Indian Journal of Agricultural Sciences **82** (9): 746–52, September 2012

Assessment of genetic relatedness among Indian mustard (*Brassica juncea*) genotypes using morphological traits and DNA marker

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Received: 9 November 2011; Revised accepted: 15 June 2012

ABSTRACT

The genetic diversity and relatedness of 17 Indian mustard (*Brassica juncea* (L.) Czern & Coss) genotypes were assessed with morpho-physiological characteristics and 10 RAPD markers. Molecular parameters, viz. total number of bands, average polymorphic band, average per cent polymorphism, average polymorphic information content (PIC), average expected gene diversity (Hi), Jaccard's similarity coefficient, principal coordinate analysis (PCA) and dendrogram generated using RAPD markers. Ten RAPD primers generated 62 loci with 50 polymorphic and 12 monomorphic showing 6.2 loci per primer. Primer OPB-01 showed maximum polymorphism (100 %) with maximum PIC (0.95) and Hi (0.33) value. Pusa Jaikisan and Kranti showed maximum similarity coefficient (0.81) while Varuna and Ogura were most distant (0.47), which was further confirmed by their morpho-physiological characteristics, viz. plant height, days to flowering (50%), days to maturity and 1000-seed weight. Seventeen Indian mustard genotypes were grouped into three major clusters I, II and III based on RAPD profiling. The cluster analysis was comparable up to some extent with Principal Coordinate Analysis (PCA) of two and three dimensional plots.

Key words: *Brassica,* DNA, Polymorphism, PIC, PCA, RAPD

Indian mustard [*Brassica juncea* (L.) Czern. & Coss.] is one of the most important oilseed species in the genus *Brassica*. It is a di-genomic amphidiploid (2n = 36, AABB), comprising the genomes of two monogenomic diploid species, *Brassica rapa* syn. *campestris* (L.) Koch (2n = 20, AA) and *Brassica nigra* L. (2n = 16, BB) (UN 1935). It is mainly self-pollinated, although up to 30% out-crossing is reported under natural field conditions, depending upon wind and bee activities. A number of methods are currently available for analyzing the genetic diversity in germplasm accessions, breeding lines and segregating populations. Accurate assessment of the levels and patterns of genetic diversity have diverse applications, viz. (i) analysis of genetic variability in cultivars, (ii) identifying diverse parental combinations to create segregating progenies with maximum genetic variability for further selection and (iii) introgressing desirable genes from diverse germplasm into the available genetic base. Farming practices, age and developmental stages of plants affect morphological characters as well (Khakwani *et al.* 2005). These features make the identification of different plants both at intra- and inter-variety level very difficult. The varietal identification based on biochemical traits such as isozymes, protein and DNA analysis offer greater scope than morphological characteristics. More recently, PCR-based markers have gained popularity because they are unaffected by environment, detectable at all stages of development, and ubiquitous in number, covering the entire genome. They also have the advantage of being abundant, highly polymorphic and analytically simple (Yildirim *et al*. 2010).

In the present research, an attempt has been made to correlate the genetic relatedness among seventeen genotypes of Indian mustard using morpho-physiological characteristics, viz. plant height, days to flowering (50%), days to maturity and 1000-seed weight and ten RAPD markers.

MATERIALS AND METHODS

Various parameters related to morphology and flowering were assessed for the 17 genotypes of Indian mustard, viz. Pusa Jaikisan, Kranti, Pusa Purak,Varuna, Pusa Mahak, Pusa Bold, Pusa Tarak, *Oxyrrhina*, EJ 19, EJ 20, NPJ 112, NPJ 124, *Ogura*, *Siifolia*, *Moricandia* (green), MJR 1 and EJ 17 (Table 1) during winter season of 2008–09 and 2009–10 at Indian Agricultural Research Institute, New Delhi following random block design (RBD) with three replications.

