

MOLECULAR CHARACTERIZATION AND CLUSTERING OF BIOTIC STRESS-RESISTANCE ALLELES IN NEPALESE RICE LANDRACES FOR BROAD-SPECTRUM RESISTANCE

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ABSTRACT

Nepal's diverse agro-ecological environments contain a rich reservoir of rice landraces with considerable potential for developing cultivars resistant to biotic stresses. However, the distribution and combination of alleles conferring resistance to multiple diseases and insect pests have not been sufficiently characterized. In this study, 188 Nepalese rice landraces were evaluated using 40 trait-associated molecular markers representing seven major biotic stress categories: brown planthopper, gall midge, rice tungro virus, bacterial leaf blight, blast, rice stripe virus, and rice yellow mottle virus. A multi-stress breadth score (ranging from 0 to 7) was calculated based on the presence of discriminating traits. Hierarchical clustering using Jaccard distance and average linkage identified five molecular clusters (C1–C5) based on 32 biotic stress-associated markers. Cluster C1, comprising 126 landraces (67.0%), represented the predominant group with moderate allele accumulation across categories and notable enrichment for blast (mean 58.9%) and rice stripe virus (mean 68.3%) resistance. Cluster C2 (23 landraces; 12.2%) contained the highest accumulation of favorable alleles (mean 10.2 ± 1.5) and was strongly enriched for bacterial leaf blight (64.3%), blast (72.5%), gall midge (34.8%), and brown planthopper (28.3%) resistance loci. Cluster C3 (29 landraces; 15.4%) was specifically enriched for brown planthopper (56.9%) and rice tungro virus (62.1%) resistance alleles. Cluster C4 (2 landraces; 1.1%) exhibited an admixed multi-stress profile with balanced allele frequencies across stresses, while Cluster C5 (1 landrace; 0.5%, *Tharu Anadhi*) demonstrated a highly specialized blast-enriched profile (92.3%) while lacking resistance alleles for brown planthopper, gall midge, rice tungro virus, and bacterial leaf blight. The distribution of resistance alleles was highly uneven: the blast-resistance allele *Pid2-Digu* was present in 99.5% of genotypes, whereas resistance to rice yellow mottle virus and the bacterial leaf blight genes *Xa21* and *Xa23* were absent. Multilocus profiling identified promising landraces, such as Balamsari, NGRC08267, Yobo, NGRC07899, Rajbhog, Tauli, Kalo Jhinuwa, and Jhinuwa Basmati, which carry favorable allele combinations across multiple stress categories. These molecular profiles provide a foundation for targeted donor selection and climate-resilient rice improvement. Nevertheless, phenotypic validation under relevant disease and pest pressures remains necessary.

KEYWORDS: Rice landraces; biotic-stress resistance; favorable alleles; molecular clustering; climate resilience; Nepal

1 INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important staple food crops worldwide, providing nourishment to nearly 50% of the global population (Fukagawa et al., 2019). With the global population projected to reach approximately 9.7 billion by 2050, rice production must increase by at least 25% to meet escalating demand. As a tropical crop, rice is predominantly cultivated in irrigated ecosystems, which contribute nearly 75% of global rice production. In Nepal, rice plays a vital role in food security and the national economy, contributing nearly 50% of total cereal production and supporting the livelihoods of smallholder farmers. Despite its nutritional, economic, and cultural importance, rice production is increasingly threatened by a wide range of biotic stresses (Pedrozo et al., 2025). Pathogens and insect pests collectively reduce attainable rice yields by approximately 30% globally, with estimated losses ranging from 24.6% to 40.9% and often being greater in food-deficit regions where pest and disease pressure is high (Savary et al., 2019). Among these constraints, major diseases such as blast, bacterial blight, and sheath blight can cause substantial yield losses, while insect pests, including stem borers and planthoppers, and weeds further reduce productivity depending on the production environment (Ghimire et al., 2026; He et al., 2023; Savary et al., 2000, 2019).

The accelerating pace of climate change has intensified the frequency, severity, and unpredictability of major biotic constraints, challenging global rice production. In South Asia alone, rice diseases collectively impose an estimated annual economic burden of US\$4–10 billion and account for an average yield gap of approximately 30 % across regional production systems (Ghimire et al., 2026; Savary et al., 2019). Among these, rice blast (*Magnaporthe oryzae*) remains the most destructive disease, with maximum documented yield losses of up to 70 % and complete crop failure (100 % loss) through neck blast at grain filling in susceptible varieties; in Nepal's

mid-hill districts, epidemics routinely cause losses of 40–60 % (Ghimire et al., 2026; Skamnioti & Gurr, 2009). Bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*), the foremost bacterial disease in the region, reduces yields by 10-60 %, averaging 20-30 % in susceptible varieties under high-nitrogen management (Ghimire et al., 2026; Niño-Liu et al., 2006). Rice tungro virus, transmitted by green leafhoppers, can inflict catastrophic losses of up to 80 % in epidemic years (Ghimire et al., 2026; Hibino, 1996). While brown planthopper (*Nilaparvata lugens*) outbreaks have become increasingly common with changing rainfall patterns, and gall midge (*Orseolia oryzae*) infestations continue to constrain production in susceptible genotypes, viral pathogens, including rice stripe virus and rice yellow mottle virus (RYMV), further compound yield instability, with combined pest and disease losses historically ranging from 20-60 % (Dean et al., 2012; Jena & Kim, 2010). Under field conditions, these biotic stresses rarely occur in isolation; a single growing season may expose a rice crop to sequential or simultaneous pest and disease pressures, meaning that varieties resistant to only one stress may not be sufficient to maintain stable yields, particularly for the more than 80 % of smallholder farmers in South Asia who lack financial buffers to absorb disease-induced income shocks (Ghimire et al., 2026). Therefore, developing or identifying rice varieties that can resist multiple biotic stresses is essential for stable crop production under changing climate conditions (Das & Rao, 2024; Ramalingam et al., 2024). However, developing rice varieties resistant to several biotic stresses is difficult because different stresses often require different, and sometimes conflicting, plant defense responses. The distribution of biotic stress-resistant genotypes is widely recognized as the most sustainable, economically viable, and ecologically sound strategy for mitigating yield constraints (Collard & Mackill, 2008). Over the past two decades, significant progress has been made in identifying, fine-mapping, and cloning major genes and quantitative trait loci (QTLs) that confer resistance to these key biotic stresses. For blast resistance, *Pi* genes such as *Pid2*, *Pid3*, *Pi33*, *Pi54*, *Pik*, and *Pikh* encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins that recognize pathogen effectors and activate hypersensitive immune responses (Hassan et al., 2022; Liu et al., 2015). For bacterial leaf blight resistance, *Xa* genes, including *Xa4*, *Xa7*, *Xa21*, *Xa23*, and *Xa26*, provide race-specific to broad-spectrum resistance through diverse molecular mechanisms (Chen et al., 2021; Jiang et al., 2019; Mew et al., 2004). Brown planthopper resistance is mediated by genes such as *Bph32*, *Bph17*, and *qBph9*, which confer antibiosis and antixenosis through phloem-based defense and defense signaling pathways (Cheng et al., 2023; Jena & Kim, 2010; Muduli et al., 2021). Gall midge resistance is associated with *Gm* genes, particularly the *Gm4* locus on chromosome 8, which triggers hypersensitive response-mediated resistance (Bentur et al., 2016; Nair et al., 2013). For viral diseases, *tsv1* confers recessive resistance to rice tungro spherical virus, while *STV11* encodes a sulphotransferase that provides durable resistance to rice stripe virus (Wang et al., 2014). The identification of these genes and the development of closely linked, gene-specific molecular markers have strengthened marker-assisted breeding, allowing breeders to identify useful genetic resources and efficiently transfer favorable alleles into improved rice varieties (Collard & Mackill, 2008).

Nepal possesses remarkable topographic and climatic diversity, ranging from the tropical lowlands of the Terai (below 100 m) to temperate hills and alpine regions above 3,000 m, which has created a wide range of micro-environments suitable for crop diversification and adaptation. This extreme environmental diversity has contributed to a rich diversity of crops and their genetic resources. Nepal is recognized as a prominent center of rice genetic diversity, harboring a vast repository of traditional landraces. It is estimated that over 8,000 rice accessions have been maintained ex situ and in situ by farming communities across these diverse agro-ecological niches (Joshi et al., 2020). For generations, Nepalese farmers have not only cultivated these landraces but have actively practiced selection and conservation, resulting in highly adapted populations that harbor unique adaptive traits ranging from exceptional cold tolerance in high-altitude areas to resistance against locally prevalent neck and leaf blast races in the humid eastern hills (Giri et al., 2020; Khadka et al., 2025; Pant et al., 2018). Notably, Nepalese rice germplasm has contributed to the understanding of stress tolerance in rice.

Despite this enormous potential, Nepalese rice landraces remain underexploited as genomic resources for breeding against biotic stresses. Large scale studies on the distribution and combination of major biotic stress-resistance genes in Nepalese rice germplasm are still limited (Joshi, 2023; K.C. et al., 2021). While individual studies have examined specific traits, such as blast resistance in the Terai or bacterial leaf blight in the hills, a comprehensive study covering seven major biotic stresses brown planthopper, gall midge, rice tungro virus, bacterial leaf blight, blast, rice stripe virus, and rice yellow mottle virus across a diverse set of Nepalese rice landraces is still lacking. Moreover, using multivariate clustering to group these genetic resources based on their multiple stress-resistance gene profiles can help identify promising donor landraces and better understand patterns of disease and pest adaptation. To address this critical knowledge gap, the present study was undertaken to systematically screen a diverse panel of traditional Nepalese rice landraces for the presence of favourable alleles associated with seven major biotic stresses using 40 trait-associated molecular markers targeting previously reported resistance genes and QTLs, including *Bph32*, *Gm4*, *TSV1*, *Xa4*, *Xa7*, *Pid2*, *Pid3*, *STV11*, and RYMV loci. The specific objectives were to: (i) comprehensively profile the allelic distribution of these biotic stress-resistance genes across the germplasm; (ii) identify elite landraces possessing favourable combinations of alleles conferring resistance to multiple biotic stresses; and (iii) employ hierarchical clustering to classify the landraces into distinct groups based on their multi-stress genetic profiles. The findings contribute to the targeted conservation and deployment of native rice genetic resources for breeding climate-resilient varieties adapted to diverse agro-ecological environments.

2 MATERIALS AND METHODS

2.1 Plant materials and seed sources

A total of 188 rice landraces were evaluated in this study. The majority of the landraces were obtained from the National Agriculture Genetic Resources Center (NAGRC), Khumaltar, Lalitpur, Nepal. At the same time, the remaining accessions were collected from the Local Seed Bank, Phulbari, Chitwan, Nepal. The complete list and source information of the evaluated landraces are provided in Appendix 1. The collected landraces were grown and maintained during 2022/23 and 2023/24 to ensure genetic uniformity and sufficient seed stock for subsequent evaluation. Field experiments were conducted at the research farm of the Department of Genetics and Plant Breeding, Agriculture and Forestry University (AFU), Rampur, Chitwan, Nepal. The experimental site was located at 27°38'39.0" N latitude and 84°21'03.5" E longitude.

