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Genetic diversity assessment in Indian mustard (*Brassica juncea* L) for Alternaria blight tolerance using SSR markers

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Abstract

Using SSR markers, genetic diversity in 20 Indian mustard [*Brassica juncea* (L.) Czern & Coss.] genotypes (14 advanced breeding lines, 4 tolerant germplasm accessions, 1 tolerant check, PAB 9511, and a susceptible check Varuna) was evaluated for Alternaria blight tolerance. Genotypes DRMR 2805 and DRMR 2806 found tolerant against Alternaria blight in the field conditions, gave the lowest AUDPC values of 10.0 and 8.25, and average lesion size of 4.5 mm and 4.2 mm, respectively. Genetic diversity evaluated among 20 Indian mustard genotypes using 25 *Brassica species*-derived-SSR markers, resulted into polymorphic amplicons producing a total of 88 alleles, ranging from 2 to 6 with an average number of 3.52 alleles/locus. The PIC values ranged from 0.1073 (MB5) to 0.8173 (BRMS-017) with an average of 0.3503. The UPGMA dendrogram divided all 20 genotypes into 2 main clusters. Genotypes DRMR 2805 and DRMR 2806 shared common cluster with PAB 9511, and were at par in their disease score indices to PAB 9511. The tolerant genotypes DRMR 2805 and DRMR 2806 identified in the present study can be used for introgression of Alternaria blight tolerance into the elite mustard cultivars in future breeding programmes.

Key words: Alternaria blight, *Brassica juncea*, genetic diversity SSR markers

Introduction

Indian mustard [*Brassica juncea* (L.) Czern & Coss.] is the major economically important oilseed crop contributing about 80 per cent of the total rapeseed-mustard acreage in India. India is the second largest rapeseed-mustard growing country after China, occupying 20.23% area and contributing 11.7% share to the global production (Kumar, 2014). The overall production and productivity of mustard is stagnated due to its susceptibility to various biotic and abiotic stresses. Among the biotic stresses, *Alternaria brassicae* (Berk.) Sacc., is the most destructive pathogen of Indian mustard causing upto 35 % yield losses (Meena *et al*., 2002). No proven source of resistance against Alternaria blight disease has been reported (Meena *et al*., 2010). Although resistance against Alternaria blight has been identified in a number of wild crucifers including *Sinapis alba*, *Camelina sativa* and *Capsella bursa-pastoris* (Sharma *et al*., 2002), transfer of genes for disease resistance from wild sources has been proven difficult due to sexual incompatibility. Ovary culture and protoplast fusion for transferring Alternaria resistance from *S. alba* to rapeseed have also proven unsuccessful (Chevre *et al*., 1991).

The utilization of a species into any crop improvement breeding programme depends not only on the degree of genetic diversity it holds, but also on the precise information regarding genetic divergence and relatedness among breeding materials. At present, molecular markers are being successfully used to decode genetic diversity among populations. Amongst the various types of molecular markers used, Simple Sequence Repeat (SSR) markers are the most preferred one because of their higher

reproducibility, co-dominance nature, abundance, wide distribution throughout the genome, easy scorability, and multi-allelic variation (Powell *et al*., 1996). Simple sequence repeat markers have been used for genetic diversity analysis in a number of crops including Indian bread wheat (Mir *et al*., 2011), rice (Rahman *et al*., 2012), and maize (Sivaranjini *et al*., 2014).

Using SSR markers, the present investigation was, therefore, carried out to evaluate genetic diversity in Indian mustard genotypes for tolerance against Alternaria blight. Identification of such genetically diverse or related genotypes will help in introgression of Alternaria blight tolerance into other high yielding cultivars.

Materials and Methods

Plant material

Plant material used in the present study comprised of 20 Indian mustard genotypes including 14 advanced breeding lines (selected from 214 single plants of F_8 generation derived from 11 crosses involving Alternaria blight tolerant genotypes), 4 tolerant Indian mustard germplasm accessions, 1 tolerant check (PAB-9511) and 1 susceptible check (Varuna, Table 1).

