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Genetic diversity in Indian mustard (*Brassica juncea*) genotypes for *Sclerotinia* stem rot (*Seclerotinia sclerotiorum*) using SSR markers

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ABSTRACT

The analysis of variance confirmed that the average sum of squares was significant for lesion length (cm). Genetic diversity as well as structure of population between genotypes of Indian mustard [*Brassica juncea* (L.) Czern. & Coss.] resistant to Sclerotinia stem rot [*Sclerotinia selerotiorum* (Lib.) de Bary] was carried out using simple sequence repeats (SSRs). A total of 114 alleles were generated via 48 polymorphic primers, with mean value of 2.38 alleles per primer. The average value of PIC and the mean gene expected heterozygosity/diversity (H_e) value from all the polymorphic primers were 0.43 and 0.50, respectively. All the 16 genotypes were categorized into three major clusters depending on Jaccard's dissimilarity coefficients. Evanno method for population structure revealed the presence of three populations (*SP1*, *SP2* and *SP3*) at maximum ΔK. *SP1* mainly comprised of resistant/highly resistant genotypes. These findings indicate the existence of ample amount of genetic variability between all the Indian mustard genotypes that could be exploited in future breeding programs to develop *Sclerotinia* stem rot resistant mustard cultivars.

Keywords: Genetic diversity, Indian mustard, Population structure, Sclerotinia stem rot, SSR markers

Brassica juncea (L.) Czern & Coss., popularly referred as Indian mustard, is a natural amphidiploid and one of the most important winter oilseeds crops (Singh *et al.* 2021). It contributes approximately 80% of the total output of rapeseed-mustard in the Indian oilseed sector. India, primarily imports the processed edible oil from other exporting countries. With the present trend of increasing population by 2030, the situation would be more challenging (Jat *et al*. 2019). Therefore, to deal with the future oil demand, there is an urgent need to boost the yield potential of Indian mustard. Productivity of Indian mustard is hampered by several diseases. Among them, *Sclerotinia* stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is considered as a serious threat to the mustard cultivation in India. In epidemic form, it causes yield losses up to 90% with significant reduction in oil content and its quality. Hence, to overcome this problem, host resistance might be the only sustainable approach. Consequently, mustard breeders are now paying attention to identify and characterize the resistance sources against *Sclerotinia* stem rot.

Awareness about the genetic diversity available in existing gene pool could help the breeders to understand complexities of germplasm, figure out which combinations would provide the good progenies and extend the genetic base of breeding material for selection (Bisen *et al.* 2015). Genetic diversity based on morphological traits in Indian mustard has previously been done by many researchers (Avtar *et al.* 2016, Singh *et al.* 2018, Kumari and Kumari 2018). However, the phenotypes of the plants can be influenced by various environmental factors. The analysis of plant genomic DNA allows the direct assessment of variation in genotypes (Govindaraj *et al.* 2015). Among different marker techniques, SSR's are more effective and comparatively decisive in distinguishing closely linked species and cultivars for genetic diversity analysis in crop plants (Bisen *et al.* 2015). Keeping above fact into consideration, the main objective of present endeavor was to evaluate Indian mustard genotypes for *Sclerotinia* stem rot resistance and diversity analysis by SSR markers.

MATERIALS AND METHODS

This study was carried out using 16 diverse genotypes of Indian mustard having Indian and exotic origin. All the genotypes were raised in two rows of 4 m length with 45 cm × 15 cm (row × plant) spacing at Research Farm of Oilseeds Section, GPB, CCS HAU, Hisar during *rabi* 2018–19 and 2019–20. Supplementary file 1 (S-1) includes an overview of the genotypes.

Culture preparation and disease assessment: *Sclerotinia sclerotiorum* inoculums preparation, artificial stem

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inoculation and disease assessment was carried out as method described in our previous work (Singh *et al.* 2020). All the genotypes were categorized into five different groups based on mean lesion length (cm) as per scale suggested by Garg *et al*. (2010), viz. highly resistant (lesion length <2.5 cm), resistant (2.5 - 5.0 cm), moderately resistant (5.0 - 7.5 cm), susceptible (7.5 - 10.0 cm) and highly susceptible (≥10.0 cm) with rating 0, 1, 2, 3 and 4, respectively.

