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Molecular screening of Bangladeshi rice (*Oryza sativa* l.) genotypes for bacterial leaf blight

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ABSTRACT

Bacterial leaf blight is a major threat to rice production worldwide, especially in Bangladesh. The best approach for plant breeders to address this issue is to develop resistant varieties using the available resistant gene pool. The current study used DNA marker technology to identify major BLB resistance genes in Bangladesh's landraces and local rice cultivars. We used reported molecular markers to assess the presence of BLB resistance genes (*Xa4*, *Xa5*, *Xa7*, *Xa13*, and *Xa21*) in genotypes. The results showed that the *Xa4* gene was present in 10 genotypes. Two of these genotypes also gave positive bands for the *Xa5* gene, while three showed positive results for the *Xa13* gene. Among these, one genotype contained *Xa13* and *Xa5* genes, and another had *Xa7* and *Xa21* genes. Additionally, two genotypes were found to carry both the *Xa7* and *Xa13* genes. The phylogenetic tree illustrates the genetic relationships among 41 rice genotypes, grouping them into four main clusters. Within these clusters, genetically similar genotypes tend to be grouped. This study offers valuable insights for identifying genotypes that carry multiple resistance genes, which could serve as a potential resource for breeding programs to develop rice genotypes resistant to bacterial leaf blight.

Contribution/Originality: This study helps breeders identify genotypes with multiple resistance genes, serving as a valuable resource for breeding programs aimed at developing rice germplasm resistant to bacterial leaf blight.

1. INTRODUCTION

Rice (Oryza sativa L.) is a crucial global staple, covering 40% of cereal crop land, with Southeast Asia producing over 90% of the world's rice. In Bangladesh, rice is the primary food for nearly the entire population. However, rice yield is threatened by various abiotic stresses (like salinity and drought) and biotic stresses, particularly bacterial blight (BB) caused by *Xanthomonas oryzae*. This disease can cause yield losses of up to 80%, influenced by susceptibility, environmental factors, and crop growth stage (Noh et al., 2007). In Bangladesh, a major disease causes yield losses of 5.8%-30.4% (Ansari et al., 2019), leading to food shortages and increased prices, affecting vulnerable households' access to nutrition. The dominant *Xanthomonas* pathotypes III, IV, and V enter through hydathodes, stomata, and wounds, causing leaf wilting and impacting photosynthesis (NIÑO-LIU, Ronald, & Bogdanove, 2006; Suparyono, 1984). Factors such as host susceptibility and environmental conditions contribute to disease severity. Effective

control requires strategies that focus on varietal resistance, as chemical methods are largely ineffective. Molecular breeding techniques can expedite the identification of lines with multiple gene resistance.

Molecular screening of rice germplasm for bacterial leaf blight (BLB) resistance through gene-specific markers can be an effective tool for assessing genetic diversity within the germplasm and providing information about resistance/susceptibility for further use in breeding practices. Numerous systematic research studies have been conducted to find resistant sources of BLB in rice.

Thirty-four genes (characterized in series from *Xa1* to *Xa29*) resistant to various strains of *Xoo* have been identified to date in rice (Chen et al., 2011; Chu et al., 2006; Gu et al., 2004). A. K. Singh, Singh, Arya, Singh, and Singh (2015) observed BLB resistance in rice landraces by testing for *Xa4*, *Xa13*, and *Xa21*, finding that *Xa4* was present in 69% of the landraces. Petpisit, Khush, and Kauffman (1977) identified the *Xa4* gene, which exhibited resistance at all stages of plant growth in several commercial rice varieties. The *Xa5* gene is extensively utilized in breeding programs due to its wide-ranging resistance against numerous *Xoo* strains. *X*a21 (locus for BLB resistance) recently identified from a wild species of rice (*Oryza longistaminata*) exhibited effective resistance against 17 *Xoo* pathotypes from Punjab.

Molecular markers and their correlation to phenotypes provide the requisite landmarks to clarify genetic diversity. Specifically, single-nucleotide polymorphism (SNP) markers aid in determining the genetic diversity of rice genotypes and facilitate the identification of genotypes that are biotically and abiotically stress-tolerant (Arif et al., 2019).

To advance rice resistance against BLB, it is imperative to explore new resistant plant materials and utilize remarkable gene pools. Hence, the study sought to identify rice varieties with yield-associated BLB resistance genes and incorporate them into stress breeding programs.

