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Screening and Estimation of Allelic Differentiation in Indian mustard Using SSR Markers for Background Selection

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A B S T R A C T

In the present study, 15 genotypes of *Brassica juncea* including white rust resistant and double low quality were characterized and allelic differentiation was determined using 453 SSRs and 139 (30.7%) showed polymorphism with 308 alleles. Polymorphic information content ranged from 0.101 to 0.668, with the average value of 0.474, revealing that much variation was present among these genotypes. The cluster analysis gave three major groups where white rust resistant genotypes were grouped in one major cluster, double low quality genotypes in second cluster while the recipients were grouped in the third cluster indicating that grouping of genotypes based on SSRs corresponded well to their known pedigree data. These observations suggested that SSRs are proficient for evaluating genetic variation and relationships among different varieties of mustard. Further, findings of this study will be useful for DNA fingerprinting, varietal identification which could help during background selection for marker-assisted backcross breeding programs.

Introduction

Genetic variation has enormous importance and its assessment and relationship in breeding material may have remarkable impact in the crop improvement programs (Chandra *et al.*, 2013). The estimation of genetic variation and genomic diversity among the varieties may be used to categorise them into diverse groups; to assess the evolutionary relationships with the wild relatives; to confirm pedigrees and remove the gaps in lineage or selection history, to detect differences in allelic frequencies within genotypes or populations and to explore new alleles at various loci of interest. Thus, genetic variation in *B. juncea* will be useful for plant breeders in understanding germplasm structure and finally judging the various combinations to generate the best progeny (Hu *et al.*, 2007) which will be ultimately helpful for selection of breeding material by widening the genetic base (Qi *et al.*, 2008). The genetic distances amongst the

parents may be due to differences in number of the genes and their functional behaviour with respect to the environment (Nei, 1976).

Various morphological, biochemical and molecular approaches have been used to estimate genetic diversity between individuals or populations (Mohammadi and Prasanna, 2003). Evaluation of genetic diversity and relatedness in *B. juncea* using phenotypic parameters has been previously done by many researchers (Singh *et al.*, 2010; Alie *et al.*, 2009). Isozyme loci have been used as markers in various genetic studies including genetic diversity in *B. juncea* (Kumar and Gupta, 1985). But, environmental factors and the developmental stage of the plant are the two limiting factors associated with these markers. Among various types of markers used for genetic diversity estimation in plants, molecular or DNA markers are more specific, proficient, precise and consistent in distinguishing closely related cultivars or species (Mishra *et al.*, 2011).

Genetic variation has been evaluated using RAPD markers in various crops such as maize (Zhang *et al.*, 1998), wheat (Liu *et al.*, 1999), Brassica (Divaret, Margale and Thomas 1999), barley (Hamza *et al.*, 2004) and sesame (Salazar *et al.*, 2006). Simple Sequence Repeats (SSRs) are the most perfect and preferred markers due to several advantages over other markers such as highly reproducible, co-dominant, easily scorable, abundance, wide distribution all the way through the genome and its multi-allelic variation (Powell *et al.*, 1996).

Moreover, they have several flanking regions which are considered as highly conserved regions in various related species thus making it convenient to use same markers in associated genomes. They have been successfully used for genetic diversity analysis in a variety of crops such as Indian bread wheat (Mir *et al.*, 2011), rice (Rahman *et al.*, 2012) and maize (Sivaranjini *et al.*, 2014).

The genetic diversity studies in *B. juncea* has been subsequently carried out using isozyme markers (Kumar and Gupta, 1985), morphological markers (Pradhan *et al.*, 1993) and molecular markers (Hopkins *et al.*, 2006). Among molecular markers, RFLP (Hallden *et al.*, 1994); RAPDs (Khan *et al.*, 2011), AFLP (Zhao *et al.*, 2005) and SSRs (Abbas *et al.*, 2009) have been used to study varietal identification, genome organization and genetic diversity estimation in Brassicas. But, due to narrow genetic base and lack of availability of proper genomic information in Indian mustard, SSRs has been used in a very limited manner in Brassica as compared to the other crops (Yadav *et al.*, 2009). Thus, keeping all these facts in view, this study was planned to characterize and assess the genetic variation and resolve the genetic relationship among these genotypes for breeding purposes using microsatellite markers.