Plant material and DNA extraction: According to Murray and Thompson (1980) and Saghai-Maroof *et al.* (1984), genomic DNA of all 16 genotypes was isolated from the fresh leaf tissue using 2% CTAB extraction protocol. Quantity and quality of genomic DNA was checked on 0.8% agarose gel and diluted to a concentration of 50 ng/μL after quantification.

Molecular marker evaluation: A total of 48 polymorphic SSR primers (*S-II*) were used in present study. PCR reactions were standardized in a 10 µl reaction volume having 50 ng genomic DNA, 1 mM PCR buffer, 1.0 U Taq DNA polymerase (Thermo Fisher Scientific), 0.2 mM dNTP mix, 1.5 mM $MgCl_2$ and 0.2 µM of each primer. DNA amplifications were performed in the T100^{TM} Thermal cycler. The configured PCR framework was as follows: an initial 2-minute denaturation at 94ºC followed by 38 cycle of 1 min at 94ºC, annealing temperature according to primer sequences for 1-minute, 2 min extension at 72ºC and a final extension of 7 min at 72ºC. The amplified fragments were resolved on 2% agarose gel along with DNA ladder (50 bp). Gel was imagined under UV-light and recorded in the program for gel documentation.

Band scoring and data analysis: Bands were scored for absence and presence as zero and one, respectively in each genotype. The polymorphism information content (PIC) and expected heterozygosity/gene diversity (H_e) values of each primer were determined according to Liu (1998) and Botstein *et al.* (1980), respectively. Duncan's test (P<0.01) was performed to estimate the difference among genotypes. Genetic dissimilarities, factorial analysis and neighbor-joining (UNJ) tree was done with the help of DARwin 6.0 program. In addition, Bayesian model-based cluster analysis was also conducted via STRUCTURE v.2.3.4 program (Pritchard *et al.* 2000). The assumed population numeral (K) was set at 1–10. For each fixed K, 10 unbiased runs were tested, and each run comprised 50000 burn-in times and 100000 iterations. Using Structure Harvester v6.0 (Earl and Vonholdt 2012), the peak value of K was estimated by evaluating ΔK statistics and L (K) (Evanno *et al.* 2005).

RESULTS AND DISCUSSION

Evaluation of genotypes: The analysis of variance confirmed that the mean sum of square was highly significant for lesion length observed suggesting ample genetic variation for this trait among the genotypes studied (Table 1). Among 16 genotypes studied, DRMR 2035 was observed with least lesion length (<2.5 cm) hence, considered as highly resistant. Two genotypes namely, RH 1222-28 and EC 597328 were considered as resistant with lesion length of 3.47 and 4.66 cm, respectively. Five genotypes, viz. RH 1657, RH 1566, RH 1658, PM 26 and RH 1899-53 were considered as susceptible one with lesion length between 7.5-10.0 cm, whereas remaining seven genotypes, viz. RH 1599-44, RH 1664, RH 1599-41, RH 1633, RH 1569, EC 766300 and EC 766123 were found highly susceptible to *Sclerotinia* stem rot having lesion length >10.0 cm.

Table 1 Analysis of variance for lesion length (cm)

Source of variation	df	Mean sum of square lesion length	F value
Replication	2	5.429	1.266
Genotype	15	48.445**	11.294
Error	30	4.289	
Total	47	18.430	

df.: Degree of freedom; **Significant at 1%.

SSR polymorphism: Forty eight polymorphic primers amplified overall 114 alleles, in which 85 alleles were polymorphic displaying clear bands size difference. The polymorphic alleles in present study is considerably higher than 54 alleles detected by Sudan *et al.* (2016), 52 alleles reported by Nanjundan *et al.* (2015) and 47 alleles reported by Avtar *et al.* (2016). This difference may be attributed to different sampling size, different genotypes and SSR primers in previous studies. The overall fragment length of PCR amplified products ranged between 50-900 bp. The PIC value and gene diversity (expected heterozygosity) both are good indicator for genetic divergence measures among the germplasm. The range of PIC value was 0.11-0.77 with mean value of 0.43 demonstrating moderate discriminating capability of the SSR markers used for this study (Table 2). This result is in agreement with previous study of Avtar *et al.* (2016) and Singh *et al.* (2017). Twenty-one SSR markers in our study have PIC values of more than 0.50 suggesting their effectiveness in distinguishing genotypes. The overall polymorphic percentage (78.51%) revealed the considerable polymorphism in the studied molecular markers though it was slightly lesser than previous study of Nanjundan *et al.* (2015). The gene diversity (H_e) provides an estimate of genetic distance among genotypes in germplasm set. The average expected heterozygosity/gene diversity (H_e) was recorded to be 0.50 with maximum value (0.80) for BG46 and minimum value (0.12) for BG24, BG60, BG61, BG66, BG71 and BG77.