2. MATERIALS AND METHODS

Seeds from 41 rice genotypes (Table 1) were obtained from the Bangladesh Rice Research Institute in Gazipur and various districts of Bangladesh. Young leaves (15 to 20 days old) from actively growing seedlings were used to isolate genomic DNA (Figure 1).

The modified Cetyltrimethylammonium bromide (CTAB) method (Malek, Islam, Mamtazul, & Sultan, 2012) was employed for DNA extraction. Fresh leaf samples (200 mg) were ground in liquid nitrogen, mixed with CTAB extraction buffer, and incubated at 65°C for one hour. Chloroform was added, and the mixture was centrifuged at 10,000 rpm for 20 minutes.

The supernatant was treated with chloroform: isoamyl alcohol (24:1) and DNA was precipitated using 80% ethanol. The pellet was air-dried and resuspended in 100 µl of Tris-EDTA, and DNA quantity and quality were assessed using a Nanodrop spectrophotometer.

2.1. PCR Conditions for DNA Amplification

Previously reported five pairs of SSR primers (Table 2) were utilized for microsatellite analysis (Yap, Hsu, Wu, Lin, & Kuo, 2016).

In total, the reaction mixture was 10 µl, containing 1 µl of each forward and reverse primer, 3 µl of DNA template, 3 µl of *Taq* Green Master Mix (Canada), and 2 µl of ddH_2O water.

Polymerase chain reactions (PCR) were performed in a programmed thermocycler with an initial denaturation of 4 minutes at 94°C, followed by 35 cycles of denaturation for 30 seconds at 94°C, annealing at 57°C for 2 minutes, and extension at 72°C for 2 minutes, followed by a final extension at 60°C for 5 minutes with a final hold at 4°C.

Table 1. List of rice genotypes used in the experiment.

Sl. No.	Germplasm name	Source	Cropping season	Sl. No.	Germplasm name	Source	Cropping season
1	Mota Kartik Sail	BRRI	Deep water rice (Aman)	22	BRRI dhan28	BRRI	Boro
2	Manik Gira			23	BRRI dhan35		
3	Laxmi Digha			24	BRRI dhan45		
4	Anguli Aman			25	BRRI dhan47		
5	Dudhsor			26	BRRI dhan50		
6	Dula Bech			27	BRRI dhan81		
7	Kartik Sail			28	BRRI dhan84		
8	Kartik Jhul			29	BRRI dhan99		
9	Dulai Aman			30	BRRI dhan100		
10	Kata Mukul			31	BRRI dhan58		
11	Ijule Khoma	Cumilla		32	BRRI dhan36		
12	Lali Khoma			33	BRRI dhan97		
13	Dholi Khoma			34	BRRI dhan29		
14	Hira	Gaibandha		35	BRRI dhan55		Aus, Boro
15	Atash			36	IRBB2277		
16	Katari			37	IRBB5		
17	BR18	BRRI	Boro	38	IRBB24		
18	BR16			39	IRBB60		
19	BR8		Boro, Aus	40	IRBB64		
20	BR7			41	IRBB65		
21	BR19		Boro				

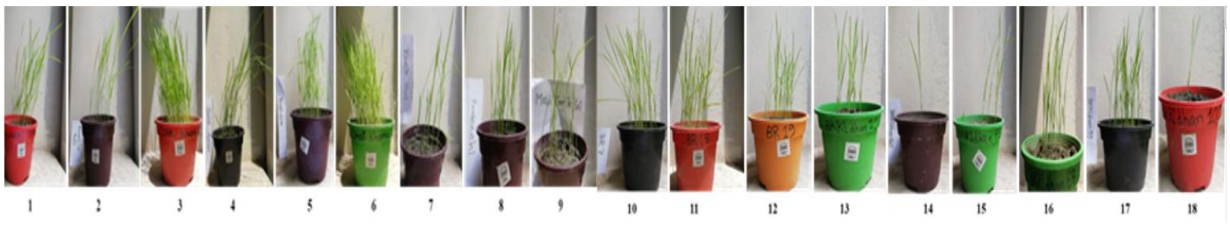

Figure 1. A representative sample of rice seedlings used for genomic DNA extraction.

The amplified products were resolved in a 2% agarose gel containing 0.5 µl ethidium bromide in 1X TBE buffer, and electrophoresis was continued for 50 minutes at 75 volts. DNA bands were visualized using an ultraviolet gel documentation system, and amplified bands were scored visually by comparing them with the standard 1500 bp ladder.

Table 2. List of SSR primers with their sequences.