Materials and Methods

Plant material used

The present study was conducted with 15 diverse genotypes of Indian mustard including white rust resistant and double low quality (Table 1). Among these genotypes Donskaja, Bio-YSR, BEC-144 were white rust resistant and PDZ-1, Heera, EC-597325, RLC-3 were double low.

Genomic DNA isolation

Fresh leaves from healthy plants at three leaf stage were collected from each mustard genotype and DNA was extracted by CTAB method (Murray and Thompson, 1980) and quantified on 0.8% agarose gel and diluted to 20 ng/µl.

SSR marker assay

A total of 453 SSR markers were used for evaluating genetic diversity among the 15 Indian mustard genotypes. PCR was performed in a 10 µl reaction having 1X buffer, 200 µM dNTPs, 0.4 µM of forward and reverse primer, 1U Taq polymerase and 1µl of template DNA (20ng/µl) in a 96-well Thermal Cycler (Eppendorf, Germany).

The PCR protocol comprised of the initial denaturation at 94°C for 5.0 min followed by 38 cycles of 30s at 94°C for denaturation, 41s at 55°C for annealing and 35s at 72°C for extension. The final extension was done at 72°C for 7 min and stored at 4°C. The PCR products were electrophoretically separated on 3.5% agarose gel and visualized under gel documentation system (Syngene, UK) (Fig. 1).

Data scoring and analysis

Gel photographs were used for scoring variations in the DNA banding patterns where presence of band was indicated '1' and absence as '0' in all the genotypes for further analysis. Variations in the bands were scored for each primer as polymorphism and the binomial data generated was further used for calculating, total alleles, total bands, number of monomorphic and polymorphic bands. In addition, the binomial data matrix was analyzed using SIMQUAL (Similarity for qualitative data) for calculation of Jaccard's similarity coefficients through NTSYSpc-2.02e software.

Further, the dendrogram was constructed using the UPGMA method. PIC (Polymorphic information content) values were calculated using the formula suggested by Anderson *et al.,* (1993): $PIC_j=1-\Sigma_i^n=_1P_i^2$, where, i = the i^{th} allele of the j^{th} marker, n = the number of alleles at the j^{th} marker and p = allele frequency.

Results and Discussion

Molecular characterization

In the present study, a good amount of polymorphic markers were detected that would be useful for genotype identification, germplasm management and genetic diversity assessment and further introgress the genes underlying them to desirable genetic backgrounds. Out of 453 SSRs, 335 (73.9%) primers gave amplified products of varying sizes in a range of 100-500 bp. Among these, 196 markers (43.2%) were found to be monomorphic while 139 SSRs were polymorphic exhibiting 30.7% polymorphism (Table 2). The polymorphism percentage obtained in this study is less as compared to (Salazar *et al.,* 2006; Abdelmigid, 2012). In other studies, percentage of polymorphic primers in brassica genotypes ranged from 21.54 to 59.36 as reported by Ali *et al.,* (2007). Similar kind of polymorphism percentage i.e. 21.54 to 59.36% was observed by Khan *et al.,* (2011).

The allelic differentiation is measured in terms of PIC value. It ranged from 0.101 to 0.668 with an average of 0.47 indicating that the level of polymorphism, as assessed by the PIC values, was quite high and varied considerably among SSR loci. Twenty five SSR markers had PIC value > 0.5 indicating that these were the most useful markers for differentiating these genotypes. The details of such markers are shown in table 3.The PIC values in the present investigation are comparable to 0.37 PIC value reported by Salazar *et al.,* (2006) and 0.5 reported by Russel *et al.,* (1997). Marker BN6A3 gave the highest PIC value (0.668) followed by BRMS-09 (0.666), C09 (0.665) and Ni3-G07 (0.664) thus revealing that BN6A3 is the most informative and best marker for identification and diversity estimation of these mustard genotypes followed by BRMS-09, C09 and

Ni3-G07 markers while the lowest PIC value (0.101) was for maker BrgMS75 indicating it as the least powerful marker. High PIC values may be observed due to the use of more number of informative markers (Akkaya and Buyukunal-Bal, 2004).