Genetic distance and cluster analysis: The dissimilarity coefficients ranged from 0.074 to 0.276 suggesting low genetic dissimilarity as compared to Abbas *et al.* (2009) in which they reported 0.05 to 0.61 values. The maximum dissimilarity (0.276) was observed between the genotypes, EC 597317 and EC 766123 while least (0.074) among RH 1899-53 and RH 1569. The genotype, EC 597317 was found to be the most diverse as it showed the highest dissimilarity coefficient values with most of the genotypes, viz. 0.233 with EC 766300; 0.223 with RH 1599-44, RH 1657; 0.209 with PM 26 and 0.207 with RH 1599-41 (*S-III*).

Table 2 List of polymorphic primers depicting total number of alleles, polymorphic alleles, per cent polymorphism, PIC and gene diversity/expected heterozygosity (H_e) values

Primer	Amplicon size (bp)	Total no. of alleles	Polymorphic alleles	Per cent polymorphism	PIC value	Gene diversity
BG1	120-800	3	2	66.7	0.575	0.650
BG2	50-500	3	3	100	0.592	0.666
BG3	180-900	3	3	100	0.577	0.652
BG4	50-250	3	1	33.3	0.592	0.666
BG5	200-620	3	3	100	0.562	0.635
BG6	50-600	3	2	66.7	0.590	0.664
BG7	225-300	2	2	100	0.375	0.500
BG8	50-700	2	1	50.0	0.355	0.461
BG10	50-800	2	1	50.0	0.372	0.495
BG11	75-450	3	2	66.7	0.469	0.536
BG12	50-250	2	1	50.0	0.318	0.397
BG17	50-900	4	1	25.0	0.703	0.750
BG18	50-450	3	2	66.7	0.592	0.666
BG19	60-400	3	2	66.7	0.536	0.604
BG20	250-850	3	3	100	0.592	0.666
BG24	50	1	1	100	0.110	0.117
BG30	50-200	3	1	33.3	0.592	0.666
BG37	110-700	3	2	66.7	0.592	0.666
BG39	110-200	2	1	50.0	0.361	0.473
BG40	60-170	2	1	50.0	0.372	0.495
BG42	50-190	2	1	50.0	0.375	0.500
BG46	50-800	5	3	60.0	0.766	0.798
BG48	50-400	3	2	66.7	0.585	0.659
BG49	50-500	3	2	66.7	0.590	0.664
BG50	50-900	3	1	33.3	0.592	0.666
BG51	50-350	3	2	66.7	0.584	0.659
BG52	75-200	3	2	66.7	0.455	0.547
BG54	100-800	3	2	66.7	0.584	0.658
BG55	90-550	2	2	100	0.368	0.486
BG57	75	1	1	100	0.258	0.305
BG58	80	1	1	100	0.195	0.219
BG60	50	1	1	100	0.110	0.117
BG61	230	1	1	100	0.110	0.117
BG62	90-480	3	3	100	0.536	0.611
BG66	70	1	1	100	0.110	0.117
BG67	120-150	2	2	100	0.374	0.497
BG68	50	1	1	100	0.258	0.305
BG71	50	1	1	100	0.110	0.117
BG72	90-330	3	3	100	0.592	0.666
BG74	65-250	2	1	50.0	0.366	0.483
BG75	350-450	2	2	100	0.371	0.492
BG77	50-300	3	3	100	0.457	0.557
BG78	500	1	1	100	0.195	0.219
BG79	50	1	1	100	0.110	0.117
BG81	50-220	2	2	100	0.371	0.493
BG82	100-200	3	3	100	0.505	0.593
BG84	300-900	3	3	100	0.327	0.355
BG86	200-320	2	2	100	0.375	0.500
Min.	50	1.00	1.00	25.00	0.11	0.12
Max.	900	5.00	3.00	100.00	0.77	0.80
Average		2.38	1.77	78.51	0.43	0.50