Sl. no.	Resistant gene	Chr. no.	Marker name	Forward primer (5′–3′)	Reverse primer (3`-5`)	Annealing temp. (°C)
1	*Xa4*	11	Xa4F/4R	GCAGCACCATCTCCATC GTTTC	CTGCTATAAAAGGCAT TCGGGTCTC	57
2	*xa5*	5	RM604F/604R	AGGTGACCATCCTCTT TCTT	TTAACGAACGAACGCG AG	
3	*Xa7*	6	Xa7F/7-1R/7-2R	GGTCGGAAGGTGAGAA AGAGGAGG	GCATGTCTGTGTCGA TTCGTCCGTACGA	
4	*xa13*	8	Xa13F/13R	TACCTCCTGATATGTG AGGTAGTGAGAG	AGAGAGAGGTAACTT GAAGAAAGGGAT	
5	*Xa21*	11	Xa21F/21R	GCTATTTCCTGATCCA GCATATCTGATC	GATCGGTATAACAGC AAAACTATTTCCG	

2.2. Data Analysis

For the quantitative trait data analysis, ANOVA (analysis of variance) and LSD (mean comparison test) were performed using Statistix 10 software. MEGA 6.0 (Ke et al., 2022; Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) software was used to construct a phylogenetic tree dendrogram.

3. RESULTS AND DISCUSSIONS

The presence, distribution, and prevalence of specific genes in certain genotypes or populations can provide prime clues for studying genetic diversity and evolutionary patterns of valuable traits. The traits could potentially be employed for breeding programs to develop disease-resistant cultivars. The present experiment aimed to screen BLB disease-resistant rice genotypes using previously reported gene-specific SSR markers.

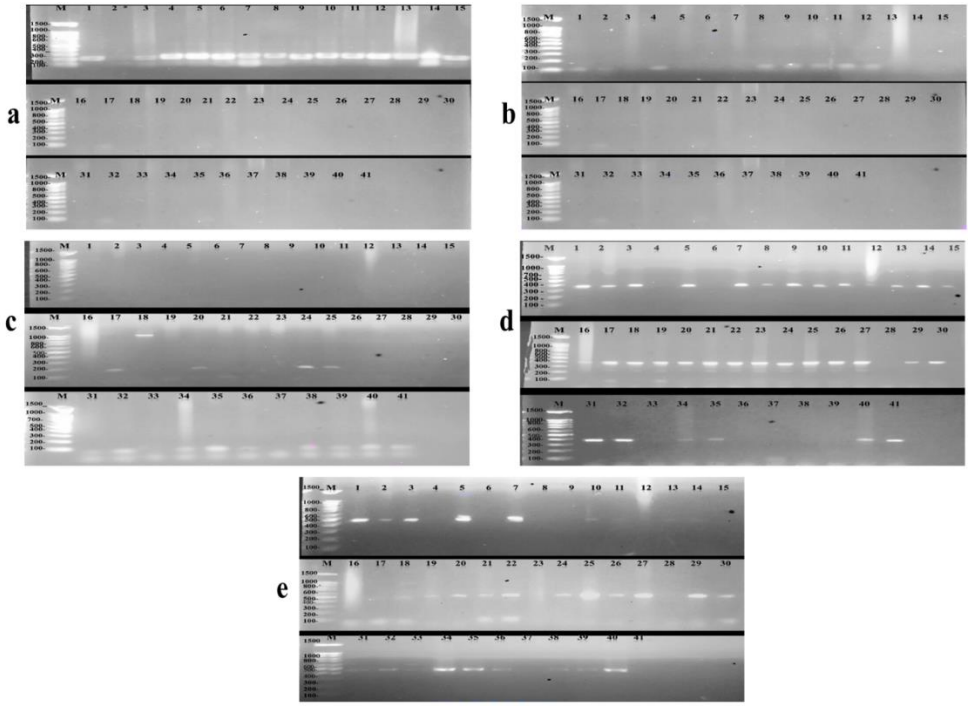

Figure 2. Identification of bacterial leaf blight disease resistance rice genotypes using functional marker **a.** Xa4F/4R, **b.** RM604F/604R, **c.** Xa7F/7-1R/7-2R, **d.** Xa13F/13R, and **e.** Xa21F/21R respectively (Table 2).

