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Identification of genomic regions linked to blast (*Pyricularia grisea*) resistance in pearl millet

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Abstract

Blast disease causes serious economic yield losses in pearl millet. Identification and introgression of genomic regions associated with blast resistance can help to develop resistant cultivars to minimize yield losses incurred from blast outbreaks. In this study, 384 advanced pearl millet genotypes were screened against six blast pathotype-isolates (major pearl millet growing agro-ecologies of India), namely, Pg 45, Pg 118, Pg 138, Pg 186, Pg 204 and Pg 232. Analysis of variance showed significant ($P < .001$) variation among genotypes for blast reaction (susceptible to resistance). ICMR 08111 and ICMR 10888 genotypes showed resistance to all six blast pathotypes. A genome-wide association study performed with 264,241 single nucleotide polymorphic markers could successfully identify 15 SNPs ($P = 1.26 \times 10^{-7}$ to 9.22×10^{-12}) underlying the genomic regions governing blast-resistance across five different chromosomes. The SNPs reported had a significant association in at least two of the three models tested (GLM, MLM and Farm CPU). These SNPs can be used in pearl millet-resistant breeding programmes after their function has been validated across different genetic backgrounds.

KEYWORDS
blast, general linear model (GLM), genome-wide association study (GWAS), linkage groups (LG), quantitative trait loci (QTL), single nucleotide polymorphisms (SNPs)

1 | INTRODUCTION

Pearl millet, a diploid ($2n = 2x = 14$) and a C_4 crop serves as one of the major staple food crops to the people living in low rainfall areas of Africa and India. Pearl millet is the sixth most important grain-producing cereal grown primarily in rainfed regions across the world (FAOSTAT, 2014). The grains of this cereal are rich sources of fat, protein, carbohydrate and minerals, especially iron and zinc. The high biomass producing potential with better quality fodder of this climate-resilient crop makes this cereal an important part of crop-livestock production system in the harsher environments of semi-arid tropics of Asia and Africa. The development of stable and high yielding hybrids and open-pollinated varieties (OPVs) in pearl millet has successfully contributed to the increase in productivity in terms of grain and fodder yield. The productivity of this crop has been challenged by various biotic (diseases and pests) and abiotic (heat, drought and salinity) stresses.

Blast or leaf spot disease, a seed and wind borne disease caused by *Pyricularia grisea* Sacc., has emerged as a severe threat in recent past across major pearl-millet-growing regions of India (Rai et al., 2012). The disease thrives well under the warm and humid conditions of major pearl millet growing regions of north western India, wherein the disease has been noticed up to the incidence of 90% (Singh et al., 2021). Blast symptoms are very much prominent that usually appear in the form of grey leaf spot on leaves and stems. Initially the symptom starts with tiny specks or lesions that broaden to form necrotic regions resulting in extensive chlorosis and ultimately end in drying of young leaves (Nayaka et al., 2017). The chemical

control of blast by spraying fungicides has contributed to a certain extent to minimize the loss incurred, but is not a cost-effective strategy. Genetic improvement of cultivar's ability through identification and introgression of resistance genes is the best cost-effective strategy to counter the loss incurred due to blast incidence. Few studies conducted up to date have indicated that single dominant gene governs the inheritance of blast resistance (Gupta et al., 2012; Singh et al., 2018; Wilson et al., 1989) in pearl millet. Hence, there is a continuous risk of the breakdown of this single resistant gene due to new pathotypes evolved because of the continuous host–pathogen interaction (Suh et al., 2009). Some of the identified resistant lines have shown susceptible reaction to newly collected blast pathotypes, whereas some new breeding lines have shown resistant reaction for them. For example, ICMR06444, ICMR11003 and IP21187-P1 were resistant to Pg 45 and Pg 118 but found susceptible to pathotype Pg 138 (Sharma et al., 2021). However, resistance sources to the highly virulent pathotype Pg 138 such as IP-13261-1-3, IP212441-2 and IP6113-2-1-2 have been identified. This differential reaction of breeding lines against blast pathotypes indicates existence of more than one gene to be responsible for blast resistance. This demands identification and introgression of such genetic regions that are resistant to one or more blast pathotypes.

Linkage mapping (QTL-mapping) based on bi-parental cross provides a great opportunity to identify the genetic regions associated with blast resistance, though its low resolution power has a significant limitation for molecular breeding. Linkage disequilibrium (LD) based mapping is a better method as it considers historical recombination and mutation in a given population along with higher resolution (Yu & Buckler, 2006), which can help to better identify the closely linked markers associated with the trait of interest. GWAS has been successfully used to identify MTAs (marker trait associations) in pearl millet for other traits, for instance, flowering time and spike morphological value (Saïdou et al., 2009), plant height, panicle length, stover and grain yield (Kannan et al., 2014), phosphorus uptake and utilization efficiency (Gemenet et al., 2015), drought (Sehgal et al., 2015) and grain mineral density (Anuradha et al., 2017; Pujar et al., 2020). However, limited information and knowledge of genetic and molecular basis of natural variation is available for blast resistance in pearl millet. In the present study, GWAS was performed for the first time to identify genomic regions associated with blast resistance in pearl millet using high density SNP markers.

2 | MATERIALS AND METHODS

2.1 | Plant material

The GWAS panel for the current study for blast (*Pyricularia grisea* (Cooke) Sacc., teleomorph: *Magnaporthe grisea*) resistance is composed of 384 (>F_6/F_7) advanced hybrid parental lines of pearl millet (*Pennisetum glaucum* (L.) R. Br.) (henceforth, are referred to as 'genotypes') involving 185 seed parents (B-lines) and 199 Pollinator/Restorer parents (R-lines) with diverse parentage developed at ICRISAT, Patancheru. IP 21187-P1 and ICMB 95444 were used as resistant (but for pathotype Pg 138) and susceptible checks, respectively, for the blast disease.

2.2 | Blast pathotype-selection

A set of six diverse pathotypes from India, namely, Pg 45, Pg 118, Pg 138, Pg 186, Pg 204 and Pg 232, maintained in Cereals Pathology section, ICRISAT, Patancheru, India, were used in the present study. These six pathotypes represent major pearl millet growing states in India and were collected from Patancheru (Telangana), Rewari (Haryana), Jaipur (Rajasthan), Aurangabad (Maharashtra), Gandhinagar (Gujarat) and Aligarh (Uttar Pradesh) (Table 1).

