattern of Different Brassica genotypes

Relationship between species and varieties were determined by UPGMA cluster analyses. On the basis of cluster study, the total genotypes were distributed into five main clusters at a similarity coefficient of 0.41.

The dendrogram (Fig. 3) constructed using the pooled RAPD loci data showed that Cluster I comprised of only two genotypes IC363713 and IC363710 (*B. rapa*). Cluster II included only one genotype Hyola-401. Cluster III included all the genotypes of species *B. napus* (AACC) and *B. carinata* (BBCC) due to the presence of a common (CC) genome. Cluster IV included all *B. rapa* genotypes *viz.,* IC398101, IC363714, JT-1 and IC346013 and are closely related to the released variety NRCYS-05-02. Cluster V was divided into two sub-clusters Va and Vb. Sub-cluster Vaincluded all the genotypes belonging to species *B. juncea viz.,* ZEM-1, PM-67, BIO34192, IC399797, Laxmi, Varuna, GM-3, IC491446, Kranti, IC560696, Vardan, GM-1 and GM-2, whereas sub-cluster Vb included only one genotype *i.e.* IC365684 of *B. rapa.* It also revealed that genotypes *viz.*, ZEM-1, PM-67, BIO34192, IC399797, Laxmi, Kranti, Vardan, IC491446, IC560696, Varuna, GM-1 and GM-2, are closely related with released variety GM-3. It was also observed that GM-3 shared a close relationship with GM-2, since Varuna is one of the commonparents of GM2 and GM-3.Most of the genotypes formed clusters according to the species they belong.Within the species, the genotypes viz., ZEM-1 and GM-2 were found to be most diverse in species *B. juncea*, Hyola-401 and HNS001 in species *B. napus* and IC363714 and IC346013 in species *B. rapa*. The results of principle component analysis (PCA) carried out with 30 genotypes, nearly was in agreement with the results of cluster analysis.

Conclusion

It can be concluded that RAPD markers, which are free from environmental influences, can be efficiently used for diversity analysis. Considering easy handling of the technique, they are especially suitable for breeding programme where large numbers of accessions are to be analyzed. Though there has been great advancement in the marker technology with the advent of different DNA markers like amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), single nucleotide polymorphism (SNPs) and diversity arrays technology (DArT), still RAPD is quite convenient to apply provided the problem of reproducibility is minimized. High level of polymorphism (95.96%) generated in these four genetically distinct species, would be useful in future breeding programme for improving yield and other characteristics of *Brassicas*.

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