2.2 Leaf sample collection and preparation for DNA analysis

Leaf samples were collected from healthy and representative plants of each rice landrace for molecular analysis. One healthy plant was selected from each experimental plot for DNA sampling (Shiokai et al., 2009). We collected six leaf discs, each approximately 6 mm in diameter, from a fully expanded, mature leaf using a sterile leaf punch. To minimize the possibility of cross-contamination among samples, the leaf surface and sampling equipment were disinfected with 80% ethanol before sampling. The leaf punch was thoroughly cleaned after collection from each plant, and gloves were used throughout the sampling procedure. Each sample was assigned a unique identification code corresponding to the respective landrace and was immediately placed in a labeled sample collection box. Duplicate sample boxes were prepared to provide an additional safeguard against sample loss or misidentification during handling and transportation.

The collected leaf samples were dried in a laboratory oven at 40°C for 48 h to reduce moisture content and minimize tissue degradation and fungal growth (Shen et al., 2022). Dried leaf tissue is suitable for DNA extraction when brittle (Till et al., 2015). Following drying, the samples were packed with silica gel and prepared for shipment to the INTERTEK Australia Laboratory (agritech.australia@intertek.com) through the INTERTEK India Laboratory (agritech.india@intertek.com) for molecular genotyping.

2.3 Genotyping using the 1K Rice Custom Amplicon panel

Genotypic characterization of the rice landraces was performed using the 1K Rice Custom Amplicon (1k RiCA) panel developed by the International Rice Research Institute (IRRI) (Arbelaez et al., 2019). The panel contains 1,022 molecular markers, comprising 800 genome-wide markers, 22 quality-control (QC) markers, and 200 trait-specific markers. The panel was validated for genotyping populations derived from the indica rice subpopulation, which is relevant since Nepalese landraces are predominantly indica (Arbelaez et al., 2019). The trait-specific component targets 87 high-value genes and quantitative trait loci (QTLs) associated with important agronomic traits and biotic and abiotic stress responses. The genotyping service was conducted by the INTERTEK laboratory using the 1k RiCA marker panel. The resulting marker dataset was used to characterize the allelic composition of the evaluated rice landraces and to determine the presence or absence of favourable alleles associated with selected biotic stress resistance loci.

2.4 Identification of biotic stress resistance loci

Trait-specific markers included in the 1k RiCA panel were used to characterize the genetic potential of rice landraces for resistance to seven major biotic stresses: brown planthopper, gall midge, rice tungro virus, bacterial leaf blight, blast, rice stripe virus, and rice yellow mottle virus. A total of 40 trait-associated markers representing genes or QTL regions previously implicated in biotic-stress resistance in rice were evaluated. These comprised seven markers associated with brown planthopper resistance, four with gall midge resistance, three with rice tungro virus resistance, eight with bacterial leaf blight resistance, 13 with blast resistance, one with rice stripe virus resistance, and four with rice yellow mottle virus resistance (Table 1). The selected markers targeted specific genes or QTL regions reported to contribute to stress adaptation in rice. The selection was based on previously established associations between the respective loci and stress-related traits.

Table 1 Biotic-stress resistance loci evaluated among 188 Nepalese rice landraces using trait-specific markers

S.N.	Biotic stress	No. of markers	Key genes/QTLs evaluated
1	Brown planthopper	7	<i>BPH9</i> , <i>Bph17</i> , <i>BPH32</i>
2	Gall midge	4	<i>Gm4</i>
3	Rice tungro virus	3	<i>TBV1</i> , <i>TSV1</i>
4	Bacterial leaf blight	8	<i>Xa26</i> , <i>Xa4</i> , <i>Xa7</i> , <i>Xa13</i>
5	Blast	13	<i>Pi33</i> , <i>Pid2</i> , <i>Pid3</i> , <i>Pi54</i> , <i>Pik</i> , <i>Pikh</i> , <i>Pb1</i>
6	Rice stripe virus	1	<i>STV11</i>
7	Rice yellow mottle virus	4	<i>rymv1</i> , <i>RYMV3</i>

Notes: A total of 16 BLB markers were included in the initial screening panel; however, eight markers (targeting *Xa10*, *Xa21*, *Xa23*, *Xa7_3*, and *SWEET14* loci) were non-informative/monomorphic for the non-favourable allele across all 188 landraces and were excluded from subsequent multivariate profiling.

2.5 Classification and scoring of favourable alleles

Genotypic data from the trait-specific markers were used to assess favourable stress-associated alleles in the 188 Nepalese rice landraces. For each marker, the previously reported favourable genotype was classified as 1 (present) and the alternative genotype as 0 (absent). Missing data were checked and, where necessary, replaced by the marker-specific mean for multivariate analyses. The original genotype data were retained separately. For each marker, the proportion of landraces carrying the favourable genotype was calculated as:

$$F_{ij}(\%) = \frac{N_{ij}}{N_j} \times 100$$

where F_{ij} is the frequency of the favourable genotype at marker j in the evaluated population, N_{ij} is the number of landraces carrying the predefined favourable genotype at marker j , and N_j is the total number of landraces with valid observations for that marker.

Favourable allele frequencies were summarized for seven stress categories: brown planthopper, gall midge, rice tungro virus, bacterial leaf blight, blast, rice stripe virus, and rice yellow mottle virus. The resulting binary matrix was used for hierarchical clustering, while the original genotype data were retained for detailed analysis and supplementary tables.

2.6 Quantification of stress-specific and multilocus favourable-allele accumulation

The accumulation of favourable alleles was assessed at both the stress-category and overall levels. For each landrace, favourable loci were counted separately for brown planthopper, gall midge, rice tungro virus, bacterial leaf blight, blast, rice stripe virus, and rice yellow mottle virus resistance. The total favourable-locus count was obtained by summing the favourable loci across all seven stress categories.

A multi-stress breadth score ranging from 0 to 7 was also calculated based on the number of stress categories for which a landrace carried at least one favourable locus. Thus, the total favourable-locus count represented the overall number of favourable loci carried by a landrace, whereas the breadth score represented the number of different stress categories covered by its favourable allele profile. These measures were used together to identify landraces with both high favourable-allele accumulation and broad multi-stress profiles.

2.7 Molecular clustering based on favourable stress-resistance allele profiles

Hierarchical molecular clustering was performed to identify groups of Nepalese rice landraces sharing similar combinations of favourable stress-associated allele states. The analysis was based on the binary matrix generated from the predefined favourable genotype states rather than on phenotypic observations. Because the blast-resistance gene *Pid2-Digu* was nearly ubiquitous among the evaluated landraces, it provided limited discriminatory power and was excluded from the clustering matrix. Clustering was therefore performed on the remaining major stress-tolerance genes to maximize resolution among landraces.

Pairwise dissimilarity among landraces was calculated using the Jaccard distance, based on the presence and absence of favourable allele states. The resulting distance matrix was subjected to hierarchical agglomerative clustering using average linkage (UPGMA). The dendrogram was divided into five molecular clusters (C1-C5), and cluster membership was assigned to each landrace.

The resulting clusters were interpreted based on their stress-associated allele composition, rather than as measures of genome-wide genetic relatedness. A circular dendrogram and heatmap were used to visualize cluster structure and the distribution of favourable alleles across landraces. Thus, the C1-C4 groups represent similarity in the predefined stress-resistance allele profiles and should not be interpreted as overall population structure.

2.8 Cluster-level characterization of favourable allele accumulation

Following hierarchical clustering, the molecular composition of each cluster was characterized using the number and frequency of favourable allele states. For each cluster, the mean number of favourable loci carried by individual landraces was calculated across the seven stress categories. For cluster c and stress category s , the mean favourable-locus count was calculated as:

$$\bar{A}_{cs} = \frac{1}{n_c} \sum_{i \in c} A_{is}$$

where n_c represents the number of landraces belonging to the cluster c .

The frequency of favorable allele states was also calculated within each cluster:

$$F_{cs} = \frac{\sum_{i \in c} A_{is}}{n_c m_s} \times 100$$

where m_s represents the number of loci evaluated for the stress category s .

Cluster profiles were compared based on the overall pattern of favourable alleles across stress categories, rather than a single stress category. This allowed identification of clusters with relatively high accumulation of favourable loci across multiple stresses and those showing more restricted or stress-specific allele combinations.

2.9 Principal component analysis of favorable stress-associated loci

Principal component analysis (PCA) was performed to visualize variation among the 188 rice landraces based on their favorable stress-associated allele profiles. The binary genotype matrix was coded as 1 for the presence and 0 for the absence of favorable alleles, with missing values replaced by marker means where required, followed by standardization. The first two principal components (PC1 and PC2) were used to visualize the landraces, while marker/QTL loading vectors indicated their contribution to the observed variation. The markers were grouped by stress category: brown planthopper, gall midge, rice tungro virus, bacterial leaf blight, blast, rice stripe virus, and

rice yellow mottle virus resistance, to facilitate biological interpretation. PCA complemented hierarchical clustering by showing the major patterns of variation in favorable allele profiles and the stress-associated loci contributing most to landrace differentiation.

2.10 Identification and ranking of broad-spectrum stress-resilient landraces

Promising landraces were identified based on the total number of favourable loci, stress-specific favourable-locus counts, and multi-stress breadth. Landraces with a relatively high number of favourable loci distributed across two or more biotic-stress categories were given greater emphasis, particularly those belonging to clusters with higher favourable-allele accumulation. The highest-ranked landraces were summarized in Table 4 by their cluster, multi-stress breadth, and number of favourable loci for each stress category. This approach captured both the magnitude and breadth of favourable allele accumulation rather than using a simple favourable/non-favourable classification. Because the assessment was based on molecular markers, the identified landraces represent potential genetic resources for multi-stress resistance and require further phenotypic validation before their stress resistance is confirmed.

2.11 Statistical analysis and data visualization

All molecular data were organized by landrace, marker/QTL, genotype state, stress category, and favourable genotype status. Data processing, transformation, descriptive analysis, hierarchical clustering, and visualization were performed in R (version 4.6.1) using the packages *vegan*, *factoextra*, *ggplot2*, *pheatmap*, *RColorBrewer*, *lme4*, *lmerTest*, *emmeans*, *multcomp*, and *multcompView*. Descriptive analyses included the frequency of favourable genotype states at individual loci, the number of favourable loci per landrace, stress-specific favourable-locus counts, total favourable-locus counts, and multi-stress breadth scores. The results were visualized using favourable-allele heatmaps, circular hierarchical dendrograms, cluster-level summaries, and ranked multilocus profiles. These complementary analyses were used to characterize individual-locus frequencies, stress-specific allele accumulation, overall favourable-locus accumulation, and the breadth of favourable alleles across stress categories.

3 RESULTS

3.1 Chromosomal distribution of biotic stress resistance genes and QTLs

The genomic distribution of the evaluated genes and quantitative trait loci (QTLs) underscores the polygenic nature of biotic stress resistance in rice and establishes a robust molecular framework for dissecting disease and pest resistance alleles within Nepalese rice landraces. Table 2 presents the chromosomal distribution, physical positions, and favourable alleles of the biotic stress resistance loci used to characterize these landraces, encompassing seven major biotic stress categories: brown planthopper, gall midge, rice tungro virus, bacterial leaf blight, blast, rice stripe virus, and rice yellow mottle virus.