The phenotyping for blast reaction against six pathotype-isolates collected from different locations was carried out at ICRISAT, Hyderabad under controlled conditions in glasshouse during the year 2018 and 2019. The completely randomized design (CRD) with two replicates; one pot/replicate and 12 seedlings/pot was followed for each pathotype, and the screening against each pathotype was conducted separately. The seeds of each 384 genotypes along with susceptible checks were sown in 10 cm diameter pots filled with sterilized soil-sand-FYM (farmyard manure) in a 2:1:1 ratio, and the pots were kept at 30 ± 1°C temperature in a glasshouse for 12 days. All six blast pathotype-isolates (Pg 45, Pg 118, Pg 138, Pg 186, Pg 204 and Pg 232) were used for the screening of GWAS panel constituting 384 genotypes. Before inoculation of the fungal spores, the spore suspension was adjusted to 1×10^5 spore/mL with the help of a haemocytometer. Tween 20 (polyoxyethylene sorbitan monolaurate) (.02% v/v) was added to the suspension just before the inoculation for the uniform dispersal of the spores. Twelve-day old seedlings were inoculated through spray with an aqueous spore suspension of each pathotype of *P. grisea* separately. The inoculated seedlings were immediately covered with polythene bags to avoid cross contamination, and the pots were incubated at 25°C for 24 h. The pots were then transferred to a glasshouse and exposed to high humidity (>90%) through mist irrigation for 4 days. The data on foliar blast severity in each line was recorded after 6 days of inoculation using 1–9 progressive scale (Figure 1) as per Sharma et al. (2020). The lines exhibiting blast score ≤3.0 were categorized as resistant, that is, R = Resistant (≤3.0), MR = moderately resistant (3.1–5.0), S = Susceptible (5.1–7.0), and HS = Highly Susceptible (>7.0). A total of 81 data-points were missing across genotype × pathotypes screening because of less plant count in any one replication.

2.3 | DNA extraction and high-throughput genotyping

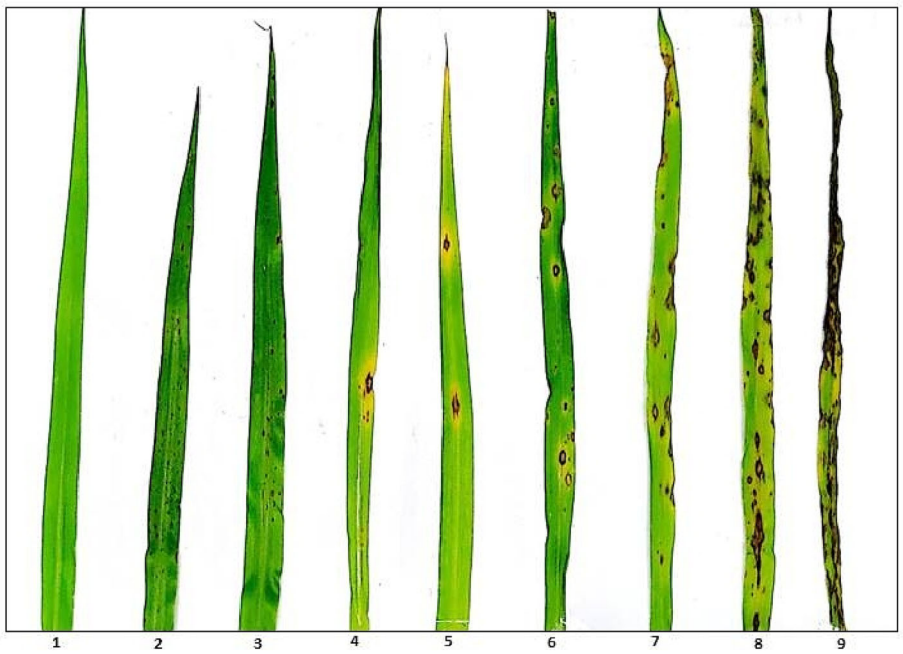
Approximately, 35 seeds of each genotype were sown in a plastic pot of 10 cm diameter filled with soil (soil type or cocopeat) at ICRISAT-Patancheru. Healthy leaf tissues were harvested 8 days after sowing using bulk strategy, where pooled leaf tissue from 20 to 25 seedlings

TABLE 1 Summary statistics of the phenotypic data of 384 pearl millet genotypes evaluated for their reaction to six pathotypes of *Pyricularia grisea*.

Blast pathotypes	Place of collection (City/state of India)	Mean	Minimum	Maximum	Standard deviation	Shapiro–Wilk test	Levene's test	H^2
Pg 45	Patancheru (Telangana)	5.7	2	9	2.001	$P < .0001$	.929	.99
Pg 118	Rewari (Haryana)	6.1	2	9	2.085	$P < .0001$	.831	.99
Pg 138	Jaipur (Rajasthan)	5.6	2	8	1.519	$P < .0001$	.774	.97
Pg 186	Aurangabad (Maharashtra)	6.1	2	9	1.4	$P < .0001$	.783	.99
Pg 204	Gandhinagar (Gujarat)	5.6	2	9	1.98	$P < .0001$	.652	.99
Pg 232	Aligarh (Uttar Pradesh)	5.6	2	9	1.908	$P < .0001$	.856	.99

Note: H^2, Heritability.

FIGURE 1 Blast scoring in pearl millet.



per genotype was collected for DNA extraction. The genomic DNA was extracted from pooled leaf tissues as per the modified DNA extraction method of Mace et al. (2003). The integrity or intactness and quality of the DNA were assessed in 0.8% agarose gel electrophoresis. The quantity of extracted DNA was evaluated using a Nanodrop ND-1000 (Thermo scientific, UK). The concentrated DNA was diluted to 100 ng/mL for further sequencing.

The genotypes were re-sequenced using restriction-site-associated DNA (RAD) sequencing, followed by SNP calling and filtering as described by Miller et al. (2007) and Varshney et al. (2017). Re-sequencing data of these pearl millet lines are available at https://www.ncbi.nlm.nih.gov//sra/?term=SRP063925. These sequences were aligned to the pearl millet reference genome (Tift 23 D2B1–P1–P5, Varshney et al., 2017). A total of 556,888 SNPs were identified after aligning to the reference genome. This set was filtered for 5% minor allele frequency (MAF) and coverage (>30%). A total of 264,241 SNPs were retained and used for analysis (Supplementary Figure Information 1). The SNP data has been imputed using BEAGLE 4.0 (Browning & Browning, 2016).

