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STUDIES ON GENETIC DIVERSITY AMONG ALTERNARIA BLIGHT TOLERANT INDIAN MUSTARD GENOTYPES USING SSR MARKERS

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ABSTRACT

Brassica species represents one of the most important oilseed crops in India; nevertheless their genetic diversity is still to be studied. An understanding of genetic diversity is essential for proper utilization of genotypes in the target oriented research programmes. 37 diverse Indian mustard genotypes including recombinant lines, indigenous lines, exotic lines with Alternaria blight tolerant (PAB 9511) and susceptible check (Varuna) were utilized. Ten A and C genome specific SSR markers were used for the development of molecular profile. A total of 41 bands were amplified that exhibited 97.56% polymorphism. Genotypes were grouped into 5 clusters. Presence of unique band helped in the identification of specific genotype. PAB-09-1, PAB-05-16, PAB 09-11, PAB-09-9, EC 399312 and EC399301shared common cluster with PAB 9511 and were at par in their disease score index to PAB 9511 as well. So these primers after validation can be used as maker for Alternaria bilght screening.

INTRODUCTION

The genetic diversity is of great significance for planning an efficient breeding programme for the improvement in any crop. *Brassica* species commonly called as rapeseed-mustard are the third most important oilseed crop of the world after soybean and plam. These are second important oilseed crops of Indian, next to soybean. India is one of the largest rapeseed-mustard growing country occupying first position with 20.23% area and second position with 11.7% share to the global production (USDA, 2012).

The maximum utilization of any species for breeding and its adaptation to different environments depend on the level of genetic diversity it holds. Genetic distance among parents may be attributed to their differences for number of genes and their functional relations in a given environment (Nei, 1976). Evaluation of genetic divergence and relatedness among breeding materials has significant implications for the improvement of crop plants. Knowledge on genetic diversity in *B. juncea* could help breeders and geneticists to understand the structure of germplasm, predict which combinations would produce the best offsprings (Hu *et al*., 2007) and facilitate to widen the genetic basis of breeding material for selection (Qi, Yang and Zhang, 2008). Among various markers available for genetic analysis in plants, molecular markers are more efficient, precise and reliable in discriminating closely related species and cultivars (Mishra *et al*., 2011). It has been reported previously that SSRs are a valuable tool for characterising germplasm in various crop species, including Brassica species (Saal *et al*., 2001). This is due to their properties of high reproducibility, co-dominance, abundance, wide dispersal throughout the genome and multi-allelic variation (Powell *et al*., 2006). Microsatellite or SSR (simple sequence repeat) markers have been developed and characterized for use with genetic studies of Brassica species (Lowe *et al*., 2004). Database derived SSR markers were used for cultivar differentiation in B. Oleracea (Louarn *et al*., 2007).

Productivity of rapeseed-mustard can be seriously affected by white rust [Albugo candida], Alternaria blight [Alternaria brassicae], powdery mildew [Erysiphe sp.] and stem rots (Pathak and Godika, 2002). Leaf blight caused by Alternaria brassicae (Berk.) Sacc. is an important disease of mustard (Brassica juncea (L.) Czern and Coss) and rapeseed (*B.campestris* L.var.toria) in the Indian sub-continent (Kolte *et al*., 1987).

The present investigation was carried out with an objective to study the diversity level among the genotypes. Identification of marker for *Alternaria* blight was another thrust area of the present study. Genetic distances will further help in identifying genetically diverse genotypes, which then can be utilized in creating valuable selectable variation.

MATERIALS AND METHODS

Plant Material

The study was carried out with 37 diverse genotypes of Indian mustard including Kranti as agronomically superior background, Varuna as susceptible check and PAB 9511 as tolerant check. The material (Table 1) comprised of 24 recombinant inbred lines (RILs), 6 exotic lines and 4 indigenous lines selected on the basis of their disease response. The experiment was conducted at Norman. E. Borlaug Crop Research Center of G. B. Pant University of Agriculture and Technology, Pantnagar in Randomised block design with three

replications.

Isolation of Genomic DNA

Fresh leaves from healthy plants of each mustard genotype were collected and DNA was extracted by following Saghai-Maroof *et al*. (1984) with slight modifications. The DNA was quantified using 0.8% agarose gel.