The analyzed markers were distributed across chromosomes 4, 6, 7, 8, 11, and 12, indicating that biotic stress resistance is governed by multiple genomic regions rather than by single chromosomal loci. Chromosome 11 harboured the highest density of biotic stress resistance loci, including Xa4, Xa26, Pik, Pikh, Pb1, STV11, and RYMV resistance genes, establishing it as a primary genomic hotspot for defence-related genes. Chromosome 8 emerged as a second major hotspot, carrying gall midge resistance genes (Gm4_1 to Gm4_4) in the 54.53-55.91 Mb regions, together with Pi33 loci (61.14-74.7 Mb) and bacterial leaf blight resistance genes (Xa13-1, Xa13-2 at ~26.72 Mb). Chromosome 4 carried Bph17 variants (9.14-19.69 Mb), the rice tungro bacilliform virus resistance gene TBV1_2 (31.24 Mb), and RYMV resistance loci (rymv1-2, rymv1-4 at 24.94 Mb). Chromosome 6 harboured brown planthopper resistance (Bph32 and Bph32-01, the latter at ~12.23 Mb) together with blast resistance genes Pid2-Digu (17.16 Mb) and Pid3 (13.05 Mb). Chromosome 7 carried rice tungro spherical virus resistance markers (TSV1_TKM6, TSV1_UM at 22.11 Mb), while chromosome 12 harboured BPH9 markers (BPH9_1, BPH9_2 at 22.88 Mb).

Table 2 Various biotic traits and their position in the chromosome, along with favourable alleles

Biotic Stress	Marker/ QTL	Chrom o- some No.	Positi on (Mb)	Favourable Allele
Brown planthopper	<i>BPH9_1</i>	12	22.88	T:T
	<i>BPH9_2</i>	12	22.88	A:A
	<i>Bph17_1</i>	4	17.59	T:T
	<i>Bph17_2</i>	4	9.14	G:G
	<i>Bph17_3</i>	4	19.69	G:G
	<i>BPH32</i>	6	-	G:G
	<i>BPH32-01</i>	6	12.23	G:G
Gall midge	<i>Gm4_1</i>	8	54.53	A:A
	<i>Gm4_2</i>	8	54.53	C:C
	<i>Gm4_3</i>	8	54.66	C:C
	<i>Gm4_4</i>	8	55.91	C:C
Rice tungro	<i>TBV1_2</i>	4	31.24	A:A

Biotic Stress	Marker/ QTL	Chrom o- some No.	Positi on (Mb)	Favourable Allele
Virus	<i>TSV1_TK</i>	7	22.11	C:C
	<i>M6</i>			
	<i>TSV1_UM</i>	7	22.11	C:C
Bacterial leaf Blight	<i>Xa26_1</i>	11	28.24	C:C
	<i>Xa4_1</i>	11	28.22	C:C
	<i>Xa4_2</i>	11	28.7	T:T
	<i>Xa4_4</i>	11	28.22	CCGGAGTGGAGCC
	<i>Xa7_1</i>	6	27.91	T:T
	<i>Xa7_2</i>	6	28.01	G:G
	<i>Xa13-1</i>	8	26.72	C:C
	<i>Xa13_2</i>	8	26.72	T:T
Blast	<i>Pi33_1</i>	8	61.14	T:T
	<i>Pi33_2</i>	8	67.13	T:T
	<i>Pi33_3</i>	8	74.7	T:T
	<i>Pid2_Digu</i>	6	17.16	T:T
	<i>Pi54_5</i>	6	22.3	G:G
	<i>Pid3-01</i>	6	13.05	G:G
	<i>Pid3-02</i>	6	13.05	C:C
	<i>Pik_1</i>	11	27.98	T:T
	<i>Pik_2</i>	11	27.98	C:C
	<i>Pikh_1</i>	11	27.98	T:T
	<i>Pikh_2</i>	11	27.98	G:G
	<i>Pikh_indel</i>	11	27.98	GTGTAGAGGATGTTAAAAA / GTGTAGAGGATGTTAAAAA
	<i>Pb1_1</i>	11	22.86	T:T
Rice stripe virus	<i>STV11</i>	11	17.98	T:T
Rice yellow mottle virus	<i>rymv1-2</i>	4	24.94	T:T
	<i>rymv1-4</i>	4	24.94	T:T
	<i>rymv1-5</i>	4	24.94	—
	<i>RYMV3-0</i>	11	26.37	C:C
	<i>1</i>			

Blast resistance exhibited the most complex genetic architecture, with favourable QTLs distributed across chromosomes 6, 8, and 11, including *Pid2*, *Pid3*, *Pi33*, *Pi54*, *Pik*, *Pikh*, and *Pb1*. This broad distribution supports the quantitative inheritance of blast resistance, involving multiple genes with complementary recognition specificities against diverse *Magnaporthe oryzae* races. The *Pid2-Digu* allele encodes a B-lectin receptor kinase that confers broad-spectrum resistance, whereas *Pid3* and *Pi33* encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins that recognize pathogen effectors and activate hypersensitive cell death. Bacterial leaf blight resistance was mediated by *Xa* genes on chromosomes 6, 8, and 11, with *Xa7* functioning as an executor-type resistance gene activated by TAL effectors, and *Xa4* and *Xa26* contributing to cell wall reinforcement and basal defence. Brown planthopper resistance was associated with loci on chromosomes 4, 6, and 12, where *Bph32* restricts phloem feeding, *Bph17* activates defence signalling, and *qBph9* modulates hormonal and metabolic responses. Gall midge resistance was concentrated on chromosome 8, where *Gm4* encodes a proline-rich protein that triggers hypersensitive responses in the rice gall midge interaction.

The favourable alleles identified across these loci, primarily homozygous nucleotide variants (A: A, C: C, G: G, and T: T), with several indel-specific favourable alleles, provide molecular signatures for enhanced biotic stress resistance. These alleles form the basis for allele frequency analysis, molecular clustering, and the identification of landraces carrying superior allelic combinations. The distribution of major biotic stress resistance QTLs across multiple chromosomes demonstrates the intricate genetic architecture underlying broad-spectrum disease and pest resistance. The identification of stable favourable alleles, particularly the clusters on chromosomes 8 and 11, offers significant opportunities for marker-assisted selection, gene pyramiding, and the development of climate-resilient rice varieties with enhanced multi-stress resistance.

3.2 Brown planthopper resistance

Genotypic analysis of brown planthopper (BPH) resistance loci among the rice landraces revealed considerable variation in the frequency of favourable alleles (Figure 1). Among the evaluated landraces, only Asorjog possessed favourable alleles for both *BPH32* and *BPH32-01* (G: G for both markers), indicating its potential as a unique donor source for *BPH32*-mediated resistance. Similarly, NGRC03435 was the only landrace found to carry favourable alleles for all evaluated *Bph17* variants (T: T for *Bph17_1* and G:G for both *Bph17_2* and *Bph17_3*) for the *BPH9* locus, three landraces Indrabeli, NGRC07875, and Shaja possessed

favourable alleles for both *BPH9* markers (*BPH9_1*: T:T and *BPH9_2*: A:A). In addition, Bayarni carried the favourable allele for *BPH9_2* (A: A), although it lacked the homozygous favourable allele for *BPH9_1*. Overall, the results indicate that brown planthopper resistance genes are relatively rare among the Nepalese rice landraces studied. However, the few landraces identified with favourable alleles at key resistance loci represent valuable genetic resources for breeding programs aimed at improving brown planthopper resistance in rice.

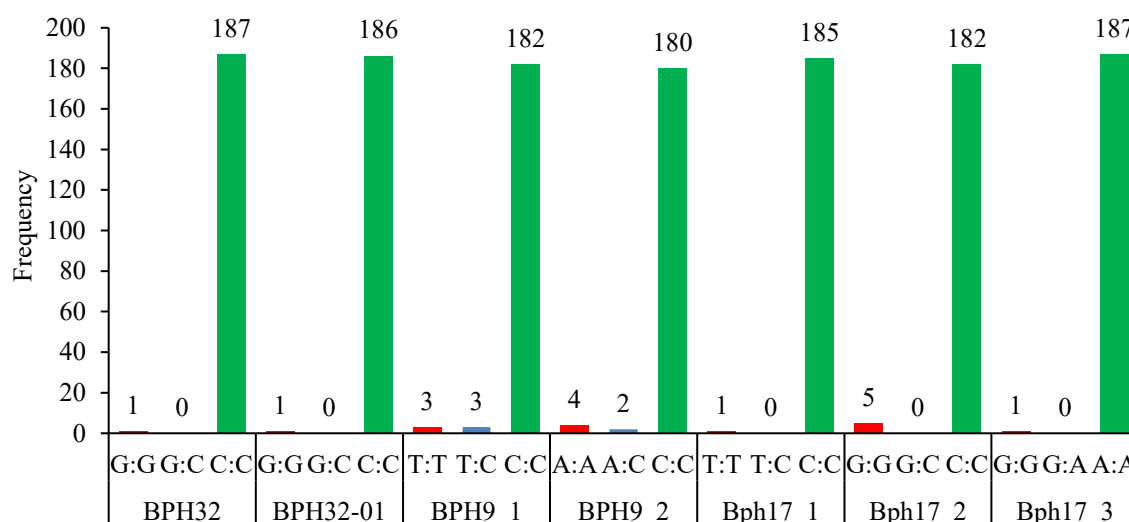


Figure 1 Distribution of brown planthopper resistance genes among Nepalese rice landraces

3.3 Gall midge resistance

The rice landraces were evaluated for the presence of gall midge resistance genes using four molecular markers, namely *Gm4_1*, *Gm4_2*, *Gm4_3*, and *Gm4_4*, all located on chromosome 8. The analysis revealed considerable variation in the distribution of favourable alleles among the studied genotypes (Figure 2). Among the evaluated landraces, only NGRC08273 possessed favourable alleles for all four gall midge resistance markers, indicating its strong potential as a donor genotype for gall midge resistance breeding. In addition, 13 landraces carried favourable alleles for three markers: *Gm4_2*, *Gm4_3*, and *Gm4_4*, suggesting the presence of substantial resistance-associated genetic variation within selected landraces. The frequency of favourable alleles varied across the four loci. Favourable alleles were detected in 22.3% of the landraces for *Gm4_1*, 10.6% for *Gm4_2*, 8.5% for *Gm4_3*, and 9.6% for *Gm4_4*. Overall, the relatively low frequency of favourable alleles across these loci indicates that gall midge resistance genes are not widely distributed among the Nepalese rice landraces studied. Nevertheless, the identified landraces with multiple favourable alleles represent valuable genetic resources for developing gall midge-resistant rice varieties.