Table 1. List of *Brassica juncea* genotypes along with their pedigree and Alternaria blight disease response

Genotype name	Parentage	Disease response			
		AUDPC 2011-12	AUDPC 2012-13	Mean AUDPC value	Average lesion Size (mm)
DRMR-2800	GSL 1 x BIO902	17.7	14.7	16.2	13.7
DRMR-2801	GSL 1 x BIO902	10.8	15.4	13.1	11.2
DRMR-2802	BEC 107 x HYOLA	13.0	17.2	15.1	10.3
DRMR-2803	EC399299 x EC 399301	20.7	18.9	19.8	12.3
DRMR-2804	PAB 9511 x PAB 9534	12.3	27.0	19.65	10.7
DRMR-2805	PAB 9511 x EC 399313	7.7	12.3	10	4.5
DRMR-2806	EC 399299 x EC 399301	4.6	11.9	8.25	4.2
DRMR-2807	JMM 915 x EC 399299	18.2	15.4	16.8	10.3
DRMR-2808	EC 399299 x EC 399313	27.0	22.1	24.55	9.2
DRMR-2809	EC 399299 x EC 399313	15.4	21.7	18.55	8.7
DRMR-2810	EC 399299 x JMM 915	32.2	30.8	31.5	9.8
DRMR-2811	PAB 9534 x EC 399301	27.0	27.0	27	10.1
DRMR-2812	JMM 915 x EC 399313	18.9	29.4	24.15	13.0
DRMR-2813	PAB 9534 x EC 399299	16.5	27.0	21.75	9.3
PAB-9511	(RC 78 x Krishna) x (PHR 1 x Poorbiraya)	9.1	13.0	11.05	3.8
EC399296	Exotic	23.5	26.6	25.05	10.0
EC399299	Exotic	23.5	30.8	27.15	11.2
EC399000	Exotic	22.1	28.7	25.4	13.4
EC399301	Exotic	21.7	29.4	25.55	8.4
Varuna	Selection from Varanasi Local	83.5	128.5	106	16.5

Disease screening

Inoculum preparation and spray

Field experiments were carried out at DRMR, Bharatpur. In both 2011-12 and 2012-13 *Rabi* seasons, seeding was done on 25th November following the standardized protocol of Meena *et al.* (2012). *Alternaria brassicae* isolated from *B. juncea*-infected plants (Bharatpur local) was used for inoculating all field experiments. Forty five and 60 days after sowing, plants were spray-inoculated with spore suspension prepared from 10-day-old freshly revived pathogen culture.

Disease scoring

On five lower leaves of each of the 10 randomly-selected plants/plot, disease incidence (appearance of symptoms) was recorded. Disease severity ratings were computed following the scale of Conn *et al*. (1990), and data statistically analysed using ANOVA.

Genotyping of Indian mustard accessions using SSR markers

Genomic DNA isolation, purification and quantification

Genomic DNA from fresh young leaves of 20 Indian mustard genotypes was isolated using the protocol reported by Thakur *et al*. (2013). After assessing the quality of DNA by gel electrophoresis on 0.8% agarose gel, DNA was quantified following the manufacturer's instructions and the protocol given by Pharmacia Biotechnology DyNA Quant Tm 200 fluorometer.

PCR amplification

A total of 65 SSR markers derived from various *Brassica species* (*B. nigra*, *B. rapa*, *B. oleracea* and *B. napus*) were used for evaluating genetic variability among the 20 Indian mustard accessions. For SSR marker analysis, the genomic DNA was amplified in a 25 µl reaction volume containing 50 ng DNA, 1X PCR buffer, 0.2 mM of each dNTP, 2.0 mM $Mgcl_2$, 1.0 U Taq polymerase (MBI Fermentas, USA) and 400 µM primer using a thermal cycler (Verity 96-w Thermal Cycler, ABI, USA). The first amplification cycle consisted of initial denaturation at 94°C for 5 min followed by 45 cycles each of 30 sec at 94°C for denaturation, primer annealing at 55°C for 30 sec, primer extension at 72°C for 45 sec, and a final extension step at 72°C for 7 min. After the completion of PCR amplification, the samples were kept at -20°C until gel electrophoresis. PCR-amplified products were electrophoretically separated on 3.5% MetaPhor agarose gel containing 0.01% ethidium bromide, prepared in 1xTAE (Tris-Acetic acid-EDTA). The gel was run for 3 hrs at 80V. After electrophoresis, the amplification products were visualized in a gel documentation system (IG/LHR, Syngene, UK). At least, two independent PCR amplifications were performed for each marker.

