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Genetic diversity analysis of *Brassica juncea* mutants through morphological and molecular characterization

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ABSTRACT

Mutation breeding is a tool to strengthen the germplasm and is being exploited for crop improvement. Mutations for morphological, yield, and yield attributes were isolated from parent variety Bio902 using -radiation and Ethyl Methane Sulphonate (EMS). Their morphological and molecular diversity analyses were performed. Morphological diversity was assessed using PCA analysis and the result revealed six principal components with Eigen values greater than one and altogether explained 74.34% of total accumulated variability. Varimax rotation was applied since the analysis without rotation of axes failed to load all the variables. The UPGMA-based cluster analysis showed the formation of 8 clusters. Thirty mutants were grouped in cluster VIII and this cluster did not contain any of the checks including Pusa bold and BIO 902 hence these mutants were diverse from the checks. For the molecular marker study, 20 SSR primers were used out of which eight primers showed polymorphism. The percent polymorphism varied from 66.67 to 100% with an average of 77%. Polymorphism Information Content (PIC) values ranged from 0.08 to 0.48 with an average of 0.34 per primer combination. Distance-based cluster analysis and dendrogram showed the presence of three major clades. The first clade consists of Bio902 along with five mutants derived from -irradiation. The second clade consists of five mutant's viz. ACNMM 23, ACNMM13, ACNMM 22, ACNMM 14, and ACNMM 7 without any checks, while the third clade consists of two genotypes viz. Kranti and ACNMM15. The findings of this study shall be useful in a breeding program to recombine desirable traits. Out of the 10 superior high-yielding mutants selected for molecular analysis, mutant ACNMM9 was similar to checks both in morphological as well as molecular studies. Mutants ACNMM 4, ACNMM 17, ACNMM 19, and ACNMM 15 were found in distinct clusters as of checks morphologically but in the same cluster as that of checks in molecular analysis.

Keywords: *Brassica juncea, mutation, genetic diversity, SSR markers, molecular characterization, cluster analysis, Eigen values, mutant, Varimax rotation, -radiation, Ethyl Methane Sulphonate*

INTRODUCTION

Indian mustard *(Brassica juncea)* belonging to the family *cruciferae (Syn. Brassicaceae)*. It is the world's third most important source of edible oil [9] and second most important in India accounting 30% of the total oilseeds production [27]. In India, the area under mustard cultivation is 6.07 million hectares producing about 8.70 million tonnes of seeds with average productivity of 1433 Kgha-1[4]. Still, there is a big gap in productivity in India compared to the world (1811 kg.ha-1: www ikissan.com). Developing high-yielding varieties is the only option to narrow down the gap. Improvement of any crop is determined by the availability of genetic variability in the germplasm and the heritability magnitude of yield-contributing traits [8]. However, most of the rapeseed-mustard varieties cultivated in India have a narrow genetic base which limits their improvement. Existing germplasm needs strengthening which could be easily used in mustard crop improvement. Mutagenesis could pave the way for generating novel variability and the development of high-yielding varieties in mustard [11, 12].

A morphological characterization is a potential tool for the classification and identification of desirable variability. Genetic diversity analysis has been carried out in Indian mustard using Principal Component Analysis [19, 18, 14] and Hierarchical Cluster analysis [23] using morphological characters. Molecular marker has been extensively used to identify the distinctness of genotype or segregating populations [6]. The precise information regarding genetic divergence and relatedness between useful mutants can be achieved through molecular markers [20]. Identification of genomic regions associated with improved traits will help to develop improved cultivars using marker-assisted selection (MAS) [22]. Simple sequence repeats (SSRs) or microsatellites have been recognized as useful molecular markers for the analysis of genetic diversity, MAS, and population analysis [17, 22]. Among Brassica species, SSR markers have been developed in B. rapa (AA), B. nigra (BB), B. napus (AACC), and B. oleracea (CC) [32]. These markers have

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been employed to detect microsatellite loci in other related species due to the conserved nature of flanking sequences [27] and their application in the assessment of genetic variability in mustard [28]. Induced mutation generates variability which could be due to new alleles. Identification of such alleles with molecular markers could help in the selection of desirable traits as well as plants. Studies on morphological and molecular diversity analysis of induced mutations have not yet been reported in mustard and therefore present study was undertaken to evaluate the extent of variability for yield and related attributes of mutants over the parent through morphological and molecular characterization.