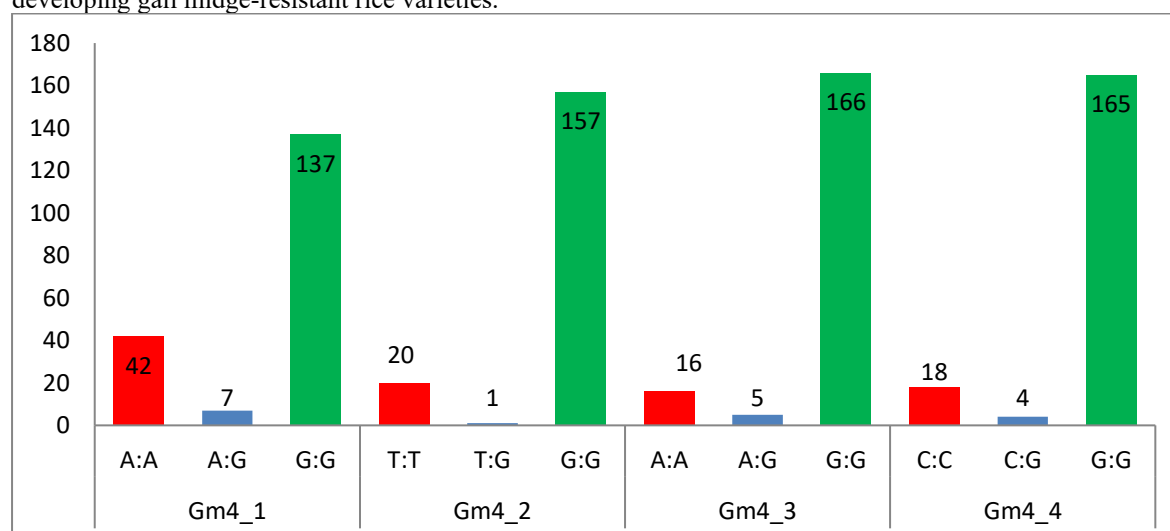


Figure 2 Distribution of rice gall midge resistance genes among Nepalese rice landraces

3.4 Rice tungro virus resistance

Genetic screening of rice landraces for rice tungro virus resistance genes was conducted using molecular markers linked to the *TBV1_2* and *TSV1* resistance loci. The *TBV* marker was linked to the *TBV1_2* gene on chromosome 4, while the markers *TSV1_TKM6* and *TSV1_UM* were associated with the *TSV1* locus on chromosome 7. The analysis revealed a low frequency of favourable alleles for tungro virus resistance among the studied landraces (Figure 3). Favourable alleles for *TBV1_2* were detected in only 3.1% of the genotypes, whereas 1.5% of the

landraces possessed favourable alleles for both *TSV1_TKM6* and *TSV1_UM*. Six landraces, Gajar Gaula, Local Jeera, NGRC03422, Rato Chucche, Seto Gede, and Tirabhul were found to carry favourable alleles for *TBV1_2*. Similarly, three landraces, namely NGRC07899, NGRC08267, and YOBO, possessed favourable alleles for both *TSV1_TKM6* and *TSV1_UM*. However, none of the evaluated landraces carried favourable alleles for all three resistance-associated markers simultaneously. Overall, the results indicate that rice tungro virus resistance genes are scarce among the Nepalese rice landraces studied. Nevertheless, the few landraces identified with favourable alleles at the *TBV1_2* and *TSV1* loci represent important genetic resources for breeding programs aimed at improving resistance to tungro virus in rice.

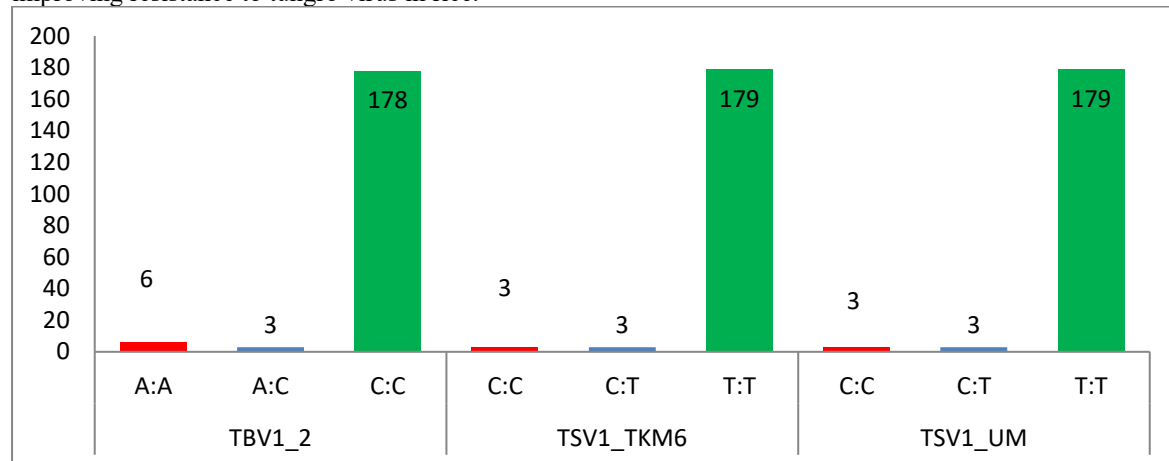


Figure 3 Distribution of rice tungro virus resistance genes among Nepalese rice landraces

3.5 Bacterial leaf blight resistance

The initial 1k RiCA panel included 16 trait-associated markers targeting bacterial leaf blight resistance. However, eight of these markers (specifically *Xa10-01*, *Xa10-02*, *Xa21-02*, *Xa23_1*, *Xa23_2*, *Xa7_3*, *SWEET14_2*, and *SWEET14_pro1*) were completely monomorphic/negative across all evaluated accessions, indicating that the corresponding resistance alleles are either absent or extremely rare in the Nepalese germplasm (Figure 4). Analysis was therefore focused on the remaining eight polymorphic markers (*Xa26_1*, *Xa4_1*, *Xa4_2*, *Xa4_4*, *Xa7_1*, *Xa7_2*, *Xa13_1*, and *Xa13_2*). The analysis revealed substantial variation in the distribution of favourable alleles among the studied genotypes. No landrace was found to possess favourable alleles for *Xa10-01*, *Xa10-02*, *Xa21-02*, *Xa23_1*, *Xa23_2*, *Xa7_3*, *SWEET14_2*, or *SWEET14_pro1* markers (Figure 4). The absence of favourable alleles at these loci suggests that several important BLB resistance genes, including *Xa21* and *Xa23*, are either extremely rare or absent in the evaluated Nepalese rice landraces. In contrast, favourable alleles were detected at several other resistance loci. The highest frequencies were observed for *Xa7_1* (56.9%), *Xa4_4* (45.7%), and *Xa26_1* (44.1%). Favourable alleles were also identified in 17.0% of the landraces for *Xa7_2*, 11.7% for *Xa4_2*, 8.5% for *Xa13_2*, 5.3% for *xa13_1*, and 4.7% for *Xa4_1*. The distribution of favourable alleles across multiple resistance loci on chromosomes 8, 11, and 12 highlights the complex genetic architecture underlying bacterial leaf blight resistance in rice. Overall, the results indicate that while certain BLB resistance genes are relatively common among Nepalese rice landraces, several highly effective resistance genes remain rare or absent, underscoring the importance of using diverse germplasm and targeted breeding strategies to enhance resistance to bacterial leaf blight.

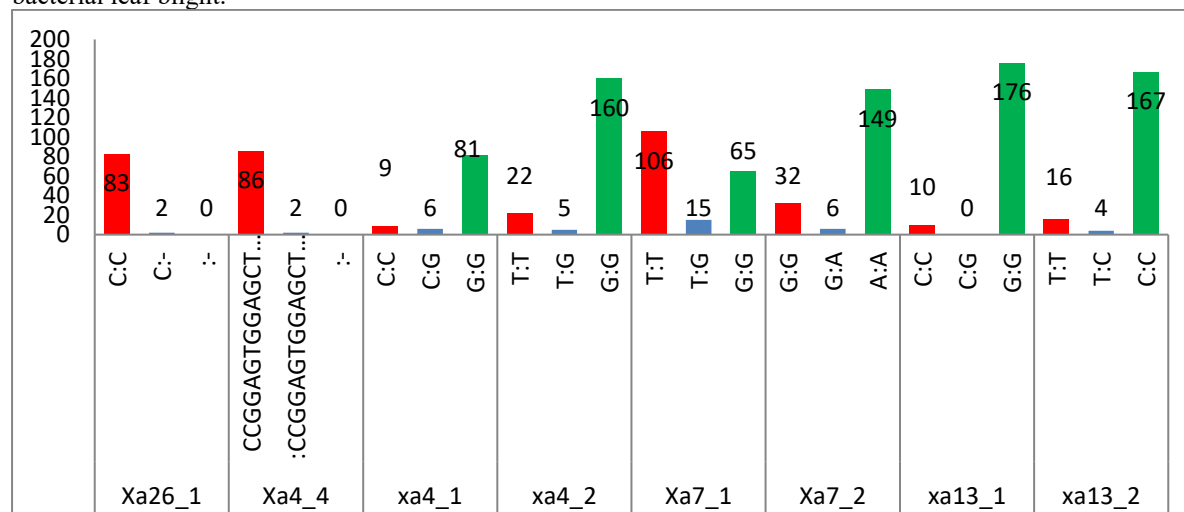


Figure 4 Distribution of bacterial leaf blight resistance genes among Nepalese rice landraces

3.6 Blast resistance

Genetic analysis of the rice landraces for blast resistance genes revealed a high frequency of favourable alleles at several important resistance loci. The studied landraces showed substantial enrichment for favourable alleles associated with the *Pid2* and *Pid3* genes (Figure 5a, b). Among the evaluated landraces, 86.1% possessed favourable alleles at all three associated markers, while favourable alleles were detected in 99.5%, 95.2%, and 92.6% of the landraces for *Pid2-Digu*, *Pid3-02*, and *Pid3-01*, respectively. Considerable variation was observed among the *Pi33* loci. Favourable alleles for *Pi33_1*, *Pi33_2*, and *Pi33_3* were identified in 1, 51, and 52 landraces, respectively. Notably, Tharu Anadhi was the only landrace found to possess favourable alleles for all three *Pi33* markers. In addition, all 51 landraces carrying the favourable *Pi33_2* allele were also found to possess the favourable *Pi33_3* allele, indicating a strong co-occurrence of these two resistance-associated markers. The analysis further revealed that 31.3% of the landraces possessed favourable alleles for *Pi54_5*, while 10.0% carried favourable alleles for both *Pik_1* and *Pik_2*. A total of 19 landraces were enriched with favourable alleles at both *Pik* loci. Similarly, favourable alleles for *Pikh_indel2* were detected in 28.6% of the evaluated landraces, and eight landraces possessed favourable alleles for both *Pikh_1* and *Pikh_2* markers. Overall, the results indicate that blast resistance genes are widely distributed among the Nepalese rice landraces, particularly the *Pid2* and *Pid3* loci, which exhibited exceptionally high frequencies of favourable alleles. In contrast, favourable alleles at the *Pi33*, *Pi54*, *Pik*, and *Pikh* loci were less common, creating opportunities for targeted use in blast resistance breeding programs.