2.4 | Association analysis

The phenotypic data for blast disease for the year 2018 and 2019 was analysed in the GAPIT programme of R package (Lipka et al., 2012) for identifying the associations between the markers and the disease scores for blast recorded with the general linear model (GLM), mixed linear model (MLM) and Fixed and random model Circulating Probability Unification (FarmCPU; Liu et al., 2016) models. LD was estimated in TASSEL (Bradbury et al., 2007). LD decay plots of r^2 values with distance in base pairs (bp) were plotted, and the LD decay curve were fitted in R (R Core Team, 2016). Three principal components (PCs) were used in the model to control the population structure to avoid the spurious associations. The PCs

Source of variation	Degree of freedom	Sum of squares	Mean squares
Replication	1	.00092	.00092
Genotypes (G)	383	6085	15.89*
Pathotype (I)	5	268.9	53.78*
G × I	1915	4232	2.21*
Residual	2222	86	.039
Total	4526	10,530	

*Significant at .001% probability.

TABLE 2 Analysis of variance of 384 pearl millet genotypes for resistance against six pathotypes of *Pyricularia grisea*.

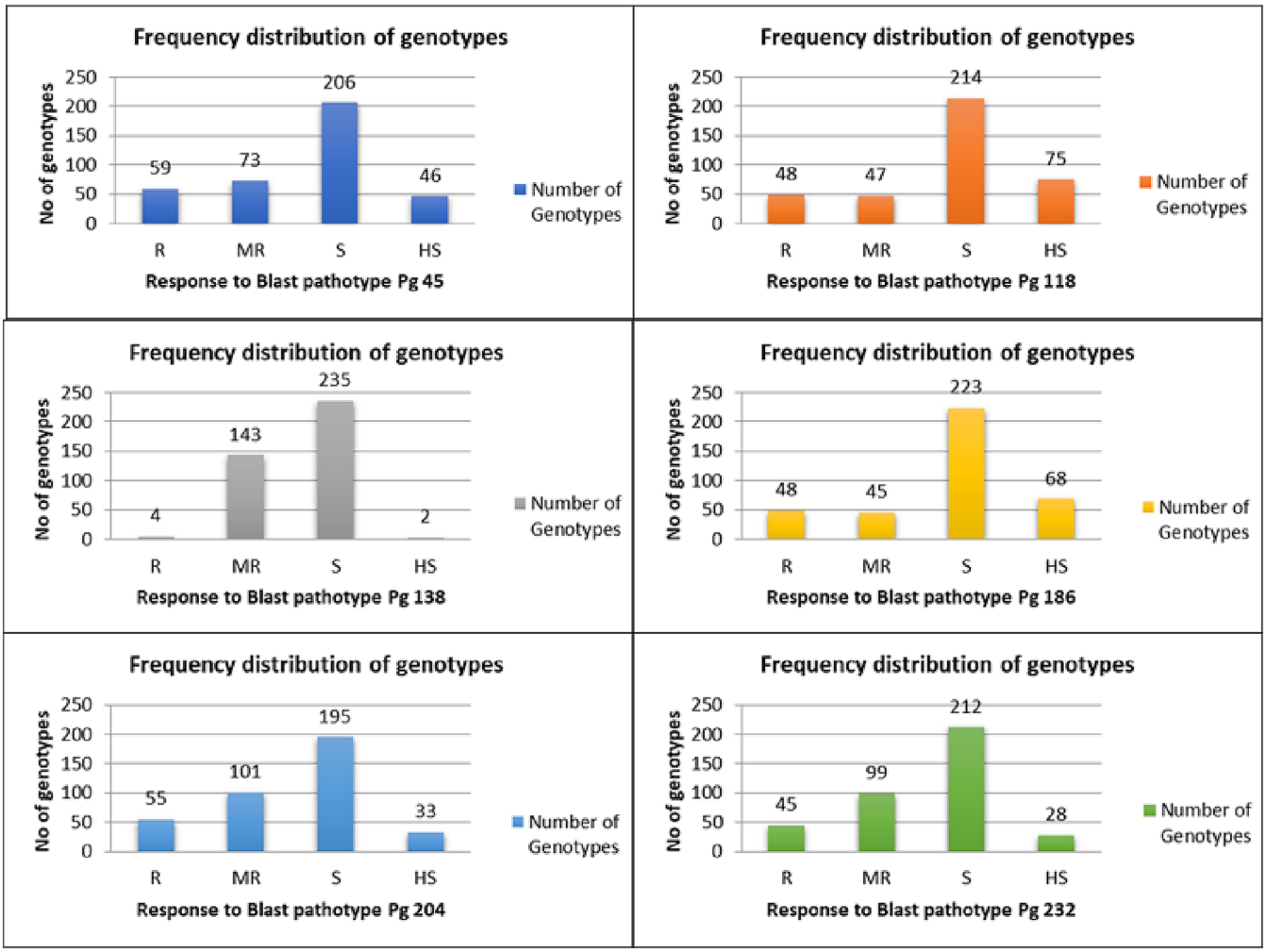


FIGURE 2 Frequency distribution of pearl millet genotypes in response to different blast pathotypes. (A) Pg 45, (B) Pg 118, (C) Pg 138, (D) Pg 186, (E) Pg 204 and (F) Pg 232. R, resistant (≤ 3.0); MR, moderately resistant (3.1–5.0); S, susceptible (5.1–7.0); HS, highly susceptible (>7).

were determined using the GAPIT programme (Lipka et al., 2012). To correct for multiple testing, the step-up procedure of Benjamin and Hochberg (1995) which controls the false discovery rate was used with a cut-off value of .05. Furthermore, Bonferroni corrected *P*-value threshold was used to compute the significant *P*-value threshold for the GWAS panel. The blast score data were analysed using the GENSTAT statistical package (version 10.1; Rothamsted Experiment Station, Herpenden, Herts, UK) to determine the significant differences among pathotypes, host genotypes and their interactions (Payne, 2002). The phenotypic and genotypic coefficient of variation for blast score against each pathotype was estimated according to Burton and Devane (1953), and the heritability against each pathotype was computed using the formula adopted by Allard (1960).