Data analysis

For disease screening, data for initial appearance of Alternaria blight symptoms, and temporal progression of disease on the leaves of all genotypes were recorded. Per cent disease severity (PDS) was recorded on leaves once a week until harvest on 10 randomly selected tagged plants in each plot, and area under disease progress curve (AUDPC) was computed using the formula of Wilcoxson *et al*. (1975) as follows:

$$AUDPC = \sum_{i=1}^{k} 1/2\ 1(yi + yi\text{-}1) \times d$$

Where yi is the disease incidence at ith day of evaluation, k is the number of successive evaluations, and d is the interval between i and i-1 evaluation of the disease.

For molecular marker analysis, scoring was done for clear, unambiguous amplicons, and their sizes were determined by comparing with 100 bp DNA ladder. Based on the presence (0) or absence (1) of amplicons, a binary data matrix was created and used to calculate Jaccard's similarity coefficient. Cluster analysis among the genotypes was based on Jaccard's similarity coefficients using UPGMA (Rohlf, 2000) and NTSYS-pc, version 2.02e (Applied Biostatistics) software.

Results and Discussion

Disease screening

In all genotypes except Varuna, disease symptoms first appeared 90-94 days after sowing; in Varuna,

symptoms first appeared 84 days after sowing. Disease severity and disease symptoms increased at a faster pace on the older leaves. Increased disease intensity in older rapeseed-mustard plants has also been reported by Sinha *et al*. (1992). Lower AUDPC values indicate slower progression of disease in leaves of tolerant genotypes. Genotypes DRMR 2805 and DRMR 2806 with AUDPC values of 7.7 and 4.6 in 2011-12, and 12.3 and 11.9 in 2012-13, respectively, were tolerant with values similar to tolerant check PAB 9511 (Table 1). Using AUDPC values as indicator of tolerance to Alternaria blight, Kumar and Kolte (2001) also reported highest AUDPC value of 106 in susceptible cultivar Varuna and lesion size of 16.5 mm. The tolerance to Alternaria blight exhibited by DRMR 2805 is because of having highly tolerant genotype PAB 9511 as one of its parents (Patni *et al*., 2005) (Table 1). In a similar study under field conditions, Kumar and Kolte (2006) also reported two Indian mustard genotypes, PR 8988 and PR 9024, tolerant to Alternaria blight.

Genetic diversity evaluation studies

Following preliminary screening of 20 Indian mustard genotypes with 65 SSR markers, 25 SSRs producing clear, distinguishable, and unambiguous bands were chosen for genetic diversity evaluation; remaining primer pairs did not generate any amplification product or stable banding patterns. A total of 88 alleles were obtained and all the primer pairs resulted into polymorphic amplicons (Table 2). The number of alleles ranged from 2 to 6 with an average number of 3.52 alleles/locus. The observed average number of alleles in the present study was lower than those reported in most other studies on Indian mustard. Using 134 SSR markers, Vinu *et al.* (2013) detected 2-8 alleles (with an average of 4 alleles) per locus in 44 Indian mustard accessions. In a genetic diversity analysis with Indian mustard genotypes and SSR markers, Parida *et al.* (2010) reported only 2 alleles per locus, where the fragment size varied from 100 to 2000 bp.