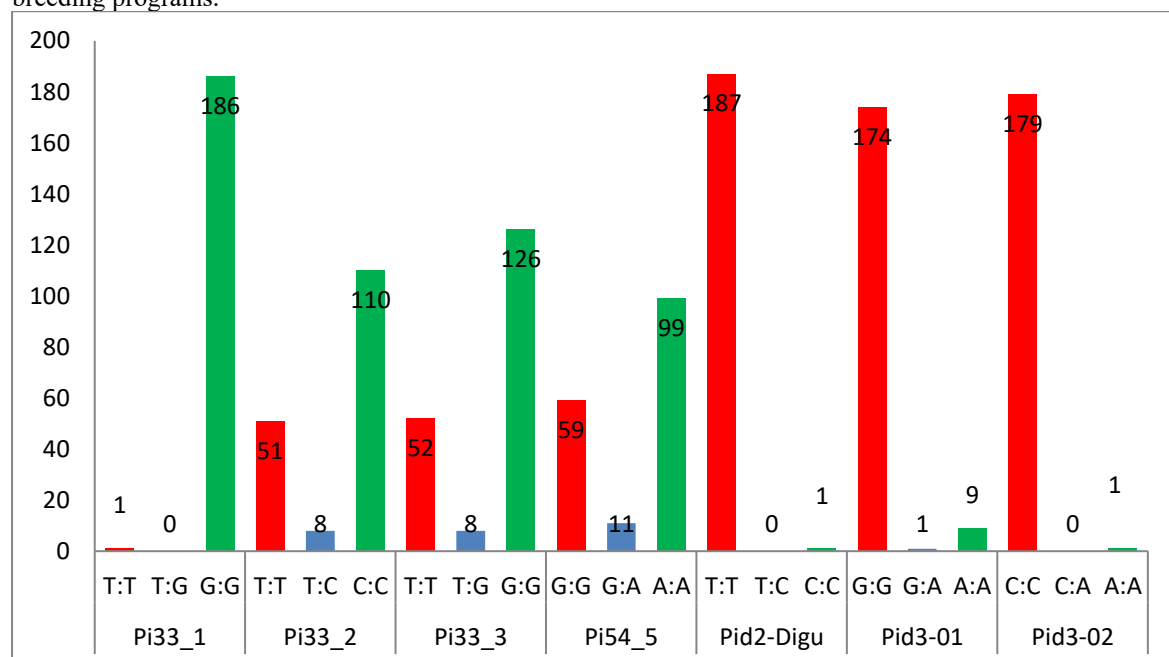


Figure 5(a) Distribution of blast resistance genes among Nepalese rice landraces

3.7 Rice stripe virus and rice yellow mottle virus resistance

The distribution of virus resistance genes varied considerably among the Nepalese rice landraces. Screening for rice yellow mottle virus (RYMV) resistance genes revealed a scarcity of favourable alleles, with little to no evidence of widespread RYMV resistance within the evaluated germplasm. In contrast, favourable alleles associated with rice stripe virus resistance were detected in 64.9% of the studied landraces. These findings indicate a substantial difference in the occurrence of resistance genes for the two viral diseases. While rice stripe virus resistance genes were relatively common among the evaluated landraces, resistance genes for rice yellow mottle virus were largely absent or occurred at very low frequencies. The limited distribution of RYMV resistance genes highlights a potential gap in the genetic resources available for breeding virus-resistant rice varieties and underscores the importance of identifying and incorporating additional sources of resistance into future breeding programs.

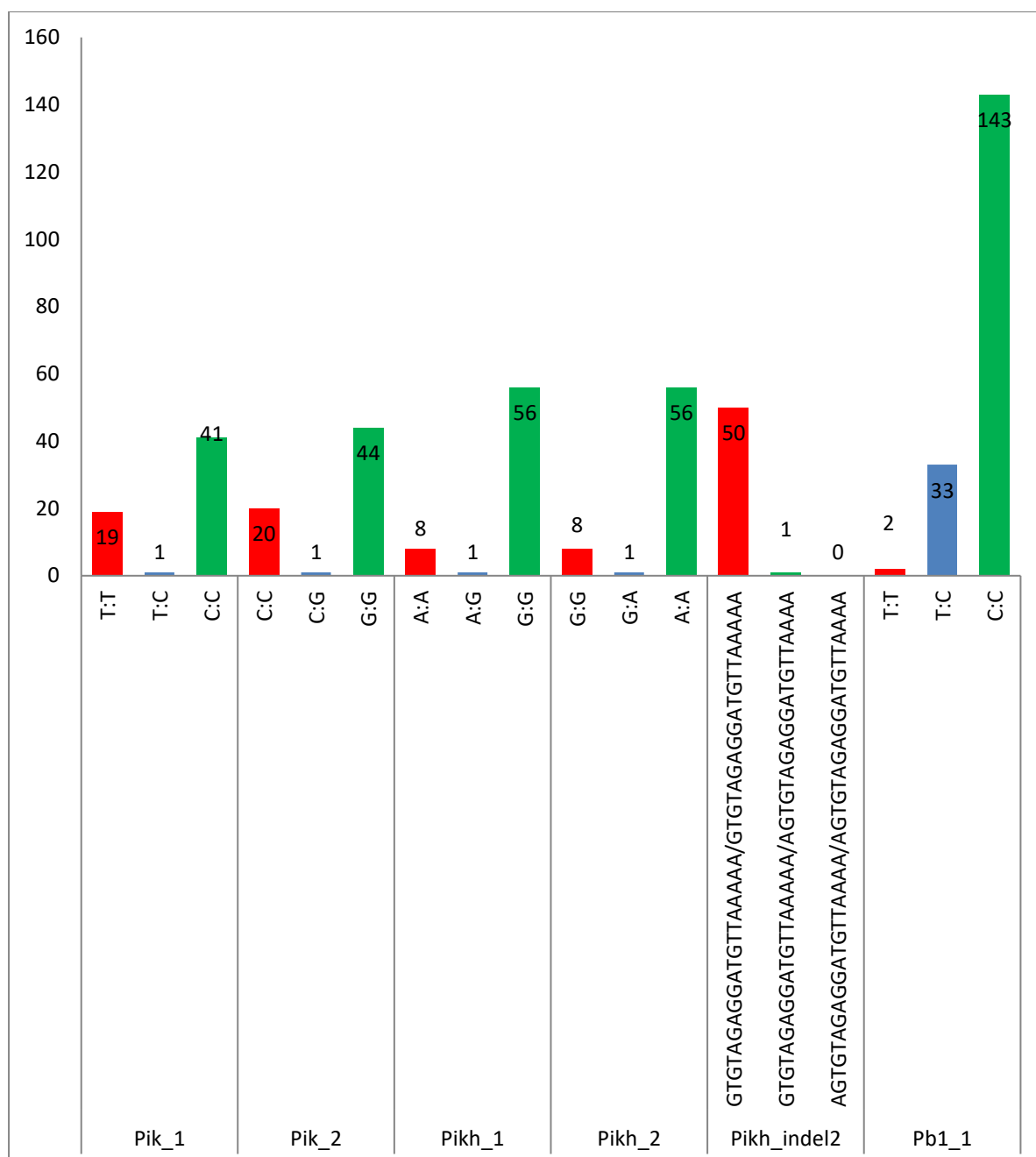


Figure 5 (b) Distribution of blast resistance genes among Nepalese rice landraces

3.8 Hierarchical clustering and cluster characterization

Hierarchical clustering based on the binary marker data grouped the 188 landraces into five distinct clusters (C1–C5), revealing clear genetic differentiation in resistance allele composition (Figure 6). Cluster C1 (126 landraces, 67.0%) represents the predominant genetic group in the Nepalese germplasm. Landraces in this cluster exhibit a balanced but moderate distribution of favourable alleles across all biotic stress categories, with notable enrichment for blast (mean 58.9%) and rice stripe virus (mean 68.3%) resistance. The relatively low frequencies for BPH (12.4%), gall midge (8.7%), and tungro virus (10.6%) indicate that these landraces possess limited resistance to these specific stresses. However, the moderate BLB frequency (32.7%) suggests a baseline level of bacterial blight resistance. Landraces such as Aachame, Aanga1, Gajali, and Jetho Budo are representative of this cluster, which serves as the foundational genetic resource for Nepalese rice breeding. Cluster C2 (23 landraces, 12.2%) comprises the most valuable genetic resources for broad-spectrum biotic stress resistance. These landraces exhibit the highest mean favourable allele count (10.2 ± 1.5) and demonstrate enrichment across multiple categories, particularly for BLB (64.3%) and blast (72.5%). The high frequencies for gall midge (34.8%) and BPH (28.3%) further underscore their broad-spectrum potential. Prominent C2 landraces include Amuhawa, Dhikatha, Khajiya, Kanak Jira, Rajbhog, Rato Dhan, and Tilaki, many of which achieved multi-stress scores of 4–5 and are top-ranked in the favourable allele summary. These landraces are ideal donors for breeding programmes targeting durable, multiple-pathogen resistance.

Cluster C3 (29 landraces, 15.4%) is distinguished by a strong BPH (56.9%) and tungro virus (62.1%) resistance profile, representing a distinct genetic group adapted to regions with high pressure from these specific stresses. The moderate BLB (38.4%) and blast (60.2%) frequencies indicate that these landraces possess resistance to multiple stresses, albeit with a different emphasis than C2. Representative landraces include Pokhara Masino, Lalayath, Tauli, Anadi Dhan, Bankae, and Seto Chuchee. Pokhara Masino is particularly noteworthy, possessing the *Bph17/xa4* allelic combination and ranking among the top landraces with 11 favourable alleles (34.38%). This cluster is valuable for breeding programmes targeting BPH and tungro virus resistance while maintaining moderate resistance to other biotic stresses.

Cluster C4 (2 landraces, 1.1%) comprises Bhatte and NGRC03110, which exhibit an admixed resistance profile with moderate frequencies across all categories. Across the evaluated stress categories, landraces in this cluster carried favorable alleles at an average frequency of 33.3% for brown planthopper (2 out of 6 loci), 50.0% for gall midge (1 out of 2 loci), 33.3% for rice tungro virus (1 out of 3 loci), 42.9% for bacterial leaf blight (3 out of 7 loci), and 61.5% for blast (8 out of 13 loci). Because Cluster C4 contains only two landraces, individual locus frequencies within the cluster are strictly discrete (0%, 50%, or 100%), whereas these percentage values reflect the average proportion of favorable loci present per stress category within the cluster. Furthermore, the presence of specific low-frequency favorable alleles—which occurred at low overall population-wide frequencies across the entire 188-landrace panel (minor allele frequencies ranging from 0.021 to 0.096)—highlights the unique genetic composition of these accessions. This distinct combination of favorable loci suggests possible heterozygosity or mixed ancestry, making Bhatte and NGRC03110 potential reservoirs of novel resistance allele combinations for gene pyramiding strategies.

Cluster C5 (1 landrace, 0.5%) consists solely of Tharu Anadhi, which exhibits a highly specialized blast-enriched resistance profile (92.3%) with negligible frequencies for all other stress categories. This landrace possesses favourable alleles exclusively for blast (*Pi33_1/2/3*, *Pid2-Digu*, *Pid3-01/02*, *Pikh_indel2*) and lacks favourable alleles for BPH, gall midge, tungro virus, and BLB resistance. The extreme divergence of Tharu Anadhi suggests a unique evolutionary history, potentially representing a landrace from a geographic region with high blast pressure but limited exposure to other biotic stresses. This landrace serves as a valuable genetic resource for blast resistance breeding and as a model for studying the genetics of specialised resistance profiles.