3 | RESULTS

3.1 | Genotype response to blast pathotypes under glasshouse screen

The susceptible check ICMB 95444 recorded ≥8 score against all the pathotype-isolates, indicating a reliable disease screen. IP 21187-P1, included as a resistant check, recorded ≤3.0 score against Pg 45, Pg 118, Pg 186, Pg 204 and Pg 232; this line is susceptible to Pg 138 and recorded a 6.0 score against this pathotype. The analysis of variance among 384 genotypes screened for six different blast pathotypes showed highly significant ($P < .001$) mean square values for genotypes (Table 2). This indicates that the genotypes differed significantly for the resistant reaction against different blast pathotypes. A highly significant mean square as recorded for different blast pathotypes indicates that all pathotypes were different in virulence. Furthermore, wide genetic variation in terms of susceptibility to resistance was observed among the genotypes screened for multiple virulent pathotypes of blast. The frequency distribution of response of all the genotypes to six highly virulent blast pathotypes is presented in Figure 2. The disease score for each blast pathotype ranged from 2 to 9 (Table 1). The trial mean for the disease scores among all the six blast pathotypes, namely, Pg 45, Pg 118, Pg 138, Pg 186, Pg 204 and Pg 232 were 5.7, 6.1, 5.6, 6.1, 5.6 and 5.6, respectively. Overall results showed that the highest number of resistant (≤3 score) genotypes were found against Pg 45 (59) followed by Pg 204 (55), Pg 118 (48), Pg 186 (48) and Pg 232 (45); whereas, relatively very few resistant genotypes were found for the pathotype Pg 138 (4). The heritability estimates for the disease resistance reaction of all the genotypes against six blast pathotypes ranged from .97 to .99. The 10 genotypes that showed resistant reaction to at least four or more pathotypes are presented in Table 3.

3.2 | Principal component analysis (PCA) and linkage disequilibrium (LD) decay

The estimates of principal component analysis (PCA) among 384 genotypes showed a clear clustering of genotypes into two major groups, each one dominated by the seed parent (B-lines, indicated by blue dots) and the pollinator or restorer parent (R-lines, indicated by red dots) on the PCA plot (Figure 3). PCA 1 and 2 together explained a total of 19.3% of variation with the maximum contribution of 13.3% by the PCA 1. The linkage disequilibrium (LD) across the whole genome was plotted as LD (r^2) between adjacent pairs of markers against the distance between adjacent markers in base pairs

TABLE 3 Ten pearl millet genotypes found resistant to at least four pathotypes of *Pyricularia grisea*.

Genotypes	Entry name	Pedigree	Origin—country name	Mean disease score					
				Pg 45	Pg 118	Pg 38	Pg 186	Pg 204	Pg 232
R18[a]	ICMR 08111	(ICMS 7704-S1–127–5-1 × RCB-2 Tall)-B-19-3-4-5-3	ICRISAT, India	**2**	**3**	**3**	**3**	**3**	**3**
R42	ICMR 10888	ICMV 93074 S1–9–1-1-1-3-B-B-B-B	ICRISAT, India	**2**	**2**	**2**	**3**	**2**	**3**
R175	Adv R lines	MDMRRC S1–1–303–2-2-3-1	ICRISAT, India	4	**3**	4	**3**	**3**	**3**
R93	Adv R lines	GB 8735-S1–15–3-1-1-3-3-1-1-2-1-3	ICRISAT, India	**2**	2	5	**2**	6	**2**
R90	Adv R lines	AIMP 92901 S1–15–1-2-3-B-1-B-14-3-1-B	ICRISAT, India	**2**	2	4	**2**	**2**	**3**
R112	Adv R lines	MDMRRC S1–1–59-3-3-1-B	ICRISAT, India	**2**	2	4	**2**	**2**	**2**
R114	Adv R lines	MDRRC-HS-28-2-3	ICRISAT, India	**2**	3	4	**3**	**3**	**2**
R119	Adv R lines	MRC S1–340–1-3-3-2-B-B-1	ICRISAT, India	**2**	2	4.5	**3**	**2**	**3**
B30	ICMB 97222	[(ICMB 88006 x ICMB 88005) x (ICMB 89111 x ICMB 88004)]-28–2-B	ICRISAT, India	**3**	3	4	**3**	**3**	**3**
R59	ICMR 12777	[(IPC 1617 × SDMV 90031-S1–84–1-1-1-1) × GB 8735-S1–25–4-4-1-1-3-1-1]-1–1–3-2-1-B-B	ICRISAT, India	**3**	2	7	**2**	**2**	**3**
No. of genotypes that score ≤3				59 (15%)[b]	48 (13%)	4 (1%)	48 (13%)	55 (14%)	45 (12%)

[a]Adv R lines = Advanced Restorer parental lines.

[b]Figure in parenthesis is percentage of lines found resistant against a particular isolate.