MATERIALS AND METHODS

Development of mutant lines

Seed Treatment: Genetically pure, dry, and healthy seeds of *Brassica juncea cv* BIO 902 were selected to prepare five lots consisting of 300 seeds each. They were irradiated with 900, 1000, 1100, 1200, and 1300 Gy doses of gamma rays using a ^{60}Co source at BARC, Mumbai. For the combination treatment of gamma rays and EMS, gamma ray-exposed seeds from each treatment were treated with 0.5% aqueous solution of EMS after pre-soaking with sterilized distilled water for 12 hours. Seeds were washed 3-4 times under running tap water to remove residual EMS and were soaked on blotting paper before sowing.

Isolation of mutants: M1 generation along with control was raised during 2014-15. Seeds from each M1 plants were harvested separately at maturity. A total of 1011M1 plants were harvested. Their plant-to-row progeny consisting of total of 51000 plants was raised in M2 generation during *rabi* 2015-16. All the recommended practices were followed to raise a good crop. Based on visual performance, twenty-six M2 plants superior for seed yield were selected and observations on yield and yield components were recorded. These putative mutants were advanced to M3 generation and sown along with 4 checks namely Pusa bold, BIO 902, Kranti, and Shatabdi during *rabi* 2016-2017 to study their breeding behaviour and yield potential. Yield and yield contributing characters were recorded at maturity. For confirmation of breeding behaviour and yield potential, identified mutants along with 4 checks namely Pusa bold, BIO 902, Kranti, and Shatabdi were raised in M4 generation during 2017-18 in randomized block design with three replications at AICRP on Linseed and Mustard farm, College of Agriculture, Nagpur.

Observations: During 2017-18, data were recorded on 5 randomly selected plants per replication for all quantitative characters namely number of lobes leaf-1, leaf length, leaf width, petal length, petal width, days to maturity, plant height, main shoot length, primary branches plant-1, siliqua length, siliqua beak length, no. of siliqua plant-1 and seed yield plant-1 in each mutant line. Observations were recorded as per DUS UPOV 2011.

Statistical Analysis: Principal component analysis (PCA) and Hierarchical cluster analysis (HCA) analysis were performed using R-software. Principle component analysis using the principal component method was carried out and a principal component with an eigen value more than one was taken. Varimax rotation was used for a clear interpretation of results.

Marker analysis: DNA extraction of ten best-performing mutants along with parent Bio 902 and check Kranti was carried out using the CTAB method and purification was performed using phenol: chloroform. DNA quantification was carried out using a spectrophotometer. Polymerase Chain Reaction (PCR) was undertaken in the thermo cycler. Initial denaturation of DNA was done at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C was performed. Twenty SSR primer sets (Table 1) were used to investigate the genetic relationships and diversity among 10 high-yielding mutants with parent Bio 902 and Kranti. All SSR fragments were scored manually. Polymorphism Information Content (PIC) value was calculated. Distance-based cluster analysis was performed and a dendrogram based on the unweighted pair group method of the arithmetic mean (UPGMA) was constructed using Jaccard's similarity coefficient with the help of DARwin [16].

RESULTS AND DISCUSSION

Genetic diversity is a pre-requisite in the crop improvement program. Seed yield potential in Indian mustard has stagnated and therefore generation of variability through induced mutagenesis looks to be a good option to isolate desirable mutants. We have isolated desirable mutations for seed yield (table 2). The seed yield of mutants was found in the range of 2536- 3276 Kg ha-1 which is 1.4 to 27% higher than the best check Kranti (2501Kg/ha). Siliqua plant-1 has a positive association with higher seed yield (table 3).

Principal component analysis (PCA):

PCA reveals the major contributor to the total variation at each distinct point and the eigen value is often used to determine the number of major principal components to be explained. PCA for the data revealed that the first six principle components (PCs) had eigen values greater than one and altogether explained 74.24% of total accumulated variability (table 4). A maximum eigen value of 2.65 was observed for the first PC and a minimum of 1.10 for sixth PC. The first principal component accounts for the maximum proportion i.e.17.17% of total variability among all variables followed by second (16.17%), third (13.91%), fourth (10.92%), fifth (8.28%), and sixth (7.36%) PCA.