SSR Marker Assay

The strains were genotyped using 10 SSR primers (Table 2). PCR was performed in a 25 μL reaction with 2.5 μL of 10X buffer, 2.0 μL of dNTPs, 1μL of primer (100ng/μL), 0.3 μL of taq polymerase and 1μL of template DNA (20ng/μL). The PCR protocol comprised of the initial denaturation of 94ºC for 5 min. This was followed by repeat of 39 cycles of denaturation at 94ºC for 1 min, annealing at 46-51ºC for 2 min and extension at 72ºC for 2 min followed by final extension at 72ºC for 10 min and stored at 4ºC. Amplification product and loading dye were mixed in 10:1 ratio and fractioned on 2.5% agarose gel. Electrophoresis was performed for 2.5h with constant voltage of 80 V.

SSR Data Scoring and Analysis

Gel photographs were used to score the data manually and independently for analysis. Scoring of gels on 0-1 pattern was done for further analysis. Presence of amplified products was scored as 1 and its absence as '0' for all the genotypes and primer combinations (Fig.1). These data matrix were entered into NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System Programme). The data were analyzed using SIMQUAL (Similarity for qualitative data) which generate Jaccard's similarity coefficients. These similarity coefficients were used to construct dendrogram using the unweighted pair-group method with Arithmetic average using (UPGMA) NTSYS program. The same programme was used for the coordinate analysis. PowerMarker software was used to calculate the PIC values. Boot strap values were calculated by using WINboot software. Disease score index of all 37 genotypes were also recorded.

RESULTS

A total of 10 simple sequence repeat primers were used to assess the molecular diversity of 37 mustard genotypes. None of the primers was found to be monomorphic and five unique bands were also observed by primers NA10-CO1a, NA10-C01c NI03-H07a, OL10-A03a and RA02-E01a. A total 41 loci were amplified that exhibited 97.56% polymorphism.

Based on the SSR marker data the Jaccard's similarity coefficients were estimated between pair of lines. The similarity coefficients were found to vary from 0.609 to 0.975. The highest value for genetic similarity (0.975) was found between EC 399302 and IC 414322 (0.975), between EC 399302 and IC 414322 (0.975), between PAB 09-6 and PAB 09-2 (0.975)

Table 1: Genotypes used for diversity analysis

S.No.	Genotype	Nature	S no.	Genotype	Nature
1.	PAB 09-1	Recombinant line	20.	PAB 2005-21	Recombinant line
2.	PAB 09-2	Recombinant line	21.	PAB 9721	Recombinant line
3.	PAB 09-3	Recombinant line	22.	PAB 2005-14	Recombinant line
4.	PAB 09-4	Recombinant line	23.	PAB 2005-11	Recombinant line
5.	PAB 09-5	Recombinant line	24.	PAB 2005-10	Recombinant line
6.	PAB 09-6	Recombinant line	25.	EC 399299	Exotic lines
7.	PAB 09-7	Recombinant line	26.	EC 399296	Exotic lines
8.	PAB 09-8	Recombinant line	27.	EC 399301	Exotic lines
9.	PAB 09-9	Recombinant line	28.	EC 399313	Exotic lines
10.	PAB 09-10	Recombinant line	29.	EC 399302	Exotic lines
11.	PAB 09-11	Recombinant line	30.	EC 399312	Exotic lines
12.	PAB 9701	Recombinant line	31.	IC 414309	Indigenous line
13.	PAB 2004-4	Recombinant line	32.	IC 414317	Indigenous line
14.	PAB 2004-19	Recombinant line	33.	IC 414306	Indigenous line
15.	PAB 2004-6	Recombinant line	34.	IC 414322	Indigenous line
16.	PAB 2005-15	Recombinant line	35.	Kranti	Agronomic background
17.	PAB 2004-16	Recombinant line	36.	Varuna	Susceptible check
18.	PAB 2004-10	Recombinant line	37.	PAB 9511	Tolerant check
19.	PAB 2005-17	Recombinant line			