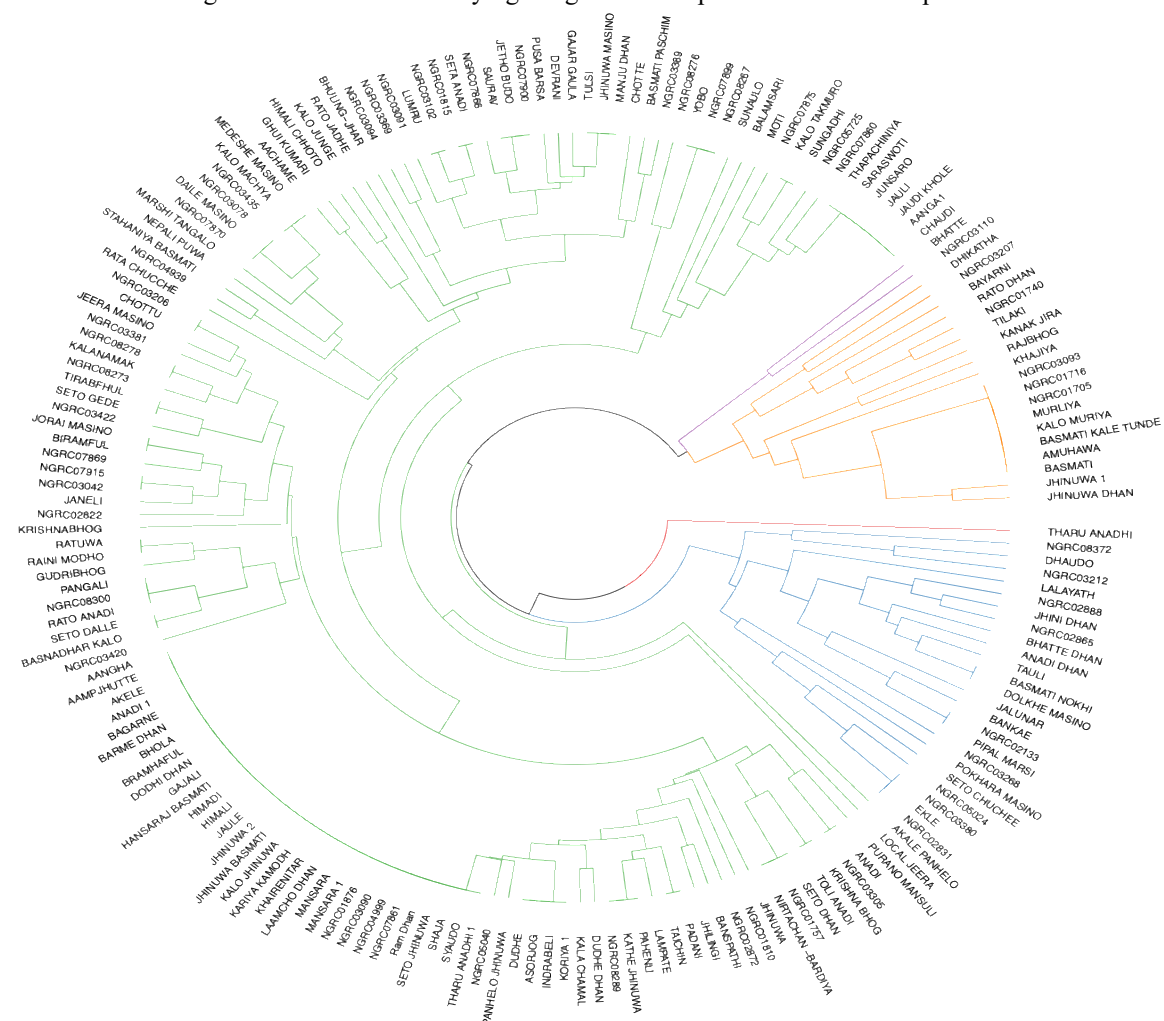


Figure 6 Hierarchical clustering dendrogram of 188 Nepalese rice landraces based on 32 biotic-stress-associated markers.

Note: Five major clusters (C1–C5) are color-coded, reflecting distinct resistance allele profiles and stress-category enrichments.

Table 3 Cluster-wise distribution, mean favourable allele counts, and stress-category enrichment profiles of 188 Nepalese rice landraces based on 32 biotic-stress-associated markers

Cluster	No. of landraces	Mean favourable alleles (±SD)	Mean frequency (%) of favourable QTLs by stress category							Interpretation
			BPH (6)	Gall midge (2)	Tungro (3)	BLB (7)	Blast (13)	Rice stripe virus (1)	RYMV (0)	
C1	126 (67.0 %)	6.4 ± 1.8	12.4	8.7	10.6	32.7	58.9	68.3	—	Broad-spectrum resistance with moderate allele accumulation across all categories; predominant cluster representing the general Nepalese germplasm. Landraces such as Achame, Aangal, Gajali, and Local Jeera exemplify this profile. Bacterial leaf blight and blast-enriched; high multi-stress allele accumulation; ideal donors for broad-spectrum resistance
C2	23 (12.2 %)	10.2 ± 1.5	28.3	34.8	8.7	64.3	72.5	65.2	—	(e.g., Amuhawa, Dhikatha, Kanak Jira, Khajiyia, Rajbhog, Rato Dhan, Tilaki). Brown planthopper and tungro virus-enriched; distinct resistance profile (e.g., Pokhara Masino, Lalayath, Tauli, Anadi Dhan, Bankae, Seto Chuchee); valuable for BPH and tungro virus resistance breeding.
C3	29 (15.4 %)	8.8 ± 1.9	56.9	8.6	62.1	38.4	60.2	58.6	—	Admixed unique resistance combination; potential for resistance gene pyramiding (Bhatte, NGR03110). Blast-specific resistance outlier; unique genetic background (Tharu Anadhi); valuable donor for blast resistance introgression.
C4	2 (1.1 %)	6.5 ± 0.7	33.3	50	33.3	42.9	61.5	50	—	
C5	1 (0.5 %)	10.0 ± 0.0	0	0	0	14.3	92.3	0	—	

Note: Values in parentheses indicate the total number of markers analysed per stress category. Percentages in parentheses indicate the proportion of the 188 landraces in each cluster. Cluster interpretations reflect patterns of molecular allele accumulation and do not represent direct phenotypic stress resistance. RYMV markers were removed due to no variation. BPH = Brown planthopper; BLB = Bacterial leaf blight; RSV = Rice stripe virus; RYMV = Rice yellow mottle virus. SD = Standard deviation. Mean frequencies represent the average percentage of favourable alleles per landrace within each cluster for each stress category.

3.9 Principal component analysis

Principal component analysis based on 32 biotic-stress-associated markers explained 22.4% of the total variation in the first two components, with PC1 and PC2 accounting for 13.0% and 9.4%, respectively (Figure 7). The QTL/marker vectors exhibited distinct orientations and contributions, revealing contrasting patterns of favorable allele distribution among the 188 Nepalese rice landraces. PC1 was primarily associated with bacterial leaf blight resistance loci, particularly *Xa7_2*, *xa13_1*, *xa13_2*, and *xa4_1*, which showed strong positive loadings. In contrast, brown planthopper resistance markers, especially *Bph17_2*, and rice tungro virus resistance markers (*TSV1_TKM6* and *TSV1_UM*) were predominantly oriented toward the positive PC2 axis. Blast resistance loci, including *Pik_1*, *Pik_2*, *Pikh_1*, *Pikh_2*, and *Pi33_2*/*Pi33_3*, showed strong negative loadings on PC1, indicating their distinct distribution patterns relative to bacterial leaf blight resistance markers.

The brown planthopper resistance markers *BPH9_1* and *BPH9_2* clustered near the origin with relatively short vectors, suggesting limited contribution to the first two principal components. Gall midge resistance marker *Gm4_1* and blast resistance locus *Pb1_1* showed moderate contributions to PC1, while *Pid3-01* and *Pid3-02* were oriented toward the negative PC1 and positive PC2 region. The rice stripe virus resistance marker *STV11* was positioned toward the positive PC2 direction, suggesting its favorable allele distribution was associated with this axis.

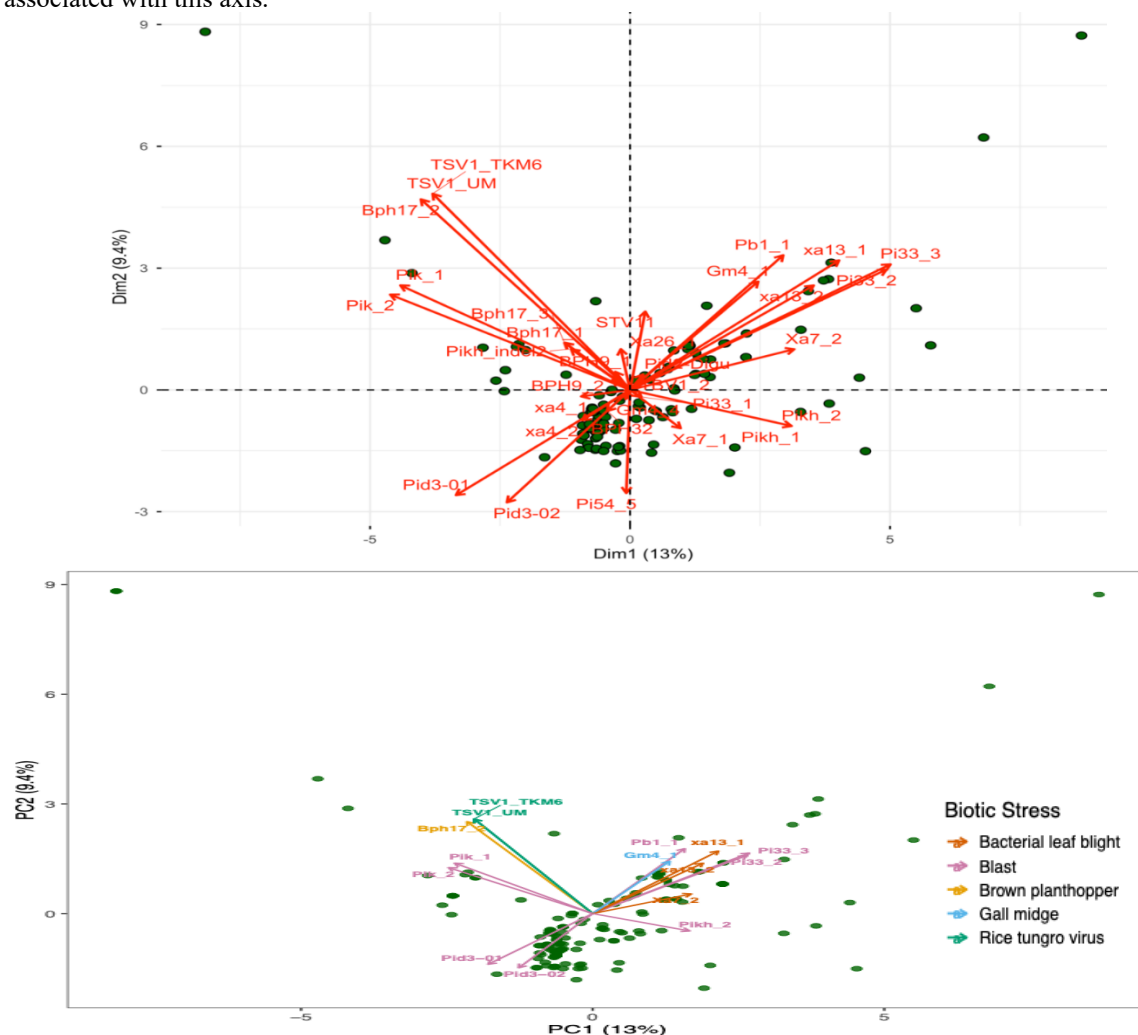


Figure 7 PCA of Nepalese rice landraces based on biotic stress markers

Note: Landraces are plotted according to PCA scores, whereas arrows represent marker loadings. Marker vectors are colored according to stress category (bacterial leaf blight, blast, brown planthopper, gall midge, rice tungro virus, and rice stripe virus). Genotypes were coded as 1 for the predefined favorable allele and 0 for its absence. Percentages indicate the variance explained by PC1 and PC2. Missing genotype values were replaced by marker means before analysis.