Number of alleles

Overall 308 alleles were detected from 139 polymorphic SSR markers across all 15 genotypes. The number of alleles per primer pair (locus) varied from 2-3 with 2.21 as an average across 139 primers (2 alleles for 109 markers and 3 alleles for 30 markers). An average of 2.21 alleles is similar to an average of 2.0-5.5 alleles per primer pair for different classes of SSRs as suggested by Wong *et al.*, (2009).The details of frequent alleles, rare alleles and unique alleles for different genotypes are shown in table 4.

Frequent, rare, and unique alleles

Out of 15 genotypes, frequent alleles were considered to be those occurring in more than 20% (3-5 genotypes) whereas those occurring in 2 genotypes (<20%) were classified as rare alleles. A similar criterion was followed by Alvarez *et al.,* (2007). In this study, 26 frequent alleles were identified among 139 loci, with an average of 0.19 alleles per locus. Genotype BEC-144 shared a common frequent allele at any given locus with a maximum frequency of 16 followed by Bio-YSR (13), PDZ-1(9), EC-597325 (9) Donskaja (8) and Laxmi (8). A total of 8 rare alleles were identified among 139 microsatellite loci, with 0.06 alleles per locus as the average. These rare alleles were present in 10 genotypes namely Donskaja, Bio-YSR, BEC-144, EC-597325, RLC-3, Heera, PM-24, PM-30, RH0749 and RLC-1 (Table 4).

A total of 5 unique alleles were detected in 5 genotypes (BEC-144, EC-597325, RLC-2, PM-30 and Laxmi). Five SSR markers-MR33, BrgMS490, Ni2D10, BrgMS75 and cnu_m602a amplified these specific unique alleles (Table 4). Presence of such unique alleles will be helpful and can be useful further in DNA fingerprinting, detection of particular genotype and discover specific differentiating genes/alleles. They are very much consistent in the estimation of genetic relationships across the genotypes. In addition, the added advantage is that they can be converted to sequence tagged site markers (STS) and sequence characterized amplified regions (SCARs) thus giving high prospective for further use. Moreover, exclusive bands can be proved valuable to distinguish the genotypes at the molecular level without using field data. Similar kind of results was observed by Sahu *et al.,* (2012) and Vinu *et al.,* (2013) in various crops.

Fig.1 Agarose gels showing amplification profiles of genotypes using the primer BrgMS334 and BrgMS399. Lane M- 50 bp ladder 1-Donskaja 2-BioYSR 3-BEC144 4-PDZ-1 5-EC597325 6-RLC-3 7-Heera 8-PM24 9-PM30 10-NRCDR02 11-DRMRIJ31 12-RH0749 13- Laxmi 14-RLC-1 15-RLC-2