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Table 1 Genotypes and pedigree of Indian mustard varieties

Genotypes	Pedigree
Pusa Jaikisan	Somaclone of Varuna
Kranti	Selection from Varuna
Pusa Purak	[{(PR 602ES 2 × BJ33) × Divya} × RH 30]
Varuna	Selection from Varansi Local
Pusa Mahak	Pusa Bold × Glossy mutant
Pusa Bold	Varuna × BK 1780
Pusa Tarak	Agra local × Poorbi Raya
Oxyrrhina	Pusa Bold background
EJ 19	(Agra local × ZEM 2) × (Agra local × Poorbi Raya)
EJ 20	EC 289602 × DIRA 313-6
NPJ 112	SEJ 8 × Pusa Jagannath
NPJ 124	SEJ 8 × Pusa Jagannath
Ogura	Pusa Bold background
Siifolia	Pusa Bold background
Moricandia (green)	Pusa Bold background
MJR 1	Unknown
EJ 17	[{(Divya × Pusa Bold) × PR 666 EPS} × (PR 704 EPS 2 × B 85)]

Table 2 Morpho-physiological characteristics of 17 genotypes of Indian mustard

Genotypes	Plant height (cm)	Days to flowering (50%)	Days to maturity	1000-seed weight (gm)
Pusa Jaikisan	165	60	152	7.0
Kranti	165	60	152	5.1
Pusa Purak	165	66	146	7.9
Varuna	148	55	150	7.6
Pusa Mahak	180	62	145	3.7
Pusa Bold	165	68	145	7.5
Pusa Tarak	165	60	146	6.6
Oxyrrhina	180	69	141	7.5
EJ 19	170	66	146	4.3
EJ 20	175	65	143	6.7
NPJ 112	160	57	143	5.1
NPJ 124	160	58	145	7.6
Ogura	190	66	140	7.3
Siifolia	200	65	143	7.3
Moricandia (green)	170	66	144	4.9
MJR 1	180	62	145	5.5
EJ 17	190	64	146	5.7
Mean	172.00	62.76	145.43	6.31
CV (%)	7.666	8.440	3.501	20.716
CD (*P*=0.05)	6.307	6.998	7.632	0.273

The crop was raised with all recommended package of practices. Observations on various characters, viz. plant height, days to flowering (50%), days to maturity and 1000-seed weight were recorded on 10 randomly selected plants in each genotype per replication. Genomic DNA was extracted from seedlings as per Dellaporta *et al.* 1983 with minor modification. Seven days old seedlings were crushed in 800 µL DNA extraction buffer [100 mM Tris-HCl, *p*H 8.0; 20 mM EDTA, *p*H 8.0; 1.4 M NaCl; 2% CTAB (w/v); 2% 2-mercaptoethanol (v/v); 1% PVP (w/v)] and transferred in 2 ml Eppendorf tubes. The mixture was incubated at 65°C in a water bath for 60 min. with intermittent shaking at 15 min. intervals. Equal volume of chloroform: isoamyl alcohol (24:1) was added in eppendorf tubes. The mixture was centrifuged at 10 000 rpm for 10 min at 4°C. The supernatant was transferred to a clean 1.5 ml tube, added 2/3 volume of isopropanol and kept it for overnight at –20°C. DNA was pelleted by centrifugation (10,000 rpm, 10 min at 4°C) and pellets washed with 70% ethanol, air dried and resuspended in 100 µl of TE buffer. The extracted DNA was quantified and purity checked using Bio-photometer (Eppendorff). RAPD profiling was carried out with 10 random primers (Table 2) following the method of Yildirim *et al*. 2010 with minor modifications. A 25 µL reaction cocktail was prepared as follows: 10 × 2.5 µL buffer, 1.0 µL dNTPs (10 mM), 1.0 µL $MgCl_2$ (25 mM), 1.67 µL primer (5 µM), 0.7 µL *Taq* polymerase (5 unit), 16.13 µL water, and 2.0 µL sample DNA (100 ng/µL). A total of 10 RAPD primers were tested in this study. The thermocycler (Quanta Biotech) was programmed as follows: 2 min. at 95°C; 2 cycles of 30 s at 95°C, 1 min. at 37°C, and 2 min. at 72°C; 2 cycles of 30 s at 95°C, 1 min. at 35°C, and 2 min. at 72°C; 41 cycles of 30 s at 94°C, 1 min. at 35°C, and 2 min. at 72°C, and a final 5 min. extension at 72°C, followed by cooling down to 4°C. The markers were checked twice for their reproducibility. The polymerase chain reaction products (25 µL) were mixed with 6X gel loading buffer (3 µL) and loaded onto an agarose (1.5%, w/v) gel in 1X TBE (Tris-borate-EDTA) buffer and electrophoresis was performed at 80 V for 150 min. The bands were visualized under UV in a Bio Doc Image Analysis System. The morphological characters were analyzed following routine statistical tests using SPSS software (version10.1). The RAPD profiling was scored for the presence (1) or absence (0) of bands of various molecular weight sizes in the form of binary matrix (Gupta *et al*. 2010).