Table 2: Simple Sequence repeat markers sequences

S. No.	Locus	Sequence (Forward primer)	Sequence (Reverse primer)
1.	NA10-CO1a	TTTTGTCCCACTGGGTTTTC	GGAAACTAGGGTTTTCCCTTC
2.	NA10-CO1c	TTTTGTCCCACTGGGTTTTC	GGAAACTAGGGTTTTCCCTTC
3.	NI02-D08a	TTTAGGGAAAGCGAATCTGG	ACAACAACCCATGTCTTCCG
4.	NI03-H07a	GCTGTGATTTTAGTGCACCG	AGCCGTTGATGGAATTTTTG
5.	NI-F02a	TGCAACGAAAAAGGATCAGC	TGCTAATTGAGCAATAGTGATTCC
6.	OL10-A03a	CTGGTTTTCTCCTTCATCAG	CTGTGTAGCTTTTAGTCTTT
7.	OL10-F11a	TTTGGAACGTCCGTAGAAGG	CAGCTGACTTCGAAAGGTCC
8.	RA02-A04a	AAAAACTCCTCTTCAACG	CCCAAAGTTAGGTTTTAATGTAATCTC
9.	RA02-A04c	AAAAACTCCTCTTCAACG	CCCAAAGTTAGGTTTTAATGTAATCTC
10.	RA02-E01a	TCTATATTAACGCGCGACGG	GCACACACACACTCAAACCC

and PAB 09-2 and PAB 09-1 (0.975). PAB 09-8 and IC 414309, Varuna and IC 414309 were associated with each other with least similarity coefficient (0.609), this exhibited the presence of maximum diversity between these group of genotypes.All the loci amplified by the primer pair were polymorphic which varied in size from <100 bp to >2000 bp. Boot strap values varied from 0.2% to 75.9%. The maximum value (75.9%) of boot strap was found between EC399296 and PAB-2005-10 followed by PAB-09-1 and PAB-09-4 (64.4) and PAB-09-8 & PAB-09-9 (58.7). The polymorphic information content (PIC) values ranged from 0.00 (single locus) to 0.2815. In Varuna a unique band of 500bp was present with the marker NA10-CO1c

Genetic Diversity among the Genotypes

The objective of the experiment was to estimate the level of genetic diversity among the mustard genotypes using SSR markers. The UPGMA (Unweighted Pair Group Method with Arithmetic mean) dendrogram was constructed using Jaccard's similarity coefficients based on SSR marker data generated on 37 genotypes (Fig.2). UPGMA ordered the populations of 37 genotypes into four clusters. Cluster I consisted of ten genotypes namely PAB 09-3, PAB 05-14, PAB 09-6, PAB 09-5, PAB 05-15, IC 414306, PAB 09-4, PAB 05-17, EC 399299 and IC 414317 and related to members of cluster II by similarity coefficient of 0.85. Cluster II consisted of twelve genotype namely EC 399301, PAB 09-10, PAB 09-1, PAB 09-2, PAB 9511, PAB 04-6, PAB 04-4, PAB 05-16, PAB 9701, EC 399312, PAB 09-11 and PAB 09-9.

Cluster III consisted of four genotypes (Kranti, PAB 05-11, PAB 05-21, PAB 09-7) .Cluster II and III had a similarity coefficient of 0.81.Cluster-IV has seven genotypes (PAB 05-10, EC 399296, PAB 04-10, Varuna, EC 399302, EC 399313 and PAB 9721) and it showed a similarity coefficient of 0.73 with cluster III. Cluster-V consisted of four genotypes (PAB 09-8, PAB 05-19, IC 414309 and IC 414322). The similarity coefficient between cluster IV and V was found to be 0.72. On the basis of disease score index the 37 genotypes were grouped into 4 groups (Table 5).

The PAB 9511 was found the tolerant whereas the Varuna was found to be susceptible genotypes. PAB-09-1, PAB-05-16, PAB 09-11, PAB-09-9, EC 399312 and EC399301 fell in the same cluster where the PAB 9511 was present. At the same time these also a moderately tolerant disease reaction.