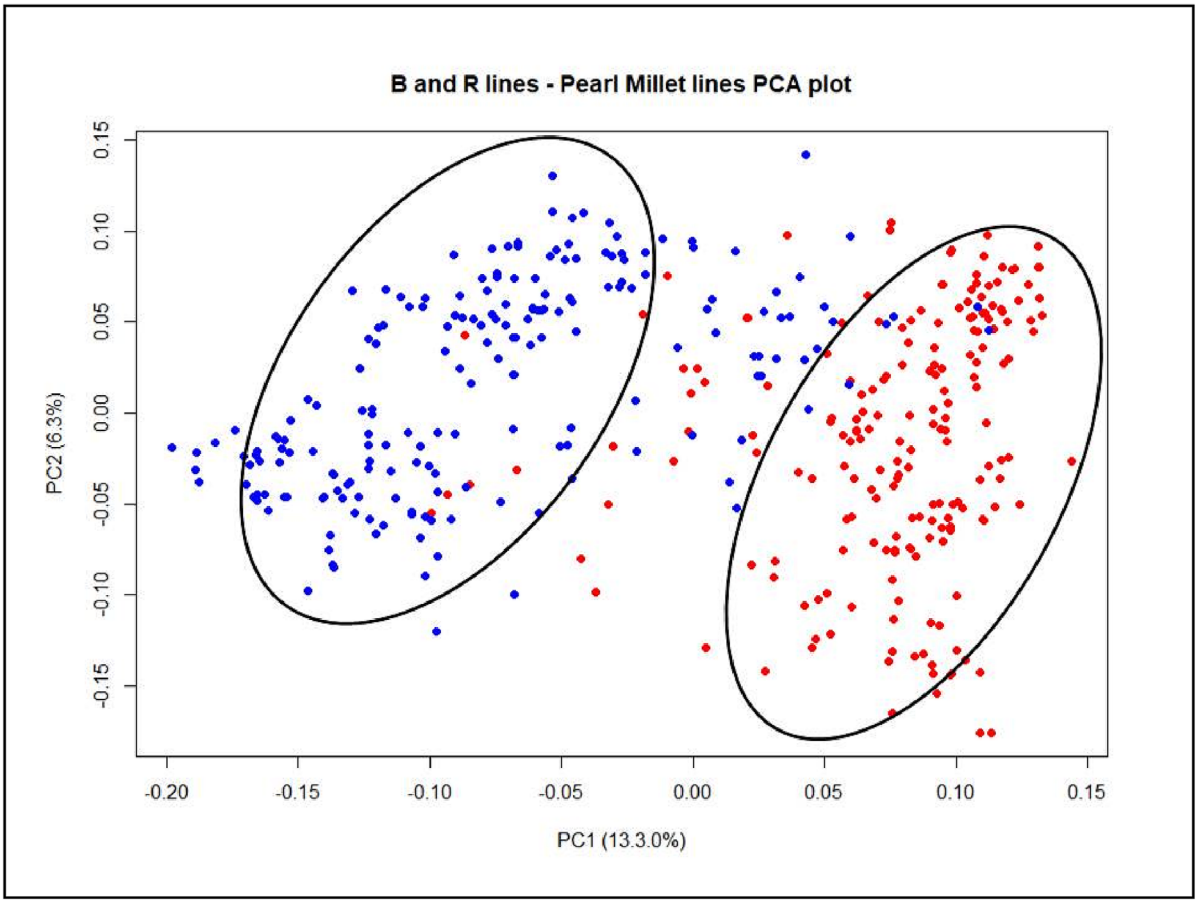


FIGURE 3 Principle component analysis (PCA) showing two clear groups among the genotypes used for the genome wide association mapping study (GWAS). (blue: B-lines; red dots: R-lines).

(bp) (Figure 4). The genome-wide LD plot unveiled a relatively higher LD-decay of about >500 Kb among the GWAS panel. This was consistent across all the seven chromosomes.

3.3 | Mapping of genetic regions governing resistance to *Pyricularia grisea*

The GWAS was performed to identify the genetic regions associated with the resistance to six blast pathotypes using the disease incidence scoring and 264,241 SNPs derived from re-sequencing of 384 pearl millet genotypes. A total of 15 SNPs with strong association (Threshold '$-\log P' = 6$) underlying genetic regions for resistances to blast were identified across five different chromosomes and are represented on Manhattan plots (Figure 5) along with their quantile–quantile (QQ) plots of the expected versus observed *P*-values that inspects the genomic inflation. The maximum number of SNPs having significant association were located on chromosome 4 (five SNPs) followed by chromosome 1 and 3 with three SNPs each. In addition, two SNPs each were located on chromosome 6 and 7. Out of six blast pathotypes tested, the current study could identify the associations for only three pathotypes. Of the 15 significant SNPs identified, eight SNPs were identified for Pg 138 followed by four SNPs for Pg 118 and three for Pg 204.

3.3.1 | Pg 118

A total of four SNPs spanning on chromosomes 4, 6 and 7 were found significantly associated with Pg 118, explaining phenotypic variance ranging from 13.31% to 16.65%. Out of four SNPs, two SNPs (S7_146512193 and S4_184208528) were found significant in all three models (GLM, MLM and FarmCPU); whereas, two SNPs (S4_30412820 and S6_144092278) were found significant in GLM and FarmCPU models (Table 4).

3.3.2 | Pg 138

A total of eight SNPs significantly associated with the genetic regions governing resistance against Pg 138 were identified. Among these, three SNPs (S3_159161939, S3_17901208 and S3_221057270) were