Data were analyzed to obtain Jaccard's similarity coefficients among the isolates by using NTSYS-pc (version 2.11W; Exeter Biological Software, Setauket, NY, Rohlf, 1993). The Hi was calculated as per De Vicente and Fulton 2003. The bands scored in the gel were assigned numbers 1 and 0 based on its presence and absence in a particular lane. The presence of band corresponds to the dominant/ heterozygous genotype (AA/Aa) and the absence to the recessive genotype (aa) for dominant marker and were scored 1 and 0, respectively. In case of Co dominant marker, the dominant genotype (AA) is represented by scoring pattern (1, 0) heterozygous (Aa) by (1, 1) and recessive (aa) by (0, 1). The polymorphic information content (PIC) that

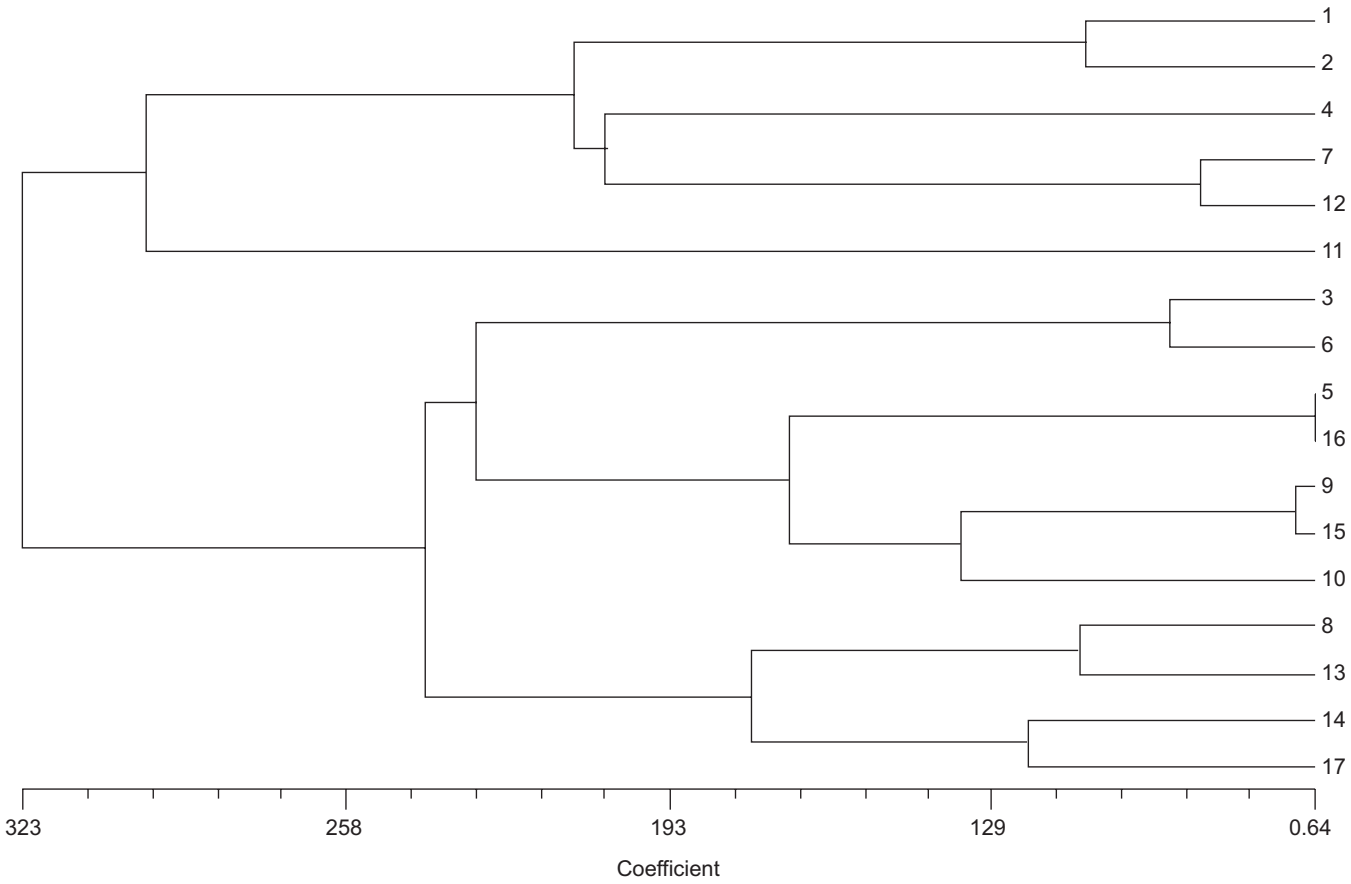

Fig 1 Dendogram derived from UPGMA cluster analysis using Euclidean dissimilarity coefficient based on morphological characteristics (1-17: Indian mustard genotypes)

provides an estimate of the discriminatory power of a locus or loci, by taking into account, not only the number of alleles that are expressed, but also relative frequencies of those alleles, was estimated using the formula:

$$PIC = 1 - 1/n \sum_{i=1}^{n} Pij$$

where Pij is the frequency of j^{th} allele in the i^{th} primer.

RESULTS AND DISCUSSION

Morphological description

All the seventeen genotypes of Indian mustard showed significant differences for the morpho-physiological traits, viz. plant height, days to flowering (50%), days to maturity and 1000-seed weight studied (Table 2). The UPGMA cluster analysis based on Euclidean dissimilarity coefficient also revealed maximum similarity between Pusa Mahak and MJR 1 (Fig 1). However, it has to be validated using more number of morphological characters.

Molecular analysis

The RAPD profile generated by each primer was analyzed using standard DNA marker (250 bp DNA ladder) and compared with their respective banding pattern (Fig 2),

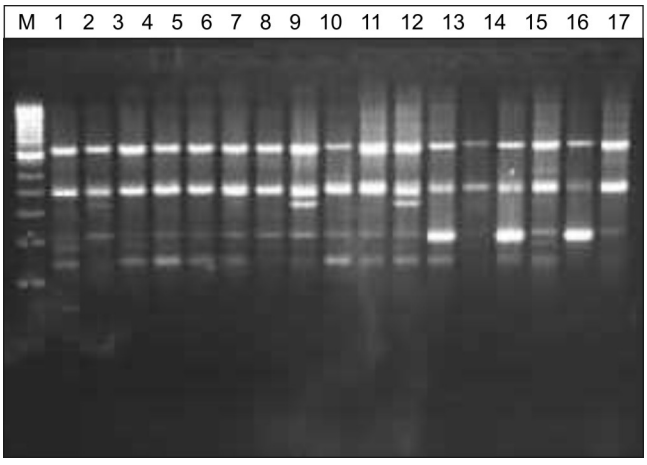

Fig 2 The RAPD profile of different genotypes of *Brassica juncea* obtained with primer OPB-18. Lane M is 250 bp DNA ladder and Lanes 1-17 represent genotypes, viz. Pusa Jaikisan, Kranti, Pusa Purak, Varuna, Pusa Mahak, Pusa Bold, Pusa Tarak, *Oxyrrhina*, EJ 19, EJ 20, NPJ 112, NPJ 124, *Ogura, siifolia*, *Moricandia* green, MJR 1 and EJ 17