PC3 (8.3%) differentiated landraces based on a specific combination of BPH and BLB resistance alleles. The positive axis was driven by the BPH markers *Bph17_1* and *Bph17_3*, together with the BLB resistance genes *xa4_1* and *xa4_2*. In contrast, negative PC3 loadings were associated with the blast resistance markers *Pik_1* and *Pik_2* (−0.387), and the BLB locus *Xa7_1*. This pattern reveals that certain landraces tend to co-inherit the *Bph17/xa4* cluster, while others carry the *Pik/Xa7* cluster, indicating possible linkage blocks or selection histories. Landraces such as Pokhara Masino, Krishnabhog, and Tharu Anadhi exhibited extreme positive PC3 scores, reflecting their enrichment for the *Bph17/xa4* combination.

PC4 (7.2%) primarily separated landraces based on blast resistance gene families and gall midge resistance. Positive loadings were driven by the blast loci *Pikh_1*, *Pikh_2*, *Pid3-02*, and the gall midge marker *Gm4_4*. The negative axis was defined by *Pbl_1*, *Xa7_1*, and *xa13_2*, along with the BPH marker *Bph17_2*. This separation highlights that PC4 distinguishes landraces harbouring the *Pikh/Pid3/Gm4* cluster (positive) from those carrying the *Pbl/Xa7/xa13* combination (negative). Notable landraces with extreme positive PC4 scores included Nepali Puwa, NGRC01740, Rato Dhan, and Tilaki, while Kanak Jira and Rajbhog showed the most negative scores.

PC5 (7.1%) captured a refined divergence within blast resistance genes and their co-inheritance with gall midge and tungro virus resistance. The positive axis was driven by the gall midge marker *Gm4_1*, the BPH markers *BPH9_1* and *BPH9_2*, and the tungro virus marker *TBV1_2*, along with the blast resistance loci *Pid3-01* and *Pikh_indel2*. In contrast, negative PC5 loadings were dominated by the blast alleles *Pikh_1*, *Pikh_2*, and *Pi54_5*, together with *xa13_1* and the tungro markers *TSV1_TKM6* and *TSV1_UM*. This bipolar structure reveals that PC5 primarily separates landraces carrying the *Gm4/BPH9/TBV1* favourable allele cluster (positive) from those enriched for the *Pikh/Pi54/xa13/TSV1* cluster (negative). Notably, while both groups possess blast resistance genes, they belong to different members of the *Pik* locus family (*Pid3* and *Pikh_indel* variants versus *Pikh* and *Pi54*), suggesting distinct evolutionary lineages or breeding histories. Local Jeera showed the most extreme positive PC5 score, consistent with its enrichment for *Gm4_1*, *BPH9*, and *TBV1_2*, while Dhikatha and Rajbhog displayed the most negative scores, reflecting their enrichment for *Pikh*, *Pi54*, and *xa13*.

Overall, the first five principal components reveal that the genetic architecture of biotic stress resistance in Nepalese rice landraces is not only structured by major stress categories (e.g., BLB vs. blast vs. BPH) but also by specific allelic combinations and linkage blocks within these categories. These patterns have direct implications for marker-assisted breeding, as they highlight existing favourable combinations and potential linkage drag that must be considered when pyramiding non-allelic resistance genes. The top-ranked landraces, as summarised in Table 4, exhibit a broad distribution of favourable alleles across multiple stress categories, with Local Jeera possessing the highest total (14 favourable markers, 40.6%) and multi-stress score of 6, while NGRC07899, NGRC08267 and Yobo achieved multi-stress scores of 5, underscoring their value as genetic resources for broad-spectrum biotic stress resilience.

Table 4 summarizes the top-ranked Nepalese rice landraces based on the breadth and number of favourable alleles associated with biotic-stress resistance. Local Jeera achieved a multi-stress score of six, indicating the presence of favourable alleles for all six evaluated biotic-stress resistance categories. It possessed a particularly broad profile, including brown planthopper (2), blast (5), bacterial leaf blight (4), gall midge (1), rice tungro virus (1), and rice stripe virus resistance, with the highest total of 14 favourable markers. NGRC07899, NGRC08267, and Yobo each achieved a multi-stress score of five, sharing favourable alleles for brown planthopper, rice tungro virus, bacterial leaf blight, blast, and rice stripe virus resistance. Gajar Gaula also scored five, but with a distinct profile encompassing gall midge, rice tungro virus, bacterial leaf blight, blast, and rice stripe virus resistance, though lacking brown planthopper alleles. A large group of landraces—including NGRC03207, Kanak Jira, Rajbhog, Rato Jadhe, Amuhawa, Balamsari, Basmati, and Kalo Takmuro—scored four, exhibiting strong blast and bacterial leaf blight resistance alongside varying contributions from gall midge and brown planthopper. In contrast, Dudhe Dhan possessed four stress-resistance categories but with a notably lower marker count (5), reflecting a narrower allelic breadth. Notably, no landrace carried any favourable allele for rice yellow mottle virus, and the rice stripe virus marker (STV11) was the most widely distributed resistance allele across the panel. These results demonstrate that both the number and cross-stress distribution of favourable alleles should be considered when identifying rice landraces with broad-spectrum biotic-stress resilience.

Table 4 Top-ranked Nepalese rice landraces based on the number of favorable alleles associated with multiple biotic-stress resistance categories

Rank	Landrace	Multi-stress score (0–7)	Brown planthopper (0–7)	Gall midge (0–4)	Rice tungro virus (0–3)	Bacterial leaf blight (0–8)	Blast (0–13)	Rice stripe virus (0–1)	Rice yellow mottle virus (0–4)	Total favorable QTLs (0–40)
1	Local Jeera	6	2	1	1	4	5	1	0	14
2	NGRC07899	5	1	0	2	1	6	1	0	11
3	NGRC08267	5	1	0	2	1	6	1	0	11
4		5	1	0	2	1	6	1	0	11
5	Yobo Gajar Gaula	5	0	1	1	1	5	1	0	9

6	NGRC03 207	4	0	1	0	5	6	1	0	13
7	Kanak Jira	4	0	1	0	5	5	1	0	12
8	Rajbhog	4	0	1	0	4	6	1	0	12
9	Rato Jadhe	4	0	1	0	2	8	1	0	12
10	Amuhaw a	4	0	1	0	3	6	1	0	11
11	Balamsar i	4	2	0	0	2	6	1	0	11
12	Basmati Basmati	4	0	1	0	3	6	1	0	11
13	Kale Tunde	4	0	1	0	3	6	1	0	11
14	Kalo Muriya	4	0	1	0	3	6	1	0	11
15	Kalo Takmuro	4	0	1	0	3	6	1	0	11

Note: Multi-stress score = number of biotic-stress resistance categories with ≥ 1 favorable allele (0–7). Category values indicate the number of favorable resistance-associated markers detected for brown planthopper (BPH; 0–7), gall midge (0–4), tungro (0–3), bacterial blight (0–8), blast (0–13), Rice stripe virus (0–1), and Rice yellow mottle virus (0–4). Total favorable markers = sum across all six categories (0–54). Missing data were not counted as favorable. Markers were scored according to their predefined favorable genotypes. The score represents the genetic potential for multiple biotic-stress resistance and should not be interpreted as direct phenotypic resistance

4. DISCUSSION

The unpredictable South Asian monsoon, evolving pathogen populations, and intensifying insect pest pressure have increased the need for rice varieties with resistance to multiple biotic stresses. Nepal's diverse agro-ecological conditions have contributed to a rich collection of farmer-selected rice landraces. In this study, 188 Nepalese rice landraces were evaluated using 40 trait-associated markers covering seven major biotic stresses. The results showed that favourable alleles were widely distributed but varied greatly among stress categories. Hierarchical clustering identified five molecular groups with contrasting favourable-allele profiles, while multilocus analysis identified several landraces carrying favourable alleles across multiple stress categories. These results provide useful information for selecting potential donors for biotic-stress-resilient rice breeding.

4.1 Brown planthopper resistance

Brown planthopper is one of the most destructive insect pests of rice in Asia, causing direct damage through phloem feeding and transmitting viral diseases such as grassy stunt and ragged stunt. In the present study, BPH resistance genes were found to be relatively rare among Nepalese landraces. Only Asorjog possessed favourable alleles for BPH32, and only NGRC03435 carried all major Bph17 variants. Previous studies have shown that Bph32 is generally rare and primarily derived from specific donor genotypes such as Ptb33 (Zhang et al., 2024). The Bph32 gene encodes an unknown SCR domain-containing protein that is expressed in leaf sheaths and vascular bundles following BPH infestation, restricting insect feeding. Bph17, by contrast, is actually a cluster of three genes encoding plasma membrane-localized lectin receptor kinases (OsLecRK1–OsLecRK3), which collectively confer broad-spectrum, durable resistance through complex defence mechanisms including phloem-based resistance and defence signalling pathways. The complementary mechanisms of Bph17 (pest recognition and defence activation), BPH9 (hormonal and metabolic modulation), and BPH32 (phloem-based defence) could be effectively combined through gene pyramiding to achieve durable BPH resistance. The identified landraces represent valuable starting material for such efforts.

4.2 Gall midge resistance

Gall midge is a serious dipteran pest that causes silvershoot or onion shoot damage in rice, leading to complete yield loss in severely infested fields. Among the evaluated Nepalese landraces, only NGRC08273 possessed favourable alleles for all four Gm4 markers, indicating a potentially complete resistance profile. Additionally, 13 landraces carried favourable alleles for three of the four markers. The Gm4 gene encodes a proline-rich LRR protein that triggers hypersensitive responses in the rice gall midge interaction, and its presence in even a small subset of landraces highlights the value of Nepalese germplasm for gall midge resistance breeding. The low overall frequency of Gm4 favourable alleles (8.5–22.3%) suggests that this resistance is not widespread, making NGRC08273 and the partial-resistance landraces particularly important for introgression programs.

4.3 Rice tungro virus resistance

Rice tungro disease, caused by the combined infection of rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV), is transmitted by leafhoppers and can cause yield losses exceeding 50%. In the present collection, resistance alleles were scarce: only 3.2% of landraces carried favourable alleles for TBV1_2, and 1.6% possessed both TSV1 markers. The TSV1 gene, originally identified in the cultivar Utri Merah, confers recessive

resistance to RTSV and disrupts the tungro disease cycle by preventing RTSV infection, thereby indirectly reducing RTBV transmission. Genetic analysis revealed that resistance to RTSV in Utri Merah is controlled by a single recessive gene (*tsv1*) mapped within an approximately 200-kb region between 22.05 and 22.25 Mb of chromosome 7. The landraces NGRC07899, NGRC08267, and Yobo, which carried favourable TSV1 alleles, together with Gajar Gaula, Local Jeera, NGRC03422, Rato Chucche, Seto Gede, and Tirabhul (TBV1_2 positive), represent critical genetic resources for tungro resistance breeding in Nepal.