The analysis without rotation of axes failed to load all the variables signifying that it could not offer much information regarding the idea of the correlation between the variables and the principal components. Varimax Rotation was applied and this resulted in the loading of all the variables on different principal components. Factors loadings of different variables thus obtained are presented in table 4. The first principal factor (PF1) is ascribed to four variables i.e. days to maturity, leaf length, leaf width, no. of lobes plant-1and could be designated as maturity factor. PF2 represents for high loadings of three variables i.e. petal length, siliqua beak length, and siliqua density. PF3 has high loadings of two variables i.e. plant height and siliqua plant-1 which could be called as siliqua factor. PF4 has four variables viz. branches plant-1, siliqua angle, siliqua texture, and seed yield plant-1 and could be designated as yield and angle factor as it enabled high loadings of seed yield along with its most important component trait viz. primary branches plant-1 as well as siliqua angle. PF5 has only one variable viz. petal width. Singh et al. (2013) also reported the loading of similar types of variables on a common principal factor (PF) in Indian mustard. It revealed that the number of primary

branches/plant, number of secondary branches/plant, seed yield/plant, days to flowering, plant height, days to maturity, seeds/siliqua, and main shoot length were the major distinct variability contributing traits which accounted for more than half (58.70%) of the total variation (66.98%) in the set of 26 mutants and 4 checks. Thus, the successful transformation of 15 morphological variables into five independent principal factors by means of a grouping of similar types of variables on different principal factors elaborated and explained 66.98% of the variability of the original set. [14] also reported similar results in Indian mustard. [30] observed that the four principal components in 18 genotypes of Indian mustard contributed 86% towards total variability.

Hierarchical cluster analysis (HCA)

The based cluster analysis was performed to see the relationship among mutants (Figure 1), parents, and checks. Total genotypes are classified in eight clusters (Fig. 1). A Maximum number of mutants fall in cluster VIII having 20 mutants (Fig. 1) followed by cluster IV with three mutants, cluster VI with two genotypes, and one each in cluster I,II,III,V,VII. Cluster II, III, VI, VII, and VIII mutants were found completely diverse from the checks and ACNMM 9 was found closely related to checks Pusa Bold and Shatabdi. Among the eight selected mutants, ACNMM 1, ACNMM 3, ACNMM 12, ACNMM 17, ACNMM 20, ACNMM 22, and ACNMM 23 were all found closely related to each other and observed in cluster VIII and ACNMM 9 found closely related with check parent variety Pusa Bold and check Shatabdi. Check Kranti was the only genotype placed in cluster I. The checks Pusa Bold and Shatabdi were placed in cluster IV along with the genotype ACNMM 9 which was a high-yielding genotype. Check BIO 902 was placed in cluster V. Those genotypes that were diverse from checks were placed in cluster VIII.

Marker analysis

Two main aspects of genetic diversity, marker informativeness (polymorphic and overall efficiency of informative fragment detection) and marker performance (overall efficacy of a primer set used in determining polymorphism level, genetic diversity, and discriminatory power) were evaluated. Out of 20 SSR primers screened for 10 mutants, parents, and checks, 12 primers viz., Na14E08, Na10G10, OI11B05, OI12E03, OI10F06, Ni4H05, Ni2E12, Ni2H06, Ni2Co1, Ni4G09b, Ra2DO4, OI10F09 were found monomorphic and eight primers viz., Na12E01, Na10E02, OI10E05, Na12A08, Na12D04, Na12F11, Ra2E12, Ra2A11 were found polymorphic. PCR Product size ranged from 100 to 350bp. 200 SSR markers for genetic diversity analysis in 87 Indian mustard varieties in that they observed unique fingerprints for varietal identification [26].

The polymorphic information content (PIC) values of eight SSR loci were calculated across 10 mutant genotypes and are presented in Table 5. Out of 18 amplified bands, four were monomorphic and 14 were polymorphic. The percent polymorphism varied from 66.67 to 100% with an average of 77%. The maximum numbers of polymorphic bands were three in the Na10E02 primer with 100% polymorphism. Polymorphic information content values ranged from 0.08 to 0.48 with an average of 0.34 per primer combination. The primer Na12A08 observed minimum polymorphism with a PIC Value of 0.27. Among the primers used in the present study, Na10E02 was highly informative since it recorded high PIC, EMR, and MI values of 0.48, 3, and 1.21 respectively. SSR marker-based genetic diversity analysis has also been performed in Indian mustard which differs in Alternaria brassicicola tolerance [7]. In this study, nineteen SSR markers were found to be highly informative out of 41 with a polymorphic information content value > 0.50.