Note: M= Molecular marker (1 kb plus DNA Ladder, Bio-Basic, Canada); Lane: 1= BRRI dhan58; 2= BR18; 3= Dholi Khoma; 4= BR16; 5= BRRI dhan36; 6= BRRI dhan97; 7= BR8; 8= IRBB5; 9= Manik Gira; 10= BRRI dhan29; 11= Laxmi Digha; 12= Anguli Aman; 13= Dudhsor; 14= Dula Bech; 15= Kartik Sail; 16= Kartik Jhul; 17= Dulai Aman; 18= BRRI dhan55; 19= Atash; 20= Katari; 21= Mota Kartik Sail; 22= Ijule Khoma; 23= Lali Khoma; 24= Kata Mukul; 25= Hira; 26= IRBB24; 27= IRBB60; 28= IRBB64; 29= IRBB65; 30= IRBB2277; 31= BR7; 32= BR19; 33= BRRI dhan28; 34= BRRI dhan35; 35= BRRI dhan45; 36= BRRI dhan47; 37= BRRI dhan50; 38= BRRI dhan81; 39= BRRI dhan84; 40= BRRI dhan99; 41= BRRI dhan100.

Lower to moderate levels of diversity for BLB disease resistance genes were observed at the molecular level, Figure 2 (a-e). Out of 41 genotypes screened, 22 genotypes showed the presence of at least one resistance gene among the five genes used (Table 3). Notably, ten genotypes possessed two resistance genes and 12 genotypes possessed one resistance gene. Interestingly, 21 genotypes did not show the presence of any of the five genes studied. *Xa13* was present in 12 genotypes corresponding to the frequency of 31.70% of the total screened genotypes, which is in highest frequency compared to other genes studied. *Xa4* was present in 10 genotypes corresponding to a frequency of 25% of the total screened genotypes. *Xa7* and *Xa5* each were found to be present only in 4 and *Xa21* was found to be present only in 2 genotypes, respectively, corresponding to the frequency of 9.75% and 4.87%, respectively, of the genotypes screened in Table 3, Figure 2 (a-e). Our results in the case of *Xa4* are on par with the outcomes of Acharya et al. (2018); Ashiba, Aiyanathan, Kannan, and Pillai (2020) and Muhammad Sabar et al. (2016) also reported the presence of *Xa4* in the highest number of genotypes compared to *Xa*21, *Xa*7, and *Xa5*. The low frequency of *Xa21* and *Xa7* indicates that the genes might be rare or specific to certain genotypes, where they could perform specialized functions or provide selective advantages. *Xa13* was found the highest number of genotypes in our study, which is contradictory to the result of Singh, Goel, Hunjan, Vikal, and Lore (2012) and Baksh et al. (2021) who did not observe the presence of the *Xa13* gene in any of the rice genotypes screened for BLB resistance.

Table 3. List of rice genotypes that carry different bacterial leaf blight-resistant genes.

Resistant gene	Rice genotypes
Xa4	10 genotypes (Dudhsor, Dula Bech, Anguli Aman, BR16, BRRI dhan29, IRBB5, Manik Gira, Laxmi Digha, BRRI dhan36, and BRRI dhan97)
xa5	4 genotypes (Anguli Aman, BR16, BR18, BRRI dhan58)
Xa7	4 genotypes (Dulai Aman, Kata Mukul, Katari, Hira)
xa13	12 genotypes (Dudhsor, Dula Bech, Dulai Aman, Kata Mukul, BR18, BRRI dhan29, BBRI dhan58, BR7, BR19, Kartik Sail, BRRI dhan55, and BRRI dhan100)
Xa21	2 genotypes (Katari, Mota Kartik Sail)

The presence or absence of particular genes in different genotypes may be a sign of the population's inherent genetic diversity. Our findings revealed several genotypes (such as Dudhsor, Dula Bech, Anguli Aman, BR16, BRRI dhan29) containing multiple resistance genes, indicating their suitability as donors for marker-assisted BLB resistance enhancement. Acharya et al. (2018) detected five genotypes exhibiting the presence of both the *Xa4* and *Xa7* genes during rice genotyping screening for BLB resistance genes.

3.1. Cluster Analysis and Phylogenetic Grouping

Cluster analysis revealed the genetic relationships and similarities among different rice genotypes based on five BLB (Bacterial Leaf Blight) resistance genes. A total of 41 rice genotypes were grouped into two main clusters, designated as I and II (Table 4). Cluster I comprised 32 genotypes, which were further divided into two sub-clusters, labeled a and b. Cluster II contained 11 genotypes, also divided into two sub-clusters, a and b. All genotypes in Cluster I possessed one or more of the BLB resistance genes, while genotypes in Cluster II did not possess any BLB resistance genes, except for BR16, BR18, BR19, and BRRI dhan 100 (Table 4). The results indicated that genotypes derived from genetically similar types clustered together, exhibiting greater dissimilarity compared to other genotypes. The grouping revealed by the cluster analysis highlighted both the variation among genotypes and the variability within individual genotypes.