size range of scorable bands were 0.1-1.5 kb. The total number of bands, average polymorphic bands, average percentage polymorphism, average polymorphic information content (PIC) and average expected diversity (Hi) was determined (Table 3). The 10 decamer primers of arbitrary sequence generated a total of 62 RAPD loci with seventeen genotypes of *Brassica juncea* used in present investigation. Out of these 62 loci, 50 were found polymorphic and twelve were monomorphic. This gave an average of 6.2 loci/primer. The primer OPB-11 generated a maximum 10 bands with three monomorphic and seven polymorphic. The primer OPB-01 and OPD-12 generated minimum three bands with all polymorphic. The maximum polymorphism (100 %) with maximum PIC value (0.95) and highest average expected gene diversity of 0.33 was achieved by primer OPB-1. Primer OPB-05 revealed minimum polymorphism of 50 % and minimum average expected gene diversity (Hi) value 0.19. The size range of scorable bands was similar to the previous reported range in *Brassica* germplasm (Ahmad *et al.* 2009, Yildirim *et al*. 2010). However, about 84.14 % polymorphic bands achieved by all primers is higher than some previous studies (81.72 %) in Chinese mustard (Fu *et al*. 2006), 76 % in *B. napus* germplasm (Cartea *et al.* 2006). PIC and Hi value are not reported yet in *Brassica* species but these values showed some level of similarity in other crops (Gupta *et al.* 2010). The reason might be that the *Brassica* varieties used in the present study are different in morphology and genome constituents. Absence of bands may be caused by failure of primers to anneal a site in some individuals due to nucleotide sequence differences or by insertions or deletions between two conserved primer sites (Saha *et al.* 2008).

UPGMA cluster analysis using RAPD

A dendogram based on UPGMA analysis with RAPD data showed (Fig 3) Jaccord's similarity coefficient ranging from 0.47 to 0.81 (Fig 4). The genotypes Pusa Jaikisan and Kranti showed maximum resemblance (81 %) while Varuna manifested least similarity (47 %) with genotype *Ogura*. The similarity between Pusa Jaikisan and Kranti is attributed to their derivation from Varuna using somaclonal and pedigree selection respectively (Table 1). At the same time these two were found to maintain higher level of dissimilarity with the parent genotype, i e Varuna showing 60 and 49% Jaccard's similarity coefficient respectively. This is useful information to differentiate and distinguish the derived varieties from an original variety. A perusal of Jaccard's similarity coefficient between Pusa Bold and Pusa Mahak, EJ 17 and CMS lines, viz. *Siifolia, Ogura, Oxyrrhina* and *Moricandia* (green) ranged from 0.51 to 0.77 revealed the efficiency of selection breeding programme undertaken on Pusa Bold. The CMS genotypes could produce higher productive hybrids if crossed with appropriate restorer line as male parent. The 17 genotypes were clustered into three major clusters. Cluster I comprises of ten genotypes, viz. Pusa Jaikisan, Kranti, Pusa Purak, MJR 1, EJ 20, NPJ 124, *Moricandia* (green), EJ 19, NPJ 112 and EJ 17. Cluster II consisted of genotypes Pusa Bold, Pusa Tarak, *Siifolia, Oxyrrhina*, Pusa Mahak and Varuna, Cluster III had only one genotype *Ogura*. The result of Principal Coordinate Analysis (PCA), two and three dimensional plot was comparable to the cluster analysis up to some extent (Figs 5, 6). The maximum similarity 81% recorded between Pusa Jaikisan and Kranti is attributed to same ancestor for them. However, they were developed following somaclonal and plant selection respectively. Similar result was reported (Ali *et al.* 2007, Yildirim *et al.* 2010) more or less similar ranges of genetic similarity in *Brassica* lines. In *Brassica,* coriander and its related genera, RAPD markers have been used successfully for studying and phylogenetic relationship among and within species (Ahmad *et al.* 2007, Pareek *et al*. 2011).