Assessment of genetic diversity on the basis of SSR markers

For enabling better exploitation of genetic resources, it is desirable to know the genetic diversity at molecular levels [13]. Thus, distance-based cluster analysis was performed and a dendrogram based on the unweighted pair group method of the arithmetic mean (UPGMA) was constructed using Jaccard's similarity coefficient (Figure 2). Similar work was also conducted by [28] where genotypes were grouped into four clusters based on genetic distances. Also, [29] used both morphological and molecular markers for diversity analysis and [24] and [5] revealed the grouping of various genotypes into various clusters. The result suggested that there are 3 clades. The first clade consists of four mutant lines along with Bio902. The second clade consists of 5 mutant lines, whereas the third clade consists of one mutant which is the variety, Kranti. The mutants were grouped into three major clusters; cluster 1 separated into two sub-clusters having ACNMM9, ACNMM4, ACNMM17, ACNMM19 in one sub-cluster and the check variety BIO-902 into separate sub clusters indicating these mutant lines were closely related to BIO-902.

Similarly, cluster 2 was also divided into two sub-clusters having ACNMM23, ACNMM22, ACNMM13, ACNMM14 in one sub-cluster and ACNMM in the other whereas ACNMM7 separated into 2 sub-clusters indicating these mutant lines were distantly related to any of the given parents and check variety due to variation caused by mutagenesis and were found independent. Cluster 3 was again separated into 2 sub-clusters one with check variety Kranti and another with ACNMM15 which indicates ACNMM15 is closely related to check variety Kranti. Similar work was also conducted by Singh et. Al. (2016) where used the 150 genic-SSR markers, among them 65 markers (43.3%) were found polymorphic and amplified products of varying sizes in the range of 100-400 bp while 85 SSRs (56.6%) were monomorphic. Diversity analysis conducted by [15] using SSR markers showed a maximum PIC of 0.252 for primer Ra1F03.

CONCLUSION

The observations suggested that SSRs are proficient in evaluating genetic variation and relationships among different mutants. Further, the findings of this study will be useful for DNA fingerprinting, and varietal identification which could help during background selection for marker-assisted backcross breeding programs. Out of the 10 superior high-yielding mutants selected for molecular analysis, mutant ACNMM9 was similar to checks both in morphological as well as molecular studies. Mutants ACNMM 4, ACNMM 17, ACNMM 19, and ACNMM 15 were found in distinct clusters as of checks morphologically but in the same cluster as that of checks in molecular analysis.

Future scope of the study

Mutants ACNMM 23, ACNMM 22, ACNMM 14, ACNMM 7, and ACNMM 13 were found in different clusters than checks both morphologically as well as in molecular studies. Hence, those

genotypes which were diverse morphologically and in molecular studies must be further be evaluated in the yield trials.

Conflict of interest

Authors has no any conflict of interest.

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Fig 1. Dendrogram showing different clusters of 30 mutants along with checks

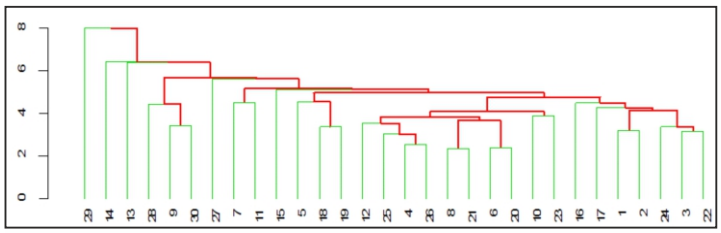

Fig 2.Dendrogram derived from banding pattern of SSR marker analysis of 12 genotypes