Table 3: Summary of SSR amplified product

1	Total number of primer tested	10
2	Number of polymorphic primers	10
3	Total number of monomorphic primer	0
4	Total number of polymorphic bands	40
5	Total number of monomorphic bands	01
6	Total number of bands	41
7	Size of amplified product (bp)	<100 to 2000
8	Percent polymorphism	97.56%

DISCUSSION

The assessment of genetic diversity is not only important for crop improvement efforts but also for the efficient management and protection of available genetic variability. Molecular profiling has been the preferred choice of breeding as these are more reliable & authentic and less influenced by environmental fluctuations (Vinu *et al*., 2013). The molecular marker in the other hand can be utilized for identification of marker for specific trait. Such diversity studies are helpful in categorizing the population into diverse group and that will help in development of gene pool.

The genetic diversity study in *B. juncea* has been done previously by using isozyme marker (Kumar & Gupta, 1985), morphological traits (Gupta *et al.*, 1991, Pradhan *et al.*, 1993) and molecular marker (Huangfu *et al*., 2009). SSR, being a potential marker system is not much used in research and breeding of *B. juncea*. Limited work considering SSR markers has been reported in *B. juncea* (Hopkins *et al.*, 2006).

In addition to microsatellite markers, other marker systems were also used by various researchers for genetic diversity studies in *Brassica* spp. Malode *et al*. (2010) analyzed 20 genotypes of *Brassica* spp. including exotic, Indian and mutants using RAPD primers and grouped the genotypes into four clusters. Similar findings have also been observed in our study with SSR markers.

The molecular marker analysis by using SSR primer successfully grouped 37 Indian mustard genotypes into 5 different groups. SSR markers have also been used for diversity analysis by the Abbas *et al*. (2003), Batley *et al*. (2003), Stephanie *et al*. (2009) and Fu *et al*. (2010). Presence of unique band helped in the identification of specific genotype. Most of the recombinant lines sharing the cluster with PAB 9511 had PAB 9511 as one of the parent in their percentage so such markers are highly reliable in the establishment of genetic relatedness among the genotypes. Similar results were reported

Table 4: Details of PCR amplified product with SSR primers

S No	Marker	Range of amplified product	Total bands	Polymorphic bands	Percent polymorphism	Unique band	PIC
1	NA10-CO1a	280-600bp	5	4	80%	-	0.0756
2	NA10-CO1c	280-600bp	6	6	100%	Varuna (500bp)	0.2163
3	NI02-D08a	<100-300bp	3	3	100%	-	0.2198
4	NI03-H07a	250-280bp	4	4	100%	-	0.1359
5	NI-F02a	250bp	1	1	100%	-	0.0000
6	OL10-A03a	200-2000bp	4	4	100%	PAB-05-10 (2000bp) and PAB-09-5 (200bp)	0.1630
7	OL10-F11a	<100-150bp	1	1	100%	-	0.2815
8	RA02-A04a	<100-300bp	4	4	100%	-	0.1709
9	RA02-A04c	<100-300bp	4	4	100%	-	0.2337
10	RA02-E01a	<100->1000bp	9	9	100%	PAB-05-10 (>1000bp)	0.1549

Table 5: Disease reaction of 37 Indian mustard genotypes

	Disease reaction Highly tolerant	Tolerant	Moderately tolerant	Moderately susceptible	Susceptible
Genotypes	Nil	PAB-9511	PAB-09-1, PAB-09-6, PAB-09-7, PAB-09-9, PAB-09-11, PAB-05-19, PAB-05-16, PAB-05-10, EC-399296, EC-399301, IC-414309, EC-399302 IC-414317, EC-399312, IC-414322	PAB-09-2, PAB-09-3, PAB-09-4, PAB-09-5, PAB-09-8, PAB-09-10, Kranti, PAB-9701, PAB-04-4, PAB-04-6, PAB-05-15, PAB-04-10, PAB-05-17, PAB-05-21, PAB-9721, PAB-05-14, PAB-05-11, EC-399299, EC-399313, IC-414306	Varuna