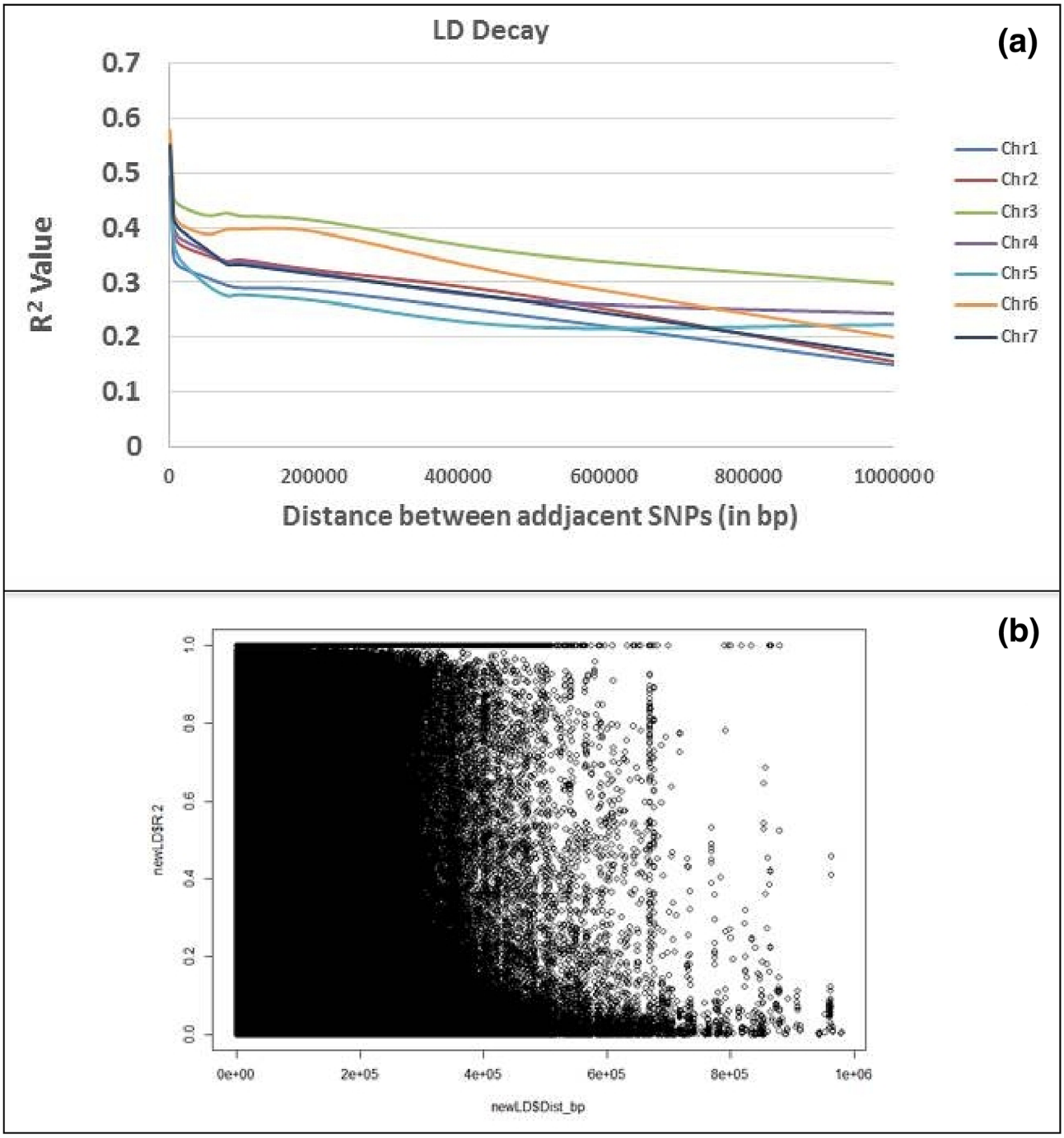


FIGURE 4 Linkage disequilibrium decay (LDD) analysis. (A) LDD plot for all the seven chromosomes. (B) LDD plot of chromosome 1.

located on chromosome 3 with a *P*-value ranging from 2.79×10^{-10} to 1.26×10^{-7}, and the proportion of total phenotypic variation explained by these three SNPs ranged from 5.9% to 13.32%. Two SNPs, namely, S4_187966579 ($P = 6.3 \times 10^{-9}$) and S4_29249234 ($P = 1.07 \times 10^{-8}$) were found, located on chromosome 4, accounted for 11.54% and 11.21% of phenotypic variance, respectively. Among the remaining three SNPs, one SNP each was found located on chromosome 1, 6 and 7. All eight SNPs reported were found significant in both GLM and MLM models.

3.3.3 | Pg 204

A total of three SNPs were found significantly associated with the genetic regions governing resistance to Pg 204. Among these, one SNP was located on chromosome 4 (S4_4224990, $P = 9.2 \times 10^{-12}$) and two SNPs were located on chromosome 1 with a *P*-value ranging from 4.2×10^{-10} to 1.45×10^{-7}, and the proportion of total phenotypic variation explained by these three SNPs ranged from 10.08% to 18.45%. All four SNPs were significant in GLM and FarmCPU models.

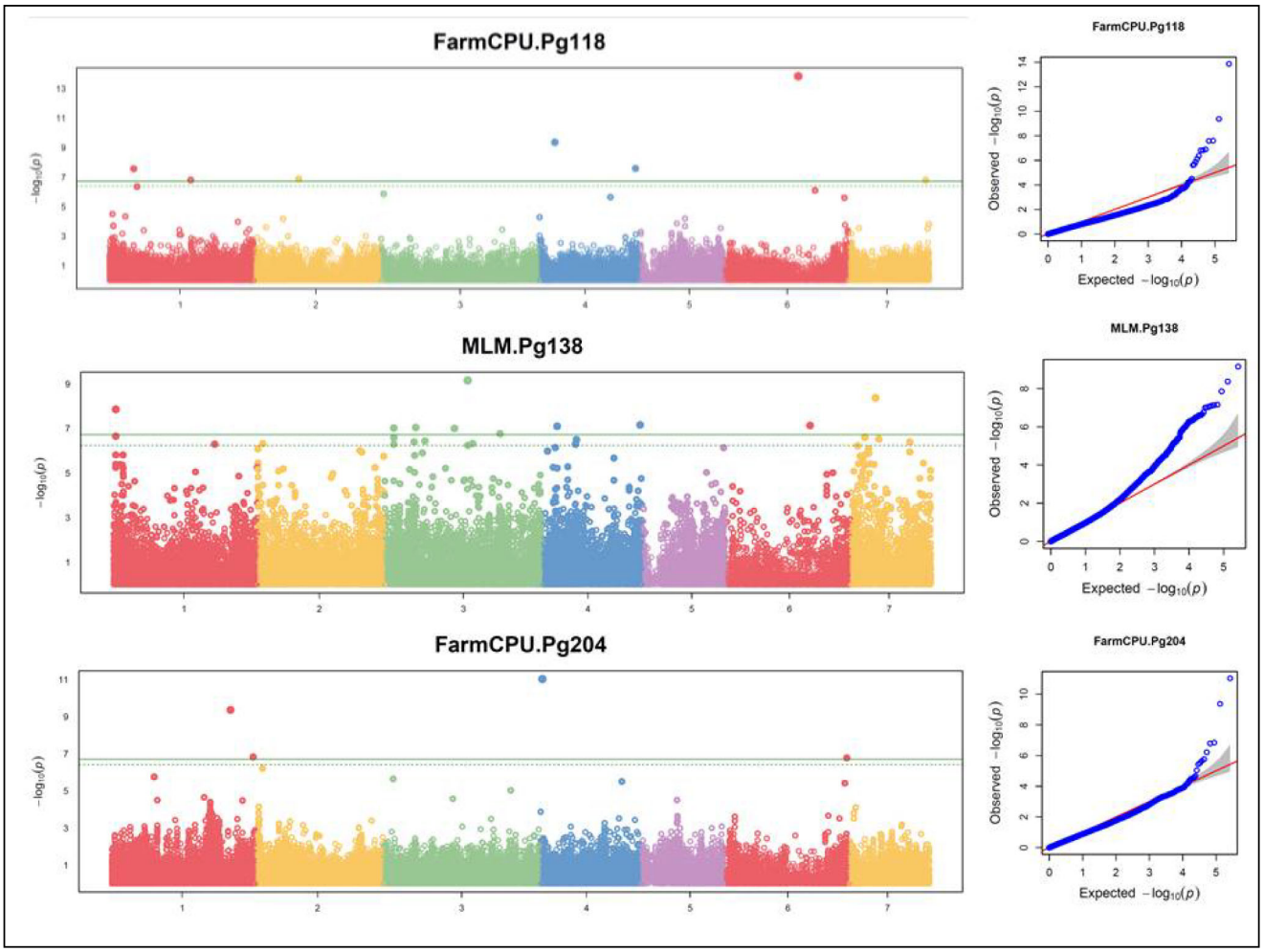


FIGURE 5 Manhattan plots and the quantile–quantile (QQ) plots for the associations detected for four pathotypes for pearl millet blast, plotted with the individual single nucleotide polymorphisms (SNPs) of all chromosomes on the *X*-axis and −log10 *P*-value of each SNP on the *Y*-axis. The different colours indicate the seven chromosomes of pearl millet. The green horizontal line shows the multiple testing threshold −log10 *P*-value.