4.4 Bacterial leaf blight resistance

Bacterial leaf blight is a devastating vascular disease of rice, particularly in irrigated and rainfed lowland ecosystems. A notable finding of this study was the complete absence of favourable alleles for Xa21, Xa23, Xa10, and SWEET14-associated markers. Xa21 and Xa23 have been reported to confer resistance to a broad spectrum of *Xanthomonas oryzae* pv. *oryzae* races across Asia, and their absence in Nepalese germplasm represents a significant gap. In contrast, Xa7 (56.9%), Xa4 (45.7%), and Xa26 (44.1%) were relatively common. The Xa7 executor-type resistance gene is activated by pathogen TAL effectors and triggers hypersensitive cell death; its effectiveness increases under high-temperature conditions, making it particularly valuable for climate-resilient breeding (Wang et al., 2021). The landraces carrying combinations of Xa4, Xa7, and Xa26 alleles—particularly those in Cluster C2—may serve as valuable donors for BLB resistance pyramiding. The identification of landraces such as NGRC03207, Kanak Jira, and Dhikatha, which possessed favourable alleles at five or more BLB loci (Table 4), underscores their potential as elite donors for multiple-gene introgression.

4.5 Blast resistance

Rice blast, caused by the hemibiotrophic fungus *Magnaporthe oryzae*, is among the most damaging rice diseases globally. A remarkable finding was the near-fixation of the *Pid2-Digu* allele (99.5%) and the very high frequencies of *Pid3* alleles (>92%). These frequencies are considerably higher than those reported for Indian rice landraces, where Singh et al. (2021) observed *Pid2* in only 60.44% of accessions. Other studies on Indian landraces have reported *Pid2* frequencies ranging from 4.96% to 100%, with *Pid3* showing the highest distribution frequency at 70.33%. The widespread occurrence of *Pid2* and *Pid3* in Nepalese germplasm suggests strong positive selection due to prolonged exposure to diverse *M. oryzae* populations. However, this uniformity may also pose a vulnerability; *M. oryzae* is a rapidly evolving pathogen capable of overcoming single resistance genes (Dean et al., 2012). Both *Pid3* and *Pi33* encode NBS-LRR proteins that recognize pathogen effectors and activate immune responses, including reactive oxygen species accumulation and defence-related signalling (Shang et al., 2009; Xu et al., 2014). *Pid2*, by contrast, encodes a B-lectin receptor kinase that confers broad-spectrum resistance. The lower frequencies of *Pi33*, *Pi54*, *Pik*, and *Pikh* favourable alleles provide opportunities for diversifying blast resistance through gene pyramiding. Interestingly, Cluster C5 (Tharu Anadhi) possessed a highly specialised blast-enriched profile (92.3% blast resistance alleles) with negligible resistance to other stresses, suggesting a unique evolutionary history under high blast pressure. Similarly, Rato Jadhe (Cluster C2) carried eight blast resistance alleles, ranking among the highest for blast resistance (Table 4), making it a prime candidate for blast resistance breeding.

4.6 Rice stripe virus and RYMV resistance

Viral diseases represent an increasing threat to rice production under climate change. In the present study, rice stripe virus resistance, mediated by STV11, was relatively common (65.4%), whereas rice yellow mottle virus resistance genes were completely absent. STV11 encodes a sulphotransferase that confers durable resistance by interfering with viral replication or movement. The resistant allele of STV11 (STV11-R) encodes a sulfotransferase that provides durable resistance to rice stripe virus. The high frequency of favourable STV11 alleles suggests that Nepalese landraces may possess intrinsic resilience to rice stripe virus. In contrast, the absence of RYMV resistance is consistent with the historical confinement of this disease to Africa. RYMV is the main virus affecting the rice crop in Africa, where it causes major yield losses in farmers' fields. Nevertheless, increasing globalization and germplasm exchange may facilitate the spread of such pathogens into new regions, highlighting the need for proactive monitoring and introgression of RYMV resistance from external donors.

4.7 Molecular clustering and multilocus resistance profiles

Hierarchical clustering using Jaccard distance and UPGMA grouped the 188 landraces into five molecular clusters (C1–C5). Because the analysis was based on predefined favourable alleles at stress-associated loci, these clusters represent similarity in resistance allele profiles rather than overall genome-wide relatedness. C1 was the predominant cluster, containing 126 landraces (67.0%) with moderate allele accumulation across all stress categories. Landraces such as Local Jeera and Aachame, which carried resistance to multiple biotic stresses, may serve as excellent donor parents for broad-spectrum resistance breeding.

C2 contained 23 landraces (12.2%) and showed a distinct allele profile, with high multi-stress tolerance and enrichment for bacterial leaf blight and blast resistance. This cluster represents the most valuable genetic resource for broad-spectrum biotic stress resistance, with landraces such as Amuhawa, Dhikatha, Kanak Jira, Rajbhog, Rato Dhan, and Tilaki achieving multi-stress scores of 4–5. These landraces are ideal donors for breeding programmes targeting durable, multiple-pathogen resistance.

C3 (29 landraces; 15.4%) was distinguished by strong BPH (56.9%) and tungro virus (62.1%) resistance, representing a distinct genetic group adapted to regions with high pressure from these specific stresses. Pokhara Masino was particularly noteworthy, possessing the *Bph17/xa4* allelic combination and ranking among the top landraces with 11 favourable alleles (34.38%). C4 (2 landraces; 1.1%) comprised Bhatte and NGRC03110, which

exhibited an admixed resistance profile with unique combinations of favourable alleles, making them potential reservoirs of novel resistance gene combinations for pyramiding strategies. C5 (1 landrace; 0.5%) consisted solely of Tharu Anadhi, which exhibited a highly specialised blast-enriched resistance profile.

The large membership of C1 indicates that a substantial proportion of the germplasm lacks pyramided resistance genes, emphasizing the need for systematic marker-assisted introgression. Overall, the clustering results indicate that favourable alleles for different stresses are not evenly distributed among the Nepalese landraces, and that broad multi-stress profiles are formed from different combinations of resistance loci rather than from a single common tolerance profile.

Multilocus profiling identified 15 top-ranked landraces (Table 4) with favourable alleles across the highest number of stress categories. Local Jeera achieved a multi-stress score of six, possessing favourable alleles for all six evaluated biotic-stress resistance categories with a particularly broad profile including brown planthopper (2), blast (5), bacterial leaf blight (4), gall midge (1), rice tungro virus (1), and rice stripe virus resistance, with the highest total of 14 favourable markers. NGRC07899, NGRC08267, and Yobo each achieved a multi-stress score of five, sharing favourable alleles for brown planthopper, rice tungro virus, bacterial leaf blight, blast, and rice stripe virus resistance. These landraces provide useful candidates for further evaluation and breeding. Because the present study is based on molecular markers, these landraces should be considered potential genetic resources rather than confirmed multi-stress-resistant genotypes until their performance is validated under controlled and field disease and pest pressure.

4.8 Principal component analysis and genetic architecture

The PCA results (PC1–PC5) provided complementary insights into the genetic architecture of biotic stress resistance. PC1 (13.0%) primarily separated bacterial leaf blight resistance from blast and tungro virus resistance, with Xa7_2, xa13_1, and xa13_2 showing strong positive loadings, while Pik_1, Pik_2, and TSV1 markers showed negative loadings. This dichotomy reflects the genetic structure of Nepalese germplasm, where landraces tend to be enriched for either BLB resistance or blast/tungro resistance, but rarely both. PC2 (9.4%) further resolved the data by distinguishing brown planthopper and tungro virus resistance from blast and gall midge resistance. Landraces with extreme positive PC2 scores, such as NGRC07899, NGRC08267, and Yobo, harboured combinations of BPH and tungro virus resistance alleles.

PC3 (8.3%) differentiated landraces based on the co-inheritance of specific BPH (Bph17) and BLB (xa4) resistance alleles. Landraces such as Pokhara Masino, Krishnabhog, and Tharu Anadhi exhibited extreme positive PC3 scores, reflecting their enrichment for the Bph17/xa4 combination. PC4 (7.2%) separated landraces based on blast resistance gene families (Pikh/Pid3 versus Pb1) and their association with gall midge resistance. PC5 (7.1%) captured a refined divergence within blast resistance genes and their co-inheritance with gall midge and tungro virus resistance, separating the Gm4/BPH9/TBV1 cluster from the Pikh/Pi54/xa13/TSV1 cluster. These patterns have direct implications for marker-assisted breeding, as they highlight existing favourable combinations and potential linkage drag that must be considered when pyramiding non-allelic resistance genes. The identification of distinct allelic combinations through PCA supports the clustering results and provides a framework for strategic donor selection in breeding programmes.

4.9 Limitations and future directions

The present study has several limitations. First, the analysis was based on 40 predefined markers and therefore represents only part of the genetic variation underlying biotic stress resistance. The effect of individual alleles may also depend on genetic background, pathogen race structure, and environmental conditions. Second, the C1–C5 clusters represent similarity in the evaluated stress-associated markers and should not be considered genome-wide population structure. Third, the study did not include controlled phenotypic screening under the seven target biotic stresses. Therefore, the identified landraces should be regarded as potential sources of stress-associated favourable alleles rather than confirmed resistant genotypes. Future studies should combine these molecular profiles with replicated phenotyping under brown planthopper infestation, gall midge pressure, viral inoculation, and pathogen challenge for blast and bacterial leaf blight. Such validation, together with broader genome-wide analyses, will help confirm the value of these landraces and identify additional loci for developing rice varieties with improved resilience to multiple biotic stresses.

5. CONCLUSION

This study systematically characterized the distribution of favourable alleles associated with seven major biotic stresses across 188 Nepalese rice landraces. The results revealed substantial variation in allele frequencies, ranging from near-fixation of Pid2-Digu (99.5%) to complete absence of Xa21, Xa23, Xa10, SWEET14, and RYMV resistance alleles. Hierarchical clustering identified five molecular groups with contrasting resistance profiles, and multilocus profiling identified 15 top-ranked landraces with favourable allele combinations across multiple stress categories. Local Jeera, NGRC07899, NGRC08267, Yobo, Gajar Gaula, NGRC03207, Kanak Jira, Rajbhog, Rato Jadhe, and Amuhawa emerged as the most promising donors for broad-spectrum biotic stress resistance breeding. These findings provide a foundation for targeted donor selection and marker-assisted breeding of climate-resilient rice varieties, while emphasizing the need for phenotypic validation under relevant disease and pest pressures. The rich genetic diversity of Nepalese rice landraces, particularly the elite clusters identified in this study, represents an invaluable resource for developing rice varieties with enhanced resilience to the increasing biotic stresses associated with climate change.

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AUTHOR CONTRIBUTIONS

S.D.: Conceptualization, Methodology, Software, Formal Analysis, Investigation, Resources, Data Curation, Writing -- Original Draft, Visualization. **M.P.P.:** Conceptualization, Methodology, Software, Formal Analysis, Investigation, Writing -- Review & Editing, Visualization, Data Curation, Supervision. **K.H.D.:** Conceptualization, Methodology, Formal Analysis, Investigation, Writing -- Review & Editing, Supervision. **S.M.:** Conceptualization, Methodology, Software, Formal Analysis, Investigation, Writing -- Review & Editing, Visualization, Data Curation, Supervision.

All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST DECLARATION

The authors declare that there are no conflicts of interest regarding the conduct of this research or the publication of this manuscript.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request and subject to the requirements of the journal or publication committee.

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DECLARATION ON LANGUAGE EDITING

As English is not the authors' first language, the authors used Grammarly Pro (Grammarly Inc.) solely for grammatical correction and language refinement to assist with language editing and improve sentence structure and clarity but not for content generation, data analysis, interpretation of results, or scientific decision-making.

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