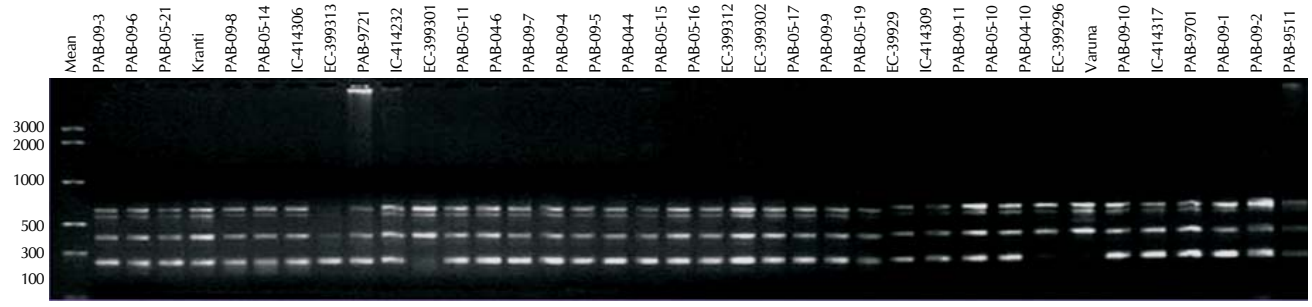

Figure 1: polymorphic SSR marker (NA10-CO1a) profile of mustard genotypes

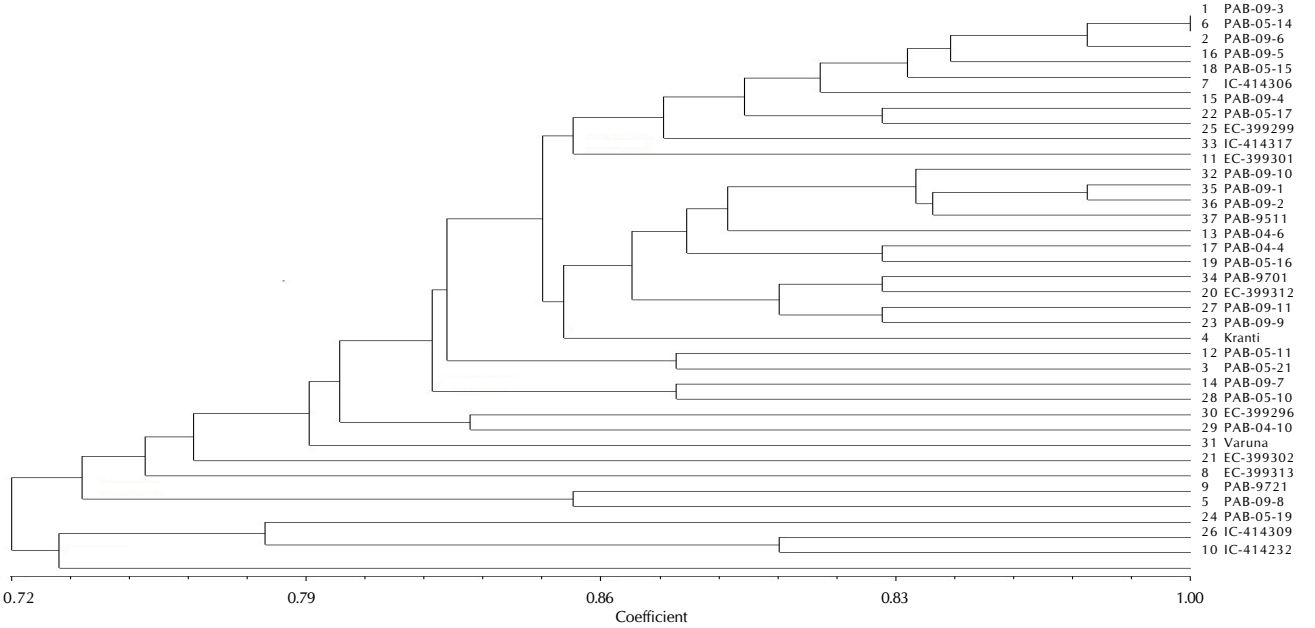

Figure 2: cluster tree analysis (NTSYS-PC) representing genetic relationships among mustard genotypes