4 | DISCUSSION

Blast caused by *P. grisea* in pearl millet has become the most severe biotic constraint across the pearl millet growing regions of India and Africa (Timper et al., 2002), causing significant reduction in grain and forage yield. Blast in pearl millet in some of the studies has been found to be governed by a single dominant gene (Gupta et al., 2012; Singh et al., 2018; Wilson et al., 1989) but the resistant lines investigated in these studies were found susceptible to several other newly collected blast isolates. Differential reaction of these and some new breeding lines towards blast pathotypes indicates the presence of more resistance alleles (Sharma et al., 2021). Very few breeding lines have been found resistant against blast across diverse agro-ecologies, thus leading to the breakdown of most of the commercial hybrids bred by major pearl millet breeding programmes at the farmers' fields. This scenario of 'blast epidemic', which is quite often in last two decades, necessitates to identify series of alleles that can be responsible for stable blast resistance in pearl millet crop. Hence, the current study focused on the identification of the genomic regions associated with resistance against different blast pathotypes through genome-wide association mapping in pearl millet.

The Shapiro–Wilk test for normality of the phenotypic data for all six blast pathotypes deviated significantly from a normal distribution (Table 1). Therefore, Levene's test was performed to test the equality among the variances within experiments. Levene's test results showed that the phenotypic variance of data within experiments was homogenous ($P = .65–.93$) for all the six blast pathotypes (Table 1); hence, the overall mean value was estimated for each of the pearl millet genotypes and used in the GWAS analysis. Significant mean squares as observed in the analysis of variance for genotypes showed presence of significant variability for the disease reaction from resistant to susceptibility against blast pathotypes drawn from different geographic origins constituting differential virulence status. The mean for the blast scores across all the six pathotypes were relatively higher (5.8), indicating that majority of the genotypes in the present GWAS panel were susceptible to blast disease. Furthermore, percent resistant lines

TABLE 4 Genomic regions found significantly associated with the resistance for different virulent pathotypes of *Pyricularia grisea* in pearl millet.

Pathotype	SNP	Chr.	Position	*P*-value	MAF	R^2	Effect	GLM	MLM	FarmCPU
Pg118	S7_146512193	7	146512193	1.77E-10	.0565	.1665	.23774	Present	Present	Present
Pg118	S4_184208528	4	184208528	1.10E-09	.0672	.1564	.21317	Present	Present	Present
Pg118	S4_30412820	4	30412820	7.80E-08	.1882	.1331	−.12198	Present	Absent	Present
Pg118	S6_144092278	6	144092278	1.17E-07	.0565	.1309	.19694	Present	Absent	Present
Pg138	S3_159161939	3	159161939	2.79E-10	.0788	.1332	−.06662	Present	Present	Present
Pg138	S7_47162813	7	47162813	1.81E-09	.0897	.1223	.05963	Present	Present	Absent
Pg138	S4_187966579	4	187966579	6.32E-09	.0924	.1151	.05718	Present	Present	Present
Pg138	S3_17901208	3	17901208	7.24E-09	.0543	.1143	.07341	Present	Present	Absent
Pg138	S4_29249234	4	29249234	1.07E-08	.0543	.1121	−.07142	Present	Present	Absent
Pg138	S1_4417247	1	4417247	1.69E-08	.0625	0.1094	−.07902	Present	Present	Present
Pg138	S6_162597659	6	162597659	9.86E-08	.0598	.0994	.06474	Present	Present	Present
Pg138	S3_221057270	3	221057270	1.26E-07	.0679	.0599	.05998	Present	Present	Absent
Pg204	S4_4224990	4	4224990	9.22E-12	.0538	.1845	.18459	Present	Absent	Present
Pg204	S1_227218820	1	227218820	4.27E-10	.0901	.1344	.13441	Present	Absent	Present
Pg204	S1_270540796	1	270540796	1.45E-07	.1263	.1008	.10087	Present	Absent	Present

Abbreviations: Chr., chromosome; FarmCPU, Fixed and random model Circulating Probability Unification; GLM, generalized linear model; MAF, minor allele frequency; MLM, mixed linear model; R^2, squared correlation coefficient; SNP, single nucleotide polymorphism.

among 384 genotypes (B- and R- lines) identified across individual pathotypes showed highest of 15% resistant genotypes against Pg 45 followed by 14% for Pg 204 then 13% each for Pg 118 and Pg 186, and the least, that is, only 1% of resistant genotypes were identified for Pg 138. To know how the individual group, that is, B- and R-lines have performed independently, the percent resistant lines were determined within 185 B- and 199 R-lines. This showed 11% of B- and 20% of R-lines were resistant for Pg 45; 8% of B- and 17% R-lines were resistant for Pg 118; only 2% of R-lines were resistant for Pg 138; 9% of B- and 16% R-lines were resistant for Pg 186; 6% of B- and 22% of R-lines for Pg 204; and 6% of B- and 17% R-lines were found resistant for Pg 232.

Overall, the blast score varied from 2 (resistant) to >7 (highly susceptible), indicating the presence of substantial variability for the blast disease reaction among the GWAS panel. This variation among genotypes for disease reaction from susceptible to resistant was thus used in the present study to identify the genomic regions associated with resistance against blast disease through GWAS. Among all the genotypes, only two R-lines, namely, ICMR 08111 and ICMR 10888 showed resistance (mean blast score ≤3.0) to all the six blast pathotypes; whereas, single B-line, namely, ICMB 97222 showed resistance to five blast pathotypes and moderately resistant to the sixth pathotype Pg 138. These genotypes showing resistance to multiple blast pathotypes can further be utilized in breeding programmes to develop blast resistant genotypes. Overall, <15% of genotypes showed resistance against any of the six blast pathotypes (Table 3). Nine of the 10 genotypes showing resistance to at least four pathotypes were from the restorer group (R-line) and only one was from seed parent group (B-line). This significantly lesser resistance in B-lines might have resulted because of the narrow genetic base of B-group in comparison to the diversity that prevails in R-line group. The B-lines bred at ICRISAT have been mostly derived from *Iniadi* germplasm (germplasm from 'Togo' region comprising of West African countries of Ghana, Togo, Benin and Burkina Faso) (Andrews & Kumar, 1996), which have traits required in seed parents, such as high productivity, earliness, bold seedness, photo-insensitive and dark grey seed colour; whereas, R lines are derived from both *Iniadi* and mostly from non-iniadi African and Asian germplasm source with traits, such as better local adaptation, taller height and good pollen production. Furthermore, molecular diversity studies conducted earlier among R- and B-lines from this breeding programme have reported wider genetic diversity among R-line groups than the B-line group (Gupta et al., 2015; Nepolean et al., 2012; Ramya et al., 2018; Singh et al., 2018).