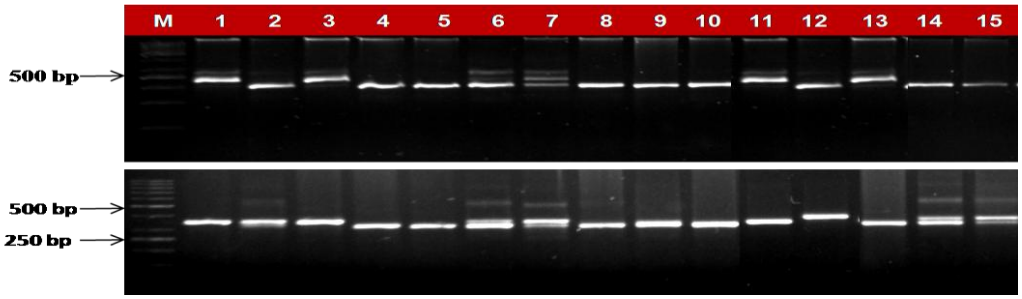

Fig.2 UPGMA dendrogram showing genetic relationships among 18 genotypes

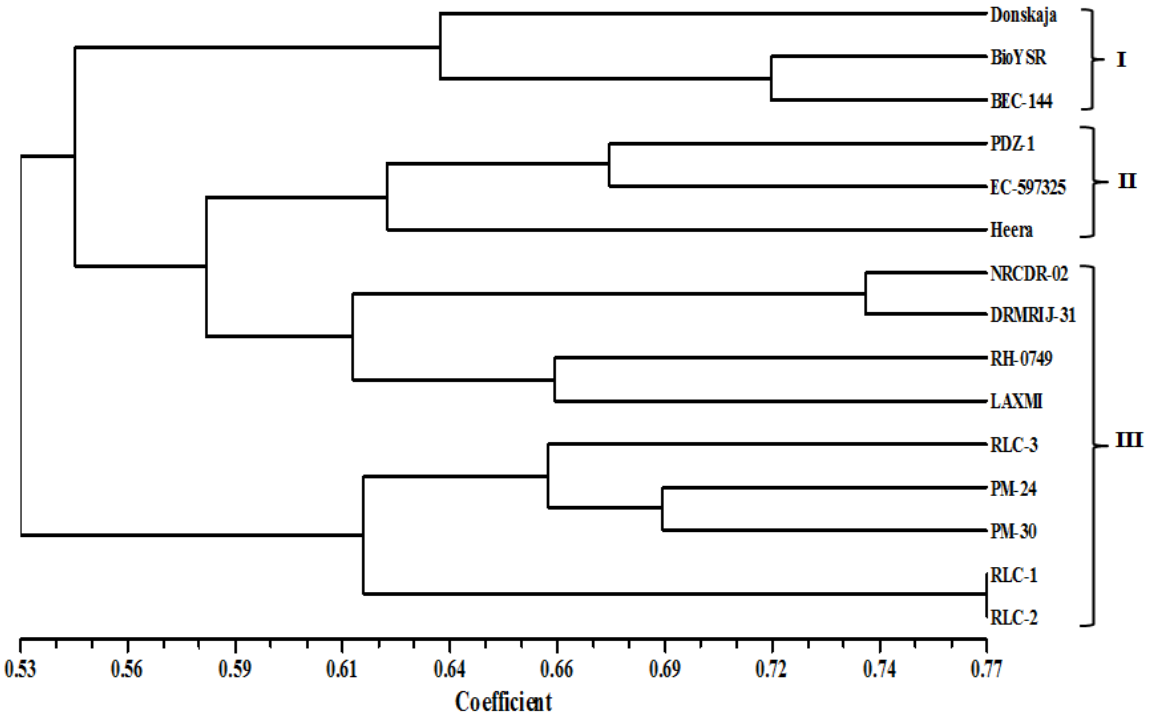

Table.1 Genotypes used for diversity analysis along with their pedigree

S.No.	Genotype	Species	Pedigree	Country/Developing institute
1.	Donskaja	*Brassica juncea*	Exotic collection from Russia	Russia
2.	BioYSR	*Brassica juncea*	Clipper/BH75/BK0019	NRCPB,IARI, New Delhi
3.	BEC-144	*Brassica juncea*	Exotic collection from Poland	Poland
4.	PDZ-1	*Brassica juncea*	LES-1-27/NUDHYJ-3	IARI, New Delhi
5.	EC-597325	*Brassica juncea*	Exotic collection from Australia	Australia
6.	RLC-3	*Brassica juncea*	JM 06003/JM 06020	PAU, Ludhiana
7.	HEERA	*Brassica juncea*	ZYR-4/BJ-1058	Nagpur University & Dhara Veg. Oil & Food Co. Ltd., Vadodara
8.	PM-24	*Brassica juncea*	(Pusa bold X LES 15) X LES 29	IARI, New Delhi
9.	PM-30	*Brassica juncea*	BIO 902/ZEM-1	IARI, New Delhi
10.	NRCDR-02	*Brassica juncea*	MDOC 43/NBPGR 36	DRMR, Bharatpur
11.	DRMRIJ-31	*Brassica juncea*	HB 9908/HB 9916	DRMR, Bharatpur
12.	RH-0749	*Brassica juncea*	RH 781 X RH 9617	CCS HAU, Hisar
13.	LAXMI	*Brassica juncea*	PR15/RH 30A	CCS HAU, Hisar
14.	RLC-1	*Brassica juncea*	QM 4/Pusa bold	PAU, Ludhiana
15.	RLC-2	*Brassica juncea*	QM 4/Pusa bold	PAU, Ludhiana