Table 3 Charaterization of total bands obtained by RAPD profiling of 17 Indian mustard genotypes using 10 random primers

Primer codes	Primer sequence	Molecular sizes of amplified products (kb)	Total bands	Monomorphic bands	Polymorphic bands	Polymorphism (%)	(PIC)	(H_i)
OPB-11	GTAGACCCGT	1.5–0.4	10	3	7	70.0	0.43	0.26
OPB-17	AGGGAACGAC	1.5–0.3	9	3	6	66.7	0.33	0.20
OPB-05	TGCGCCCTTC	1.4–0.5	6	3	3	50.0	0.44	0.19
OPB-07	GGTGACGCAG	1.2–0.4	7	0	7	100.0	0.64	0.32
OPB-18	CCACAGCAGT	1.2–0.3	7	2	5	71.4	0.44	0.20
OPB-04	GGACTGGAGT	1.0–0.3	6	1	5	83.3	0.74	0.26
OPB-15	GGAGGGTGTT	1.5–0.1	7	0	7	100.0	0.64	0.32
OPB-20	GCACCCTTAC	0.9–0.1	4	0	4	100.0	0.59	0.28
OPD-12	CACCGTATCC	0.9–0.5	3	0	3	100.0	0.74	0.29
OPB-01	GTTTCGCTCC	1.0–0.1	3	0	3	100.0	0.95	0.33
		Total	62	12	50	841.4	5.94	2.65
		Average	6.2	1.2	5	84.14	0.594	0.26