The SNPs identified through the whole genome re-sequencing (RAD-seq) in the present study would be best suited for exploitation of the genome-wide linkage disequilibrium as RAD-seq uses reduced-representation library sequencing technique that helps to decrease the complexity of genomes, leading to deep sequence coverage of the fragments adjacent to the restriction sites subsequently helping in SNP detection. However linkage disequilibrium (r^2), a non-random association of alleles present at different loci across the genome, largely contributes to structuring a population (Slatkin, 2008). Furthermore, LD-decay (linkage disequilibrium decay), the change in pairwise LD plotted against genetic distance, together with the magnitude of LD, ultimately determines the mapping resolution and the number of markers required for the desired marker density (Myles et al., 2009). Linkage disequilibrium decay observed in present GWAS panel is >500 Kb. This may be attributed to the fact that the current GWAS panel includes a set of genotypes (B- and R-advanced hybrid parental lines) having large linkage blocks. Similar higher LD decay was also

reported earlier by Serba et al. (2019) among pearl millet accessions from India, southern Africa, the Middle East and breeding population from the United States. Such differences in LD decay might arise, as LD is population specific, and the rate of recombination between loci and the number of generations of recombination influences the decay of LD in a population over time (Flint-Garcia et al., 2003). In addition, estimates of LD and the extent of decay with distance will be influenced by factors such as non-random mating, selection, mutation, migration or admixture, genetic drift and effective population size for any population. Such contrasting results with respect to LD decay were also observed in sorghum (Bouchet et al., 2012; Hamblin et al., 2005). In the present study, the population structure determined through PCA analysis could differentiate R- and B-lines into two distinct groups with some admixtures. This information was (population structure matrix) used in association mapping to avoid spurious associations arising out of such structuring of breeding populations. Furthermore, such distinct grouping of ICRISAT bred B- and R-lines have also been reported earlier in pearl millet (Gupta et al., 2015, 2020; Nepolean et al., 2012; Ramya et al., 2018; Singh et al., 2018).

GWAS was performed using 264,241 SNP markers after filtering for 5% minor allele frequency and >30% of genome coverage. We have tested associations using three models, namely, general linear model (GLM), mixed linear model (MLM) and FarmCPU available in GAPIT programme. We have identified 15 significantly associated markers (SNPs) across the three blast pathotypes that were used to screen the GWAS panel (Table 4). Present study indicated that chromosomes 1, 3, 4, 6 and 7 have significant QTLs linked to blast resistance in pearl millet. Previously, QTL mapping study on high-density GBS generated linkage map also reported genetic regions associated with leaf spot or blast resistance on chromosome 7 (Punnuri et al., 2016) and 1 (Sanghani et al., 2018). Also, a previous study identified random amplified polymorphic DNA markers associated with blast resistance, but they could not be assigned to any linkage group (Morgan et al., 1998). All the SNPs reported here are identified as significant SNPs across different models (overlapping) presenting the more confidence on these associations to investigate further in marker assisted breeding programme. The SNPs identified on chromosome 4 (five SNPs) had a high confidence and phenotypic variation explained (>11% R^2), and hence, this chromosome can further be targeted for marker assisted breeding for resistance against blast. Eventually, based on all three models, two SNPs (S7_146512193 and S4_184208528) for Pg 118 and three SNPs (S3_159161939, S4_187966579 and S1_4417247) for Pg138 can be designated as more robust and can be targeted during introgression breeding to improve the blast resistance in pearl millet. All these SNPs that have R^2 value more than 10%. GWAS has been intensively applied till date on the performance per se of the inbred genotypes in most of the crops including hybrid crops like maize (Xiao et al., 2017). These studies have shown that this methodology is efficient at finding candidate genes from which desirable alleles can be selected by breeders. Though, the lack of covariance between the phenotype of inbred lines and their test-cross in cross pollinated crops (like in maize or pearl millet) raises questions on the applicability of these findings in a hybrid breeding context (Galli et al., 2020). There have been few reports of mapping traits in a hybrid test-cross background that allows dominant alleles to be detected (Farfan et al., 2015). The SNPs identified based on performance per se of parental lines in our study should be further validated through test cross performance to improve the prediction accuracy to strengthen the breeding pipelines to develop blast resistant pearl millet hybrids.

The blast caused by *Pyricularia grisea* has been a severe concern in pearl millet, which causes significant yield losses. The present study identified two R-lines (ICMR 08111 and ICMR 10888) showing resistance to six blast pathotypes and one B-line (ICMB 97222) that showed resistance against five blast pathotypes. These genotypes can be used as a resistant source to develop new blast-resistant R- and B-lines. The genetic map and identification of genomic regions associated with blast resistance would be the best possible sustainable approach in developing a novel blast resistance hybrid. Furthermore, for the identification of SNPs associated with blast resistant regions, GWAS was performed, which revealed 15 SNPs to be linked with blast resistance. These SNPs will play a pivotal role in screening and identification of blast resistance genotypes in pearl millet germplasm after their validation across different genetic backgrounds. The SNPs identified on chromosome 4 (five SNPs) had a high confidence and phenotypic variation explained (>11% R^2), and hence, this chromosome can further be targeted for marker assisted breeding for resistance against blast.

AUTHOR CONTRIBUTIONS

Study concept and design: Shashi K. Gupta, Rajan Sharma and Raman Babu. *Contribution to experimental materials*: Shashi K. Gupta. *Conducting of experiments*: Shashi K. Gupta and Rajan Sharma. *Analysis and/or interpretation of data*: Shashi K. Gupta, Punna Ramu, Sushil Kumar, Mahesh Pujar, Raman Babu and Rajan Sharma. Drafting of the manuscript: Mahesh Pujar, Sushil Kumar, Punna Ramu, Rajan Sharma and Shashi K. Gupta. *Critical revision of the manuscript*: Shashi K. Gupta, Rajan Sharma, Punna Ramu, Sushil Kumar, Mahesh Pujar and Raman Babu. *Reading and approval of the final manuscript*: Shashi K. Gupta, Rajan Sharma, Punna Ramu, Sushil Kumar, Mahesh Pujar and Raman Babu.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests or competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in [NCBI, US National Library of Medicine) at https://www.ncbi.nlm.nih.gov//sra/?term=SRP063925.

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