Table.2 Summary of SSR amplified products

Total number of markers used	453
Number of polymorphic markers	139
Number of monomorphic markers	196
Number of not amplified markers	118
Size of amplified products (bp)	100-500
Percent polymorphism	30.7%
Total number of alleles	308

Table.3 Details of most informative markers based on their high PIC values

S. No.	Primer Name	Sequence	No.of Alleles	Range of Amplified Products	T_m (°C)	PIC value
1	BN6A3	GCTACCCACTCATGTCCTCTG CCAAGCTTATCGAATCTCAGCTA	3	150-220	55	0.668
2	BRMS-019	CCCAAACGCTTTTGACACAT GGCACAATCCACTCAGCTTT	3	200-250	55	0.666
3	C09	AGCATCAATCTTTTGCTCTGC TGCACACAAACTCCTTCTCC	3	200-260	55	0.665
4	Ni3-G07	CACTCTCTCCGCCATTTTTC CTTGAAGCGTTAAAGCCGAC	3	200-250	55	0.664
5	nia_m026a	AATGAGTAATGTCCCACACGA TGAAATTGCGGATTCTTTAGC	2	170-230	55	0.663
6	cnu_m60a	CTCCTTCATTTGATCCCCAA CTTCTTCAGGGTTTCCAACG	3	150-220	55	0.661
7	cnu_m62a	GCAGAAGCCTGAGAGTCTGG AACAAGGCTGAATGCTACCG	3	200-260	55	0.659
8	BrgMS802	TCCCACCCTCAAAATATACAGC TGCTCGTTGGAAGAGGACAT	3	350-400	54	0.657
9	EJU1	GGTGAAAGAGGAAGATTGGT AGGAGATACAGTTGAAGGGTC	3	200-300	55	0.655
10	Ol10B01	CCTCTTCAGTCGAGGTCTGG AATTTGGAAACAGAGTCGCC	3	200-250	54	0.653
11	Ol10B11	AAAATGTGAGGCTGTTTGGG TTTCGCAGCAGTAAACATGG	3	180-230	52	0.651
12	E05	CTCGTCTCAGGGATTATGTCG CAGACAGAGGATAGACCGAACC	3	130-200	55	0.646
13	nia_m043a	CCATTCGAGGTGGTCGTAAA AGAAAACGGACCTCGATTCA	3	250-300	55	0.643
14	cnu_m583a	TTGTAGAGAGAGAGAGAGGGCA CCTTCAAAAGAAAGGAGGGG	3	200-250	55	0.642
15	BrgMS787	CCATCTCAGCTCTATCTACCAAAA TCAAAACACCGAGTAAACTGGA	3	250-330	54	0.634
16	BrgMS732	GCGCCGACGAAACAATTA ATGCTCGTGCCCACAAAA	3	300-350	50	0.632
17	Ni3-B07	GGAGAAGAGGAAGAAGAAGCC CGACTTCTAGAGGAACCCCC	3	100-200	55	0.631
18	Ni2A07	GGAACCCAACAAGTGAGTCC AGAGCTTGAGACACATAACACC	3	200-250	55	0.630
19	Ra2-H10	GCGCGTGTAGGCTACGTC CGGCCGCGGCAACTG	3	120-170	55	0.627
20	E07	GAGCGAGTCGATTACTTTTGC GAATGGATTTCCGATGATGG	3	120-170	55	0.626
21	cnu_m625a	AGAGTCCGATGACAGACACG CTTTTCGGGCACTTCTTCAC	3	200-270	55	0.625
22	Ra2-H07	ATCATCAATCCTGACGAGGC CGCGCACACACACACAC	3	180-250	55	0.613
23	Ni3C05	TTTCGTGCTTTGGTGTGAAG TCCCCAAATCGAACCATAAG	3	150-200	52	0.612
24	G02	TTGGTGTCAGAAACAACG ACACACGACGGATCTCTGC	3	200-260	55	0.599
25	ENA17	CAGTTATTTCGCCTCGTCT TATTTGTGGTCTGTTATTGGA	3	250-320	55	0.598