Table 2: Summary of SSR amplified products

Total number of primer pairs tested	25
Number of polymorphic primers	25
Total number of polymorphic bands	81
Total number of monomorphic bands	7
Total number of bands	88
Size of amplified products (bp)	100-2000 bp
Percent polymorphism	92.04%

The mean polymorphic information content (PIC) values provide an estimate of the discriminatory power of a locus by taking into account the number of alleles that are expressed, as well as, the relative frequencies of the alleles. In our studies, the PIC values ranged from 0.1073 (MB5) to 0.8173 (BRMS-017) with an average of 0.3503 (Table 3);

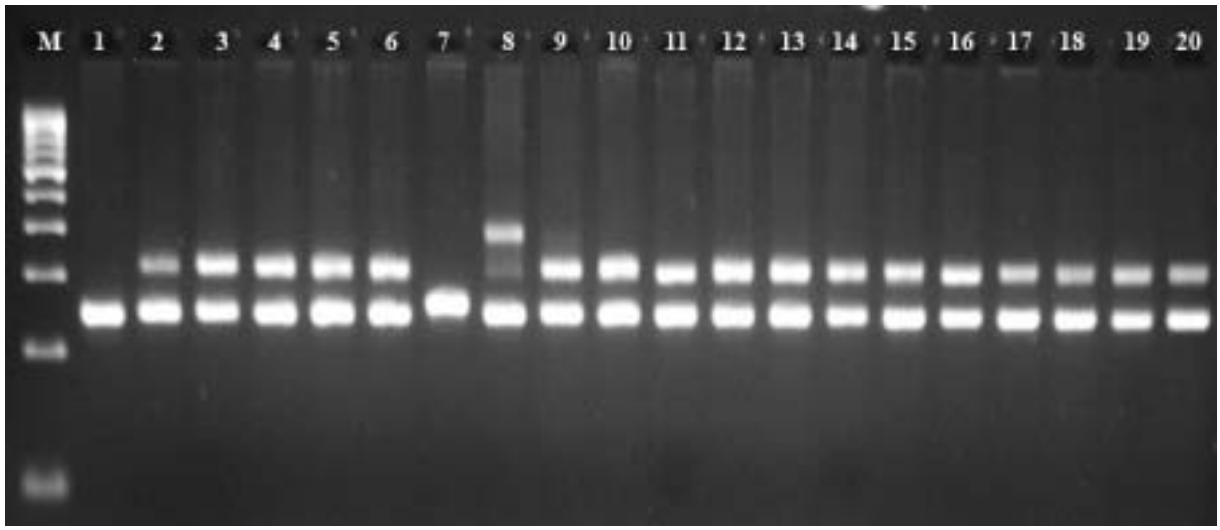

Figure 1. A typical banding pattern of SSR marker (BrgMS339) in 20 Indian mustard genotypes M-100bp DNA ladder, 1-20: *Brassica juncea* genotypes in the order as given in Table 1.

Table 3. SSR markers along with their sequence, Tm, number of bands produced, size range, percent polymorphism and PIC values