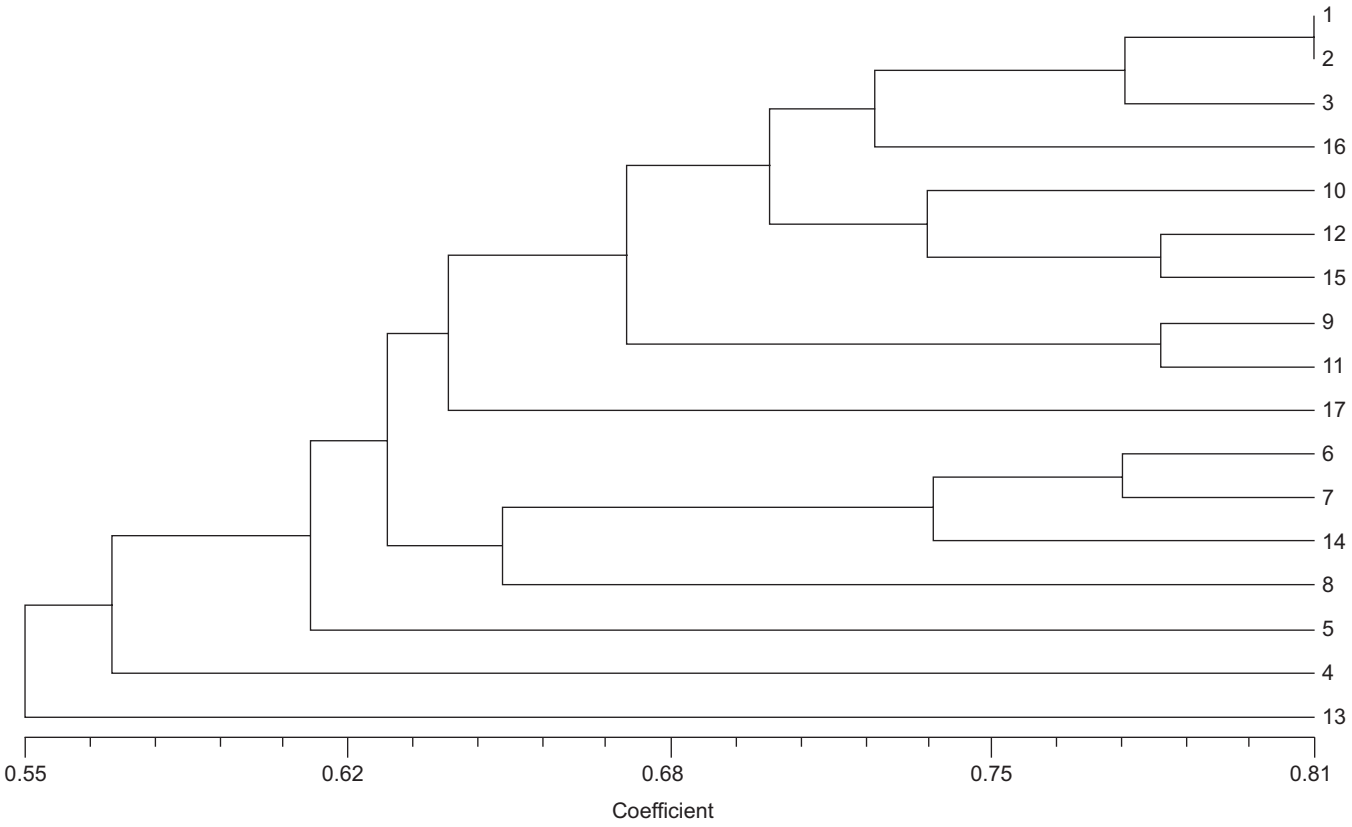

Fig 3 Dendogram derived from UPGMA cluster analysis using Jaccard's similarity co-efficient based on 10 RAPD markers. (1-17: Indian mustard genotypes)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	1.00																
2	0.81	1.00															
3	0.80	0.76	1.00														
4	0.60	0.49	0.58	1.00													
5	0.57	0.51	0.59	0.57	1.00												
6	0.60	0.53	0.55	0.60	0.61	1.00											
7	0.64	0.57	0.67	0.54	0.59	0.78	1.00										
8	0.65	0.58	0.64	0.58	0.56	0.66	0.72	1.00									
9	0.69	0.58	0.64	0.55	0.63	0.55	0.57	0.60	1.00								
10	0.73	0.62	0.67	0.67	0.60	0.52	0.64	0.68	0.64	1.00							
11	0.75	0.68	0.70	0.55	0.66	0.59	0.63	0.67	0.78	0.74	1.00						
12	0.70	0.66	0.72	0.56	0.67	0.56	0.61	0.61	0.62	0.77	0.75	1.00					
13	0.54	0.51	0.56	0.47	0.55	0.51	0.63	0.49	0.60	0.53	0.63	0.51	1.00				