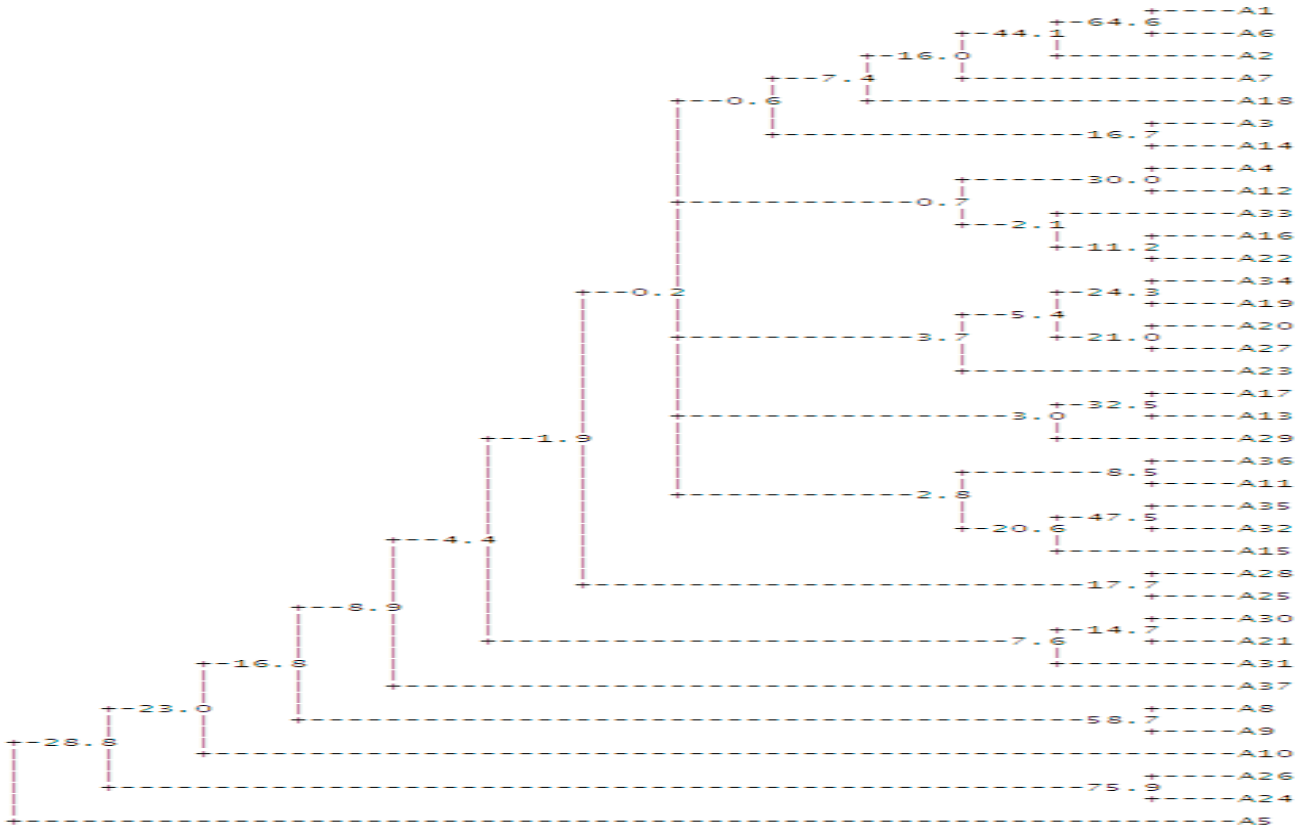

Figure 3: Cluster tree analysis (WinBoot) with bootstrap values representing genetic relationships among mustard genotypes

by Jain *et al*. (1994) and Srivastava *et al*. (2001) and Vinu *et al*. (2013).

The recombinant lines those shared common cluster with the either tolerant parent or susceptible parent were also showed almost similar type of disease response except cluster IV. So after proper validation these markers can be further used for the screening of Alternaria bilght. A similar result regarding effectiveness of SSR markers in monitoring genetic diversity for yield component traits as well as quality traits have also been reported by Charters *et al*. (1996) and Plieske and Struss (2001) respectively.

It can be concluded that SSR markers, which are free from environmental influences, are the stronger tools than quantitative trait data in discriminating *B. juncea* genotypes based on pedigree and origin. Information on genetic distances based on microsatellite markers shall be preferred in creating selectable genetic variation using genotypes which are genetically apart (Vieira *et al.,* 2007, Vinu *et al.,* 2013). The diversity analysis can further be utilized for the development of diverse gene pool. The hybridization among the diverse gene pool will result into more heterotic combinations.

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