Marker Name	Forward Primer sequence (5'-3')	Reverse Primer sequence (3'-5')	Tm (°C)	Total no. of bands	No. of poly-morphic bands	Range of amplified products (bp)	Percent poly-morphism	PIC value
BRMS-003	ACGAATTGAATTGGACAGAG	CAGATGGGAGTCAAGTCAAC	54	5	5	280-600	100%	0.4432
Ol10B11	AAAATGTGAGGCTGTTTGGG	TTTCGCAGCAGTAAACATGG	53	3	3	280-600	100%	0.5421
MB5	AACATCTTTTTGCGTGATAT	AATAGCATTGAAGCCTTAC	55	6	5	200-2000	83.3%	0.1073
NA10-C01c	TTTTGTCCCACTGGGTTTTC	GGAAACTAGGGTTTTCCCTTC	54	3	3	280-600	100%	0.5674
BRMS-017	GGAAAGGGAAGCTTCATATC	CTGGAAAGCATACACTTTGG	54	3	3	200-350	100%	0.8173
BRMS-030	TCAGCCTACCAACGAGTCATAA	AAGGTCTCATACGATGGGAGTG	52	2	1	250-280	100%	0.2138
BN6A3	GCTACCCACTCATGTCCTCTG	CCAAGCTTATCGAATCTCAGCTA	54	5	4	250-280	80%	0.6796
BRMS-033	GCGGAAACGAACACTCCTCCCATGT	CCTCCTTGTGCTTTCCCTGGAGACG	51	5	5	250-300	100%	0.6943
Ni2B03	ACTTCTTGCCCTCCTCACC	AAATACTCACTGCAATACCCAGG	52	3	3	250-350	100%	0.5301
MR52a	TCGACATGGATTCTACCAAA	GAACTTGCAAGCTGCAATTA	55	4	4	280-600	100%	0.6796
BRMS-011	GAACGCGCAACAACAAATAGTG	CGCGTCACAATCGTAGAGAATC	54	4	4	200-2000	100%	0.6802
Ni2D10	GATGCCCCAAATCTGTTACG	CAATTCGTGAAAAATAGCCG	53	2	2	250-600	100%	0.6637
Ni3C05	TTTCGTGCTTTGGTGTGAAG	TCCCCAAATCGAACCATAAG	54	4	4	200-600	100%	0.1356
NI02-D08a	TTTAGGGAAAGCGAATCTGG	ACAACAACCATGTCTTCCG	54	2	2	250-280	100%	0.5282
BrgMS710	ATCATCATCGTCTTCCTCTTCC	CTGCCCAAAATAAACACAGTCA	55	3	2	280-600	66.6%	0.1785
NI03-H07a	GCTGTGATTTTAGTGCACCG	AGCCGTTGATGGAATTTTTG	53	3	2	250-300	66.6%	0.6637
BrgMS339	CTACCTGAAGATGACCCAGACG	GCATACAAACCTCGTCCTAAGC	53	4	4	200-2000	100%	0.3452
BrgMS329	TCATCATCATAGCTTTCGCTTC	AAAACCTCCTCCTCCTCCTC	51	3	3	100-300	100%	0.2342
BrgMS1237	ATCAAAAGATGCAGGGAGAGAG	GTCCTCAATGGATTACACATGC	51	2	1	250	100%	0.2138
BrgMS787	CCATCTCAGCTCTATCTACCAAAA	TCAAAACACCGAGTAAACTGGA	55	2	1	100-300	100%	0.2138
NA10-C01a	TTTTGTCCCACTGGGTTTTC	GGAAACTAGGGTTTTCCCTTC	54	2	2	250-350	100%	0.3645
BN25A	CACGTGGTATGTTGGTATTGGG	TGATTCTCCTCCGACGCATGC	53	4	4	100-300	100%	0.6894
sORA43	GCGCGTGTGGGATCAGAA	CTTCTCCACCGTCGATCG	53	5	5	250-350	100%	0.1268
BrgMS70	TACAATGAAGATGTGATCCCGA	CGTGCGTGAGCTTATCAATACA	52	5	5	200-2000	100%	0.2127
NI-F02a	TGCAACGAAAAAGGATCAGC	TGCTAATTGAGCAATAGTGATTCC	54	4	4	250-350	100%	0.1379
			Average	3.52	3.24		92.04	0.3503

BRMS-017 was found to be the most informative marker depicting the highest PIC value of 0.8173 followed by BRMS 033, BN25A and BRMS 011. Typical banding pattern generated by the representative SSR primer (BrgMS339) is presented in Fig. 1. The average PIC values are lower than those reported by Turi *et al.* (2012) in *B. juncea* germplasm collections (0.46). The markers reported in this study are more discriminating than those reported by Gupta *et al*. (2014); the mean PIC value was higher (0.3503) in our study than those in the later (0.281), where they used EST-SSRs to estimate the genetic diversity in 27 Indian mustard genotypes cultivated in northern India. Lower number of alleles per locus, and lower PIC values may be attributed either to the use of less informative SSR markers, or the presence of lesser genetic diversity among the tested genotypes. Several other researchers have also used SSR markers for diversity analysis in *Brassica species* (Abbas *et al*., 2009; Celucia *et al*., 2009).