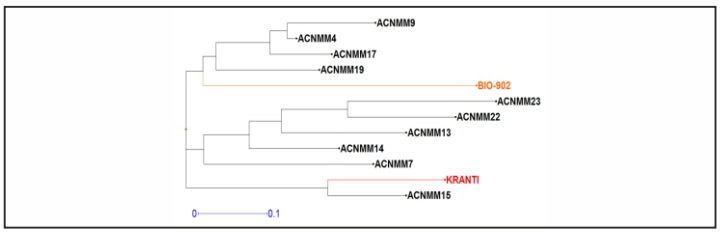

Table 1: List of 20 SSR markers used in the present study

S. No.	Primer name	Forward primer	Reverse primer
1	Na12E01	ATTCCATGACTCCATTGTC	AAATCCCTTGTCTCTGTCG
2	Na10E02	TCGCGCATGTAATCAAAATC	TGTGACGCATCCGATCATAC
3	Na14E08	TTACTATCCCCTCTCCGCAC	GCGGATTATGATGACGCAG
4	Na10G10	TGGAAACATTGGTGTTAAGGC	CATAGATTCCATCTCAAATCCG
5	OI10E05	GCCAGAAACAGGAGAAATGG	GAAGCCGAAGAAAATAAGCG
6	OI11B05	TCGCGACGTTGTTTTGTTC	ACCATCTTCCTCGACCCTG
7	OI12E03	CTTGAAGAGCTTCCGACACC	GACGGCTAACAGTGGTGGAC
8	OI10F06	CATTGGTTTAGTCATTTCGTCG	AATTCAAAAACTGCCGAACG
9	Ni4H05	GAAAACACACCACCAAACCC	CCATAGAGTTCTTGTTTCTCTCTC
10	Ni2E12	TTATCTGCTTGTCTTGGGGC	AAGGAAATCGTCTCACTTGG
11	Ni2H06	CATCAGATCCGACGAAATCC	TCCTTTGGACTGTGAAAAACG
12	Ni2C01	GAGTATGAGAGATGGGAATCCG	GACTGAGCAGCTTGGAGACC
13	Ni4G09b	AAAAACTGGACCCAATTCC	GGTTAGGTCATAAACCCAAAGC
14	Na12A08	AACACTTGCAACTTCATTTTCC	CATTGGTTGGTGAATTGACAG
15	Na12D04	ACGGAGTGATGATGGGTCTC	CCTCAATGAAACTGAAATATGTGTG
16	Na12F11	CCTCACATCGTCTTCTTCATCC	TCACATCAGTCCATGGTTCC
17	Ra2DO4	TGGATTCTCTTTACACACGCC	CAAACCAAAATGTGTGAAGCC
18	Ra2E12	TGTCAGTGTGTCCACTTCGC	AAGAGAAACCCAATAAAGTAGAACC
19	Ra2A11	GACCTATTTTAATATGCTGTTTTACG	ACCTCACCGGAGAGAAATCC
20	OI10F09	AGAGAGCGAGATTGATTGGC	AAACGACCACGAGTGATTCC

Table 2: Seed yield and its attributes of stable mutant lines compared with parent and checks

Sr. No.	Name of mutant / varieties	Pedigree of mutant	Yield kg/ha	Days to maturity	Plant height (cm)	No. of branches	No. of siliquae	1000 Seed Wt (g)	Oil content (%)
1.	ACNMM1	1000GY M-2-7-5-1	2406	106	176	7	210	6	37.95
2.	ACNMM2	1000GY M-2-7-5-2	1481	105	175	6	140	5	39.07
3.	ACNMM3	1100GY M-3-104-23-3	**3166 II**	106	175	5	282	5	37.53
4.	**ACNMM4***	1100GY M-3-104-23-4	2663	106	188	7	233	5	37.04
5.	ACNMM5	1200GY M-4-12-41-1	**2956 III**	107	178	5	262	6	37.18
6.	ACNMM6	1300GY M-5-17-11-1	2639	105	189	6	226	5	37.09
7.	**ACNMM7***	1300GY M-5-17-3-5	2536	104	172	6	220	5	36.08
8.	ACNMM8	1300GY M-5-18-31-1	2302	104	194	6	195	5	38.54
9.	**ACNMM9***	1300GY M-5-18-31-4	2191	103	190	7	186	4	36.44
10.	ACNMM10	1300GY M-5-107-28-5	2633	103	199	5	230	5	35.94
11.	ACNMM11	1300GY M-5-107-34-3	2156	106	190	6	184	6	34.20
12.	ACNMM12	(900GY+EMS) M-6-109-12-8	2840	105	187	6	259	4	38.75
13.	**ACNMM13***	(1200GY+EMS) M-9-35-60-1	2549	104	167	6	221	5	35.16
14.	**ACNMM14***	(1200GY+EMS) M-9-38-38-4	**3276 I**	104	162	6	294	6	39.41
15.	**ACNMM15***	(1300GY+EMS) M-10-44-34-5	1499	102	277	6	145	6	37.14
16.	ACNMM16	900GY M-11-47-14-5	1707	105	175	6	167	5	37.49
17.	**ACNMM17***	900GY M-11-51-36-1	2655	101	166	6	238	5	38.91
18.	ACNMM18	900GY M-11-51-36-2	1564	104	157	5	150	5	37.36
19.	**ACNMM19***	900GY M-11-51-36-6	1730	100	170	5	172	4	39.15
20.	ACNMM20	1100GY M-12-60-23-12	2001	100	184	6	177	5	38.94
21.	ACNMM21	1300GY M-15-70-47-15	2451	99	185	6	217	5	37.89
22.	**ACNMM22***	1300GY M-15-68-51-5	2788	97	162	5	243	5	37.78
23.	**ACNMM23***	1300GY M-15-68-51-7	2837	97	187	6	235	6	38.17
24.	**ACNM 24**	1300GY M-15-68-51-12	1474	100	158	5	169	5	37.79
25.	ACNMM25	(900GY+EMS) M-16-74-34-11	2019	101	170	5	181	6	37.34