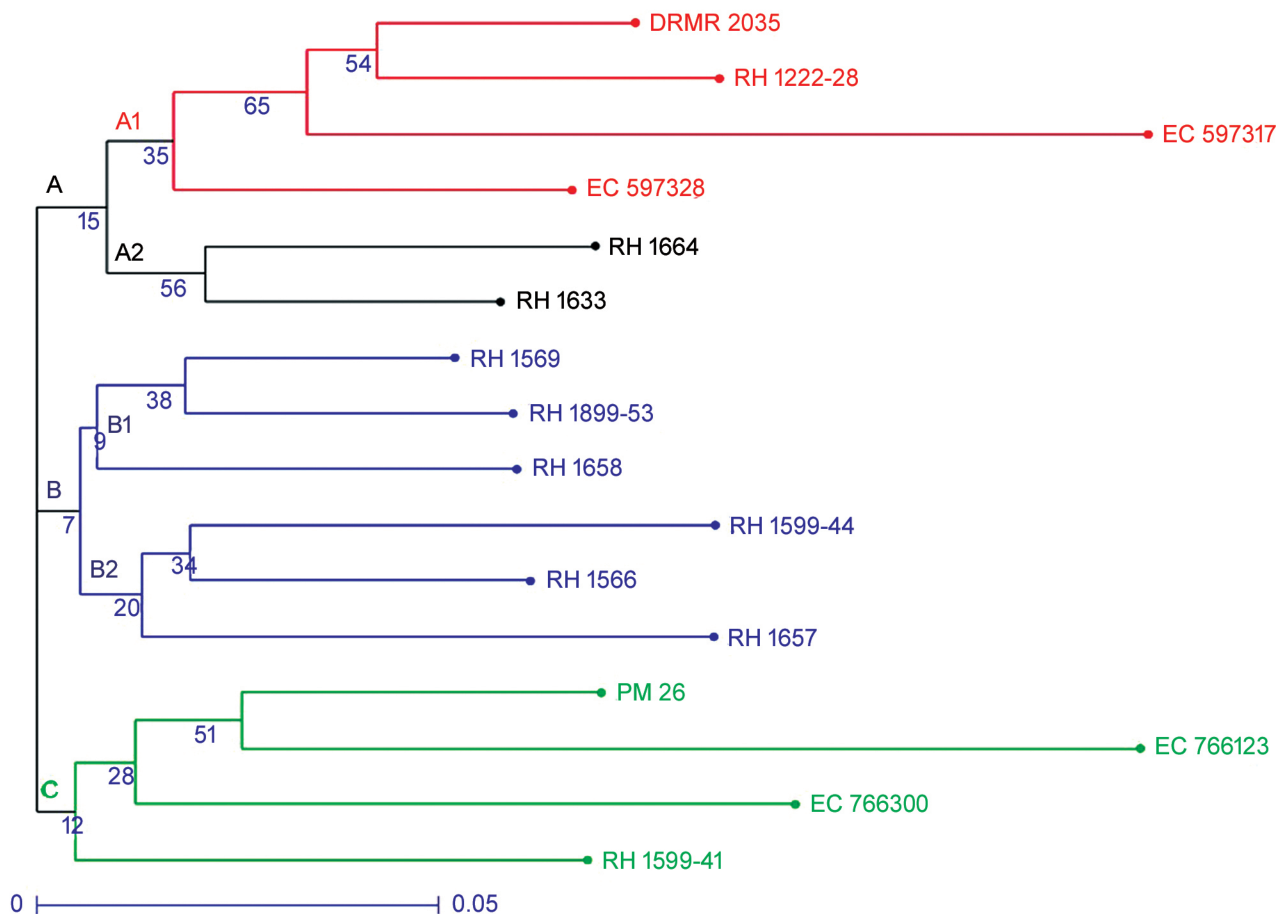

Fig 1 Genetic relationship among 16 genotypes of Indian mustard revealed by NJ tree constructed using genotypic data of 48 polymorphic SSRs primers. The numbers on branches indicate bootstrap values.