14	0.72	0.61	0.63	0.66	0.70	0.77	0.70	0.57	0.67	0.67	0.73	0.72	0.60	1.00			
15	0.71	0.71	0.77	0.54	0.62	0.55	0.59	0.60	0.57	0.70	0.73	0.78	0.53	0.69	1.00		
16	0.73	0.73	0.71	0.50	0.61	0.60	0.65	0.62	0.62	0.69	0.71	0.69	0.52	0.71	0.78	1.00	
17	0.70	0.70	0.62	0.52	0.61	0.67	0.61	0.58	0.59	0.55	0.65	0.60	0.61	0.65	0.61	0.70	1.00

Fig.4 Jaccard's similarity co-efficient of 17 Indian mustard genotypes, viz. Pusa Jaikisan, Kranti, Pusa Purak, Varuna, Pusa Mahak, Pusa Bold, Pusa Tarak, *Oxyrrhina*, EJ 19, EJ 20, NPJ 112, NPJ 124, *Ogura, siifolia*, *Moricandia* green, MJR 1 and EJ 17 using RAPD primers

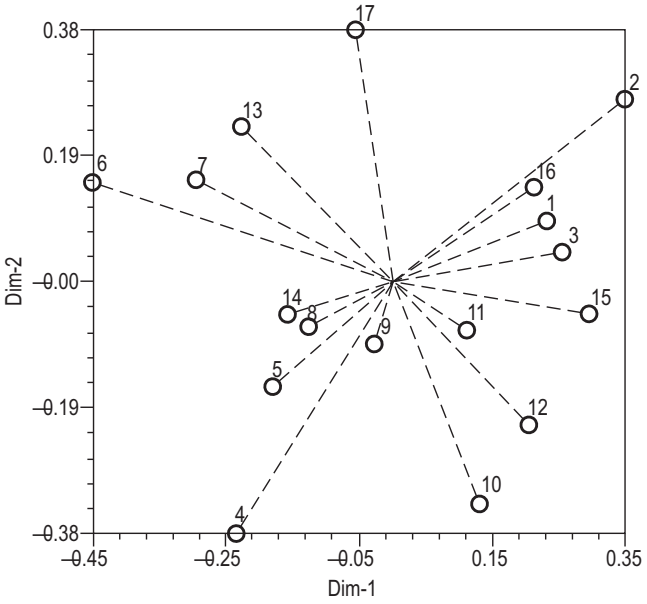

Fig 5 Two dimensional plot (with vector) of 17 genotypes of *B. juncea* obtained using principal coordinate analysis. The numbers plotted represent individual genotype and correspond to those listed in Table 1