Table.4 Details of frequent, rare and unique alleles

S. No.	Pattern of alleles	Locus name	Tm (°C)	No. of alleles	Size (bp)	PIC value	*Brassica juncea* varieties													
							Donskaja	BioYSR	BEC-144	PDZ-1	EC-597325	RLC-3	Heera	PM-24	PM-30	NRCDR-02	DRMRIJ-31	RH-0749	LAXMI	RLC-1	RLC-2
1	Unique	MR33	49	2	180-230	0.244	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—
2		BrgMS490	54	2	200-250	0.188	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—
3		Ni2D10	52	2	170-200	0.117	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+
4		BrgMS75	54	2	120-180	0.099	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—
5		cnu_m602a	55	2	240-270	0.197	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—
6	Rare	sORA43	55	2	120-150	0.218	—	—	—	—	—	+	—	—	+	—	—	—	—	—	—
7		BrgMS792	55	2	200-240	0.218	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—
8		BrgMS344	53	2	220-250	0.197	—	+	—	—	—	—	+	—	—	—	—	—	—	—	—
9		BrgMS778	54	2	180-260	0.180	—	—	—	—	+	+	—	—	—	—	—	—	—	—	—
10		BRMS-027	50	2	200-240	0.336	—	—	—	—	—	+	—	—	+	—	—	—	—	—	—
11		BRMS-029	55	2	200-220	0.277	+	—	—	—	—	—	—	—	—	—	—	—	—	+	—
12		ENA4	55	2	150-200	0.459	—	+	+	—	—	—	—	—	—	—	—	—	—	—	—
13		H01	55	3	100-150	0.581	—	—	—	—	—	—	+	—	—	—	—	+	—	—	—
14	Frequent	BRMS-006	49	2	150-200	0.290	—	—	—	—	—	—	—	+	—	—	—	+	—	—	+
15		BrgMS783	54	2	310-340	0.320	—	—	—	—	—	—	+	—	—	—	+	+	—	—	—
16		BrgMS388	56	2	370-400	0.320	—	—	—	+	+	—	—	—	+	—	—	—	+	—	—
17		BrgMS794	53	2	250-270	0.345	+	—	+	—	—	—	+	—	—	—	—	—	—	+	—
18		Ni2A12	54	2	80-130	0.332	+	—	+	—	—	—	+	—	—	—	—	+	—	—	—
19		MR176	50	3	150-230	0.244	—	—	—	+	—	+	—	—	—	—	—	—	+	—	+
20		BRMS-005	50	2	200-250	0.362	—	+	+	—	—	—	+	+	+	—	—	—	—	—	—
21		cnu_m584a	55	2	200-250	0.408	+	+	—	+	—	—	—	—	—	—	—	—	—	—	+
22		cnu_m605a	55	2	200-230	0.426	+	+	+	—	—	—	—	—	—	+	—	—	—	—	—
23		cnu_m613a	55	2	160-210	0.375	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—
24		cnu_m625a	55	3	200-270	0.625	+	+	+	—	—	—	—	—	—	—	—	—	+	—	—
25		Ra2-G08	55	2	300-350	0.415	—	—	+	—	—	—	—	—	—	+	—	+	—	—	—
26		Ra2-H07	55	3	180-250	0.613	—	+	+	+	—	—	—	+	—	—	—	—	+	—	—
27		Ra2-H10	55	3	120-170	0.627	—	+	—	—	+	—	—	—	—	—	+	—	—	+	+
28		GOL3	55	2	120-150	0.359	—	—	—	+	+	—	—	—	—	—	—	+	+	—	—
29		ENA18	55	2	100-130	0.473	—	—	—	—	—	—	—	—	—	+	—	—	+	+	—
30		ENA20	55	2	140-160	0.375	—	—	—	—	+	—	+	—	—	—	—	—	—	+	—
31		A11	55	2	250-270	0.444	—	+	+	—	+	+	—	—	—	—	—	—	+	—	—
32		B03	55	2	200-230	0.480	—	—	+	—	+	—	—	—	—	+	+	—	—	—	—
33		E07	55	3	120-170	0.626	—	+	+	+	+	+	—	—	—	—	—	—	—	—	—
34		Ni3-F01	55	2	150-200	0.489	—	+	+	—	+	—	—	—	—	—	—	—	—	—	—
35		A09	55	2	100-170	0.473	+	+	+	+	—	—	—	—	—	—	—	—	+	—	—
36		G02	55	3	200-260	0.598	—	—	—	—	+	—	+	+	—	—	—	—	—	+	—
37		G09A	55	2	100-150	0.387	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—
38		G10	55	3	175-250	0.580	—	—	+	+	—	—	—	—	—	—	+	—	—	—	+
39		G11	55	2	175-200	0.415	—	+	+	+	—	—	—	—	—	—	+	—	—	—	—