The Unweighted Pair Group Method with Arithmetic mean (UPGMA) dendrogram was constructed using Jaccard's similarity coefficients based on SSR marker data generated on 20 Indian mustard genotypes (Fig. 2). The similarity coefficients varied from 0.75 to 1.0. UPGMA grouped all 20 Indian mustard genotypes into two main clusters at a similarity coefficient of 0.75. Cluster I is further divided into two sub clusters: sub-cluster IA consisted of thirteen genotypes and sub-cluster IB consisted of four genotypes namely EC 399296, EC 399299, EC 399000 and EC 399301. Cluster II consisted of three genotypes namely DRMR 2805, DRMR 2806 and PAB 9511. The highest values for genetic similarity coefficient (0.988) were found between genotypes DRMR 2804 and DRMR 2812. The least similarity coefficient value (0.90) was found between DRMR 2805 and DRMR 2806 and PAB 9511; this exhibited the presence of maximum genetic diversity between these groups of genotypes. DRMR 2805, DRMR 2806 and PAB 9511 fall into the same group of genotypes which showed highly tolerant reaction to Alternaria blight. DRMR 2805 and DRMR 2806, identified as the potential Alternaria blight tolerant genotypes, can now be used as donors for introgression of Alternaria blight tolerance in elite mustard cultivars.

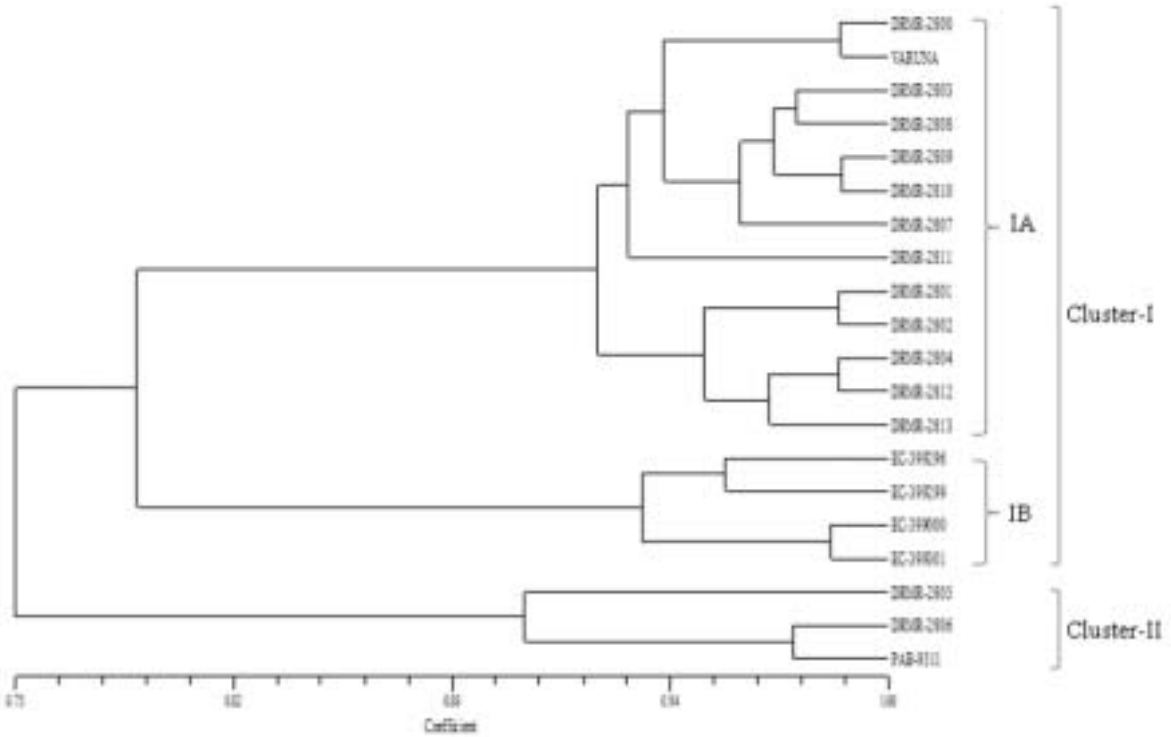

Figure 2. UPGMA dendrogram showing genetic relationship among 20 Indian mustard genotypes

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