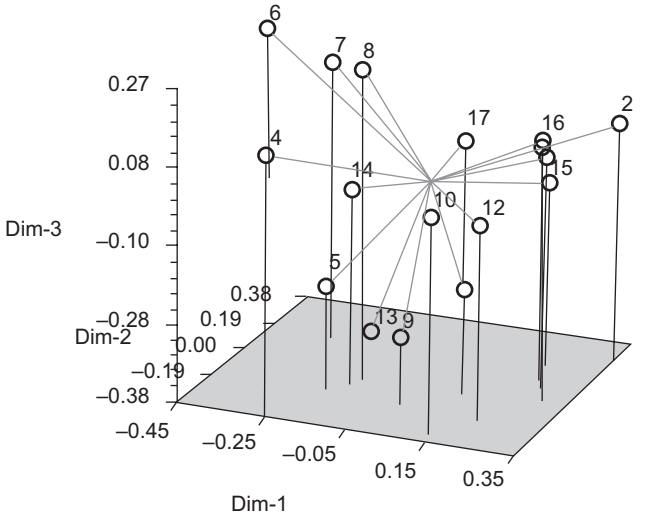

Fig 6 Three dimensional plot (with vectors) of 17 genotypes of *B. juncea* obtained using principal coordinate analysis. The numbers plotted represent individual genotype and correspond to those listed in Table 1

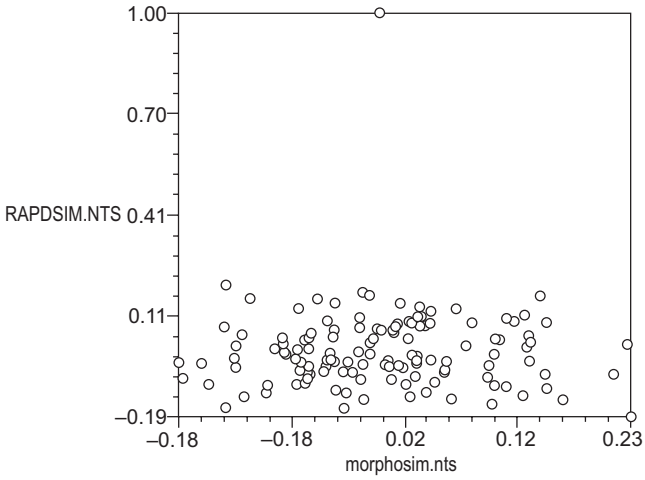

Fig 7 The matrix correlation between morphological descriptors and RAPD profiling

Correlation on genetic variability of morphological descriptors and RAPD markers

The correlation between indices of morphological descriptors and RAPD markers was found to be non-significant (Fig 7). Non-significant correlation between morphological characters and AFLP markers in sorghum was reported (Geleta and Labuschagne 2005). The lack of significant correlation between morphological descriptors and molecular markers could partially be explained by the fact that different coefficients, i e Euclidean dissimilarity coefficient for morphological descriptors and Jaccard's similarity co-efficient for molecular markers were used in the study. Similar views were held for varietal discrimination in pea by morphological and molecular markers (Smýkal *et al.* 2008). This might also be due to the fact that molecular markers measure genetic variation mainly in non-coding sequences which probably have a minor impact on the phenotype. Morphological descriptors on the other hand are affected by environmental conditions and show considerable variation.

It is concluded that the information generated in these 17 genetically distant lines studied would be useful in future breeding programme for improving yield and other characteristics of *Brassicas*. Further, it was observed that PCR based assay like RAPD can be used effectively to estimate genetic variability in *B. juncea* and considering easy handling of the technique, they are especially suitable for breeding programme where large number of accessions are to be analyzed. It is also suggested that more *Brassica* genotypes and molecular markers should be used for better understanding of genetic variability present in Indian mustard genotypes.

ACKNOWLEDGEMENT

The authors thankful to Dr D C Joshi, Scientist, IGFRI, Jhanshi for morphological and molecular data analysis

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