Cluster analysis based on SSRs

The UPGMA dendrogram was constructed using 15 genotypes based on SSR marker data and it gave three distinct groups (Fig. 2). The cluster analysis discriminated well between the 15 genotypes and the relationships detected between different genotypes were in agreement with their known pedigree relationships. The genetic relationships among the 15 genotypes exhibited variation. The similarity coefficients were found to vary from 0.447 to 0.767 revealing the presence of maximum diversity between these genotypes. Similar kind of genetic variation has also been observed by Alie *et al.*, (2009) and Singh *et al.,* (2010) in *B. juncea*. The highest value for genetic similarity (76.7%) was found between RLC-1 and RLC-2 followed by 73.8% between NRCDR-02 and DRMR IJ-31 and 71.5% between Bio-YSR and BEC-144. RLC-3 and BEC-144 were associated with each other with least similarity (44.7%).

Cluster I consisted of 3 donors for white rust resistance namely Donskaja, Bio-YSR, BEC-144 where Bio-YSR and BEC-144 exhibited highest similarity (71.5%) followed by Donskaja and BEC-144 (63.7%), Donskaja and Bio-YSR (63.3%). Cluster II comprised of three double low donors viz. PDZ-1, Heera, EC-597325. Here, PDZ-1 and Heera showed least similarity (58.1%) while PDZ-1 and EC-597325 were close to each other with 67.6% similarity while Heera and EC-597325 had 66.3% similarity. One donor RLC-3 and eight recipient genotypes were grouped together in Cluster III. In this cluster, RLC-1 and RLC-2 were grouped together at a highest similarity of 76.7% as they had a similar plant type, yield and grain characters and were developed from the same cross (QM4 x Pusa bold) while RLC-3 was grouped with PM-24 as it has different parental lineage than RLC-1 and RLC-2. Similarly, PM-24 and PM-30 were closely associated with each other and both are single zero and clustered together at a lower similarity of 68.9%. NRCDR-02 and DRMRIJ-31 showed closer relationship with similarity of 73.8% while two genotypes RH0749 and Laxmi showed similarity of 66.3%.

This depicts the importance of SSR markers in estimating the close pedigree relationships in breeding material. They are highly polymorphic, reproducible, co-dominant, PCR-based markers and are the most preferred one and thus considered to be very potent in genotype discrimination. It has been reported previously that SSR is an important tool for germplasm characterization in a variety of crops, including oilseed Brassica (Saal *et al.*, 2001). A similar result regarding effectiveness of SSR markers in monitoring genetic diversity have also been reported by Hopkins *et al.*, (2006) and Fu and Gugel, (2010). Thus, in this study, the dendrogram constructed using the UPGMA method depicted that genotypes which were genetically similar were grouped together and explained the relationship between these genotypes. Genetic variation existing among selected genotypes of *B. juncea* will be further helpful in developing and planning breeding strategies by estimating genetic relationships among different genotypes and crop improvement programs will be implemented taking them into consideration.

This study confirmed that the tested genotypes possessed a good level of microsatellite variation. The markers used here were of value for characterizing the mustard genotypes and thus further can be used for background selection in breeding programs. It can be concluded that as SSR markers are free from environmental influences they are the stronger tools than quantitative trait data in distinguishing *B. juncea* genotypes based on pedigree and origin.

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Conflict of interest

We declare that no conflict of interest exits among authors in the submission of this manuscript.

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