26.	ACNMM26	(1300GY+EMS) M-19-126-60-14	2779	104	193	6	234	5	37.59
27.	**BIO-902***	Check	2345	106	183	6	191	5	37.20
28.	Pusa Bold	Check	2445	100	185	5	214	6	46.4
29.	**Kranti***	Check	2501	101	177	5	226	5	37.18
30.	Shatabdi	Check	2334	98	176	6	196	4	37.18
G. Mean	1934								
SE ±	100.62								
CD	285.05								
CV 5%	12.60								

Mutants and varieties used for molecular marker analysis.

Table 3. **Estimates correlation coefficients of yield and yield contributing traits**

Characters	Seed yield	Days to maturity	Plant height at maturity	Number of branches plant^{-1}	Number of siliqua plant^{-}	1000 seed weight	Oil content (%)
Seed yield	**1.00**	0.061	-0.253	-0.021	0.980**	0.108	0.019
Days to maturity		1.00	0.024	0.262	0.080	0.107	-0.303
Plant height at maturity			1.00	0.206	-0.274	0.192	-0.076
Number of branches plant^{-}				1.00	-0.094	-0.106	-0.217
Number of siliqua plant^{-}					**1.00**	0.096	0.059
1000 seed weight						**1.00**	0.140
Oil content (%)							**1.00**

Table 4. **Total variance of different principal components**

Principal Component (PC)	Eigen value	Percent variability	Cumulative % variability
1	2.65	17.70	17.70
2	2.42	16.17	33.87
3	2.08	13.91	47.78
4	1.63	10.92	58.70
5	1.24	8.28	66.98
6	1.06	7.36	74.24

Table 5 . **Factor loadings of characters with respect to different principal factors (verimax rotation)**

Traits	PF1	PF2	PF3	PF4	PF5
Branches^{-1} plant	-0.343		0.211	0.761*	-0.109
Days to maturity	0.391*	0.211		-0.423	
Plant height			0.578*		0.150
Leaf length	0.994*				
Leaf width	0.923*			-0.132	0.165
Siliqua length	-0.235	-0.118			-0.720
Number of lobes	0.102*	-0.989			
Petal length	-0.289	0.159*		-0.535	0.123
Petal width	0.112	-0.221	-0.165	0.318	0.510*
Siliqua angle	0.106			0.158*	-0.445
Siliqua beak length		0.677*	0.194		-0.162
Siliqua density	0.398	0.406*			0.153
Siliqua^{-1} plant	-0.266	0.151	0.938*	0.147	
Siliqua texture			-0.319	0.367*	0.247
Seed Yield	-0.226	0.107	-0.405	0.502*	0.171

Table 5 . Details of informative markers based on PIC, EMR and MI

Sr. No.	Marker	Number of Fragments				PIC	EMR	MI
		Total	Monomorphic	Polymorphic	%Polymorphism			
1	Na12E01	2	0	2	100	0.38	2	0.76
2	Na10E02	3	0	3	100	0.40	3	1.21
3	OI10E05	2	1	1	50	0.48	1	0.48
4	Na12A08	3	1	2	66.67	0.27	2	0.54
5	Na12D04	2	0	2	100	0.39	2	0.79
6	Na12F11	2	1	1	50	0.48	1	0.48
7	Ra2E12	2	1	1	50	0.08	1	0.08
8	Ra2A11	2	0	2	100	0.27	2	0.53

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