A dendrogram constructed based on UNJ method using Jaccard's dissimilarity coefficient matrix, grouped all 16 Indian mustard genotypes into three major groups (Fig 1). Our findings are consistent with preceding studies where Iqbal *et al.* (2015) also obtained 3 main clusters while evaluating 16 Indian mustard genotypes for selection of short duration genotypes and Singh *et al.* (2017) also grouped 15 Indian mustard genotypes into three major clusters. Group A, comprised six genotypes with further subdivision into two subgroups, i.e. A1 and A2. Subgroup A1 includes four resistant genotypes of Indian and Exotic origin (DRMR-2035, RH 1222-28, EC 597317 and EC 597328) having lesion length <5.0 cm, whereas subgroup A2 comprised two highly susceptible genotypes of Indian origin (RH 1664 and RH 1633) having lesion length >10.0 cm. Group B also consisted of six susceptible genotypes (lesion length >7.5 cm) and had two subgroups namely B1 and B2. In this group, all the six genotypes originated from India (Hisar location) and derived from the bi-parental crosses. Each sub-group contains three genotypes including one highly susceptible genotype to *Sclerotinia* stem rot (lesion length >10.0 cm). The group C includes four genotypes of exotic and Indian origin. The genotypes of this group were characterized as highly susceptible to *Sclerotinia* stem rot having mean stem lesion length >10.0 cm (except PM 26, lesion length <10.0 cm). Though, grouping of few susceptible genotypes into group A may be due to the fact that allelic composition among these genotypes was identical at some of the SSR marker loci considered for this experiment. In a similar study, clustering of genotypes of Oilseed *Brassica* was reported based on their reaction to *Sclerotinia* stem rot (Sharma *et*

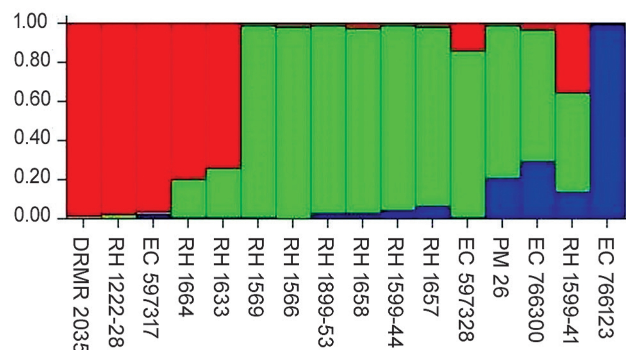

Fig 2 Estimated population structure of 16 Indian mustard genotypes as revealed by 48 SSR markers at ΔK = 3. Red, Green, and Blue color represents SP1, SP2 and SP3, respectively.

al. 2018). The principal coordinate analysis (PCoA) further confirmed the places and clustering of genotypes. So, PCoA based on SSR markers data supported the differentiation of resistant and susceptible genotypes.

Population structure analysis: Model based cluster study was conducted to determine the genetic structure. The maximum likelihood and ΔK (ΔK = 3) values suggested that all the 16 genotypes of Indian mustard were predominantly allocated into three subpopulations, viz. SP1, SP2 and SP3 (Fig 2). Most of the genotypes maintained their identity with admixture of alleles of other accessions. The three sub-populations were separated mainly on the basis of their disease reaction to *Sclerotinia* stem rot. The *SP1* mainly comprised resistant/highly resistant genotypes. The genotypes RH 1664 and RH 1633 of SP1 exhibited susceptible reaction to stem rot. It may be due to fact that these genotypes were having admixture of alleles of SP2. The SP2 and SP3 contained all the genotypes which had susceptible/highly susceptible reaction to *Sclerotinia* stem rot (except EC 597328). Grouping of 16 Indian mustard genotypes based on the structure analysis was identical with the obtained dendrogram and it supported the accuracy of the clustering. Similar to our study, Sudan *et al.* (2016) identified an optimal number of three sub-populations at maximum likelihood and ΔK values.

In the present study, wide range of genetic variation has been observed for lesion length, an important criterion for phenotyping of *Sclerotinia* stem rot. Out of the 16, four genotypes (DRMR-2035, RH 1222-28, EC 597317 and EC 597328) were categorized as resistant for *Sclerotinia* stem rot. Moreover, SSR markers can be effectively used to discriminate resistant/susceptible genotypes for *Sclerotinia* stem rot. Resistant genotypes reported in our study might be exploited for development of *Sclerotinia* stem rot resistant cultivars in Indian mustard breeding programmes.

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