Table 4. List of 41 rice germplasm with their cluster.

Cluster	Sub cluster	Genotype
I	a	Dholi Khoma, Manik Gira, Dula Bech, Kartik Sail. Lali Khoma, Kartik Jhul, Anguli Aman, Dulai Aman, Katari, Laxmi Digha, Hira, Mota Kartik Sail, Dudhsor, Ijule Khoma, Kata Mukul.
	b	BRRI dhan58, BRRI dhan55, IRBB5, IRBB65, IRBB24, IRBB65, IRBB60, BR7, IRBB2277
II	a	BRRI dhan28, BR19
	b	BR18, BRRI dhan35, Atash, BRRI dhan 100, BRRI dhan47, BRRI dhan81, BR16, BRRI dhan50, BRRI dhan99

Neighbor-joining uprooted phylogenetic tree was constructed (based on the MEGA 6.0 version of the phylogenetic tree) to evaluate the genetic and evolutionary relations among the genotypes (Figure 3). The phylogenetic tree grouped 41 genotypes into four major clusters. Clusters I and IV were the largest groups, each comprising 15 rice genotypes. Remarkably, most of the members of cluster I comprised rice cultivars that carried more than one resistance gene for bacterial blight. This information would be important for enhancing the stability and resistance potential of rice genotypes. Cluster II was the second largest group, comprising nine rice genotypes, and cluster III was the smallest group, comprising only 2 genotypes. The evolutionary analysis speculated that the genotypes present in the same cluster might be genetically similar, while different clusters are genetically dissimilar. Singh et al. (2015) and Nachimuthu et al. (2015) grouped different blast-resistant rice genotypes into different clusters for the assessment of genetic diversity.

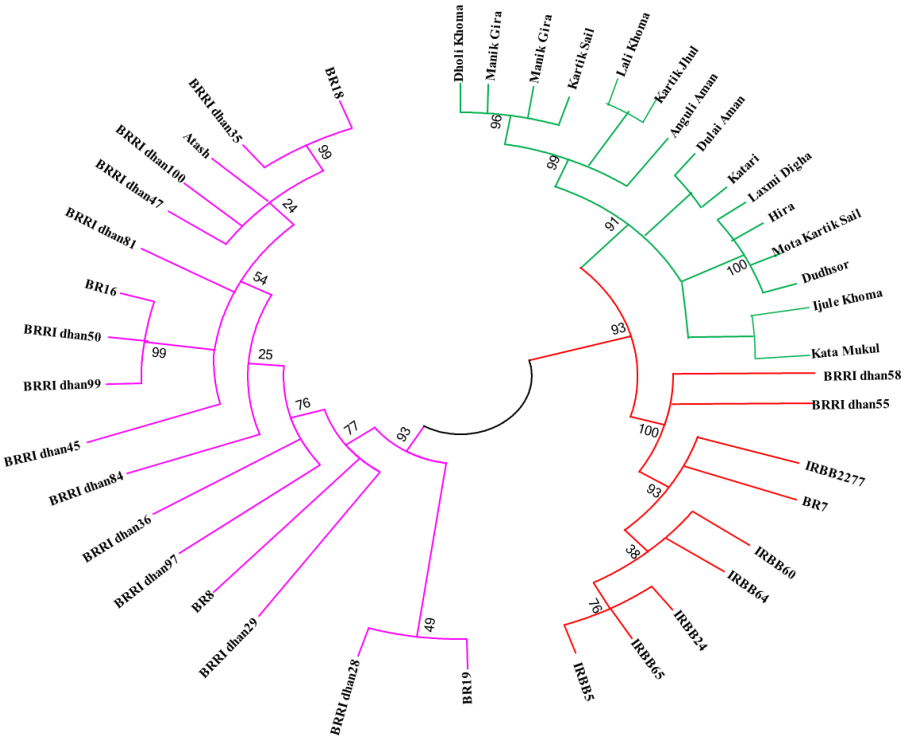

Figure 3. The phylogenetic tree of 41 rice germplasm was constructed using the neighbor-joining method by MEGA 6.0 version. The number at the branch indicates the percent bootstrap value based on 1000 replications.

4. CONCLUSION

The current study demonstrates that out of all the genotypes screened, around 12 exhibited the presence of multiple resistance genes against bacterial blight. These genotypes can be utilized in developing BLB-resistant rice varieties through a marker-assisted breeding program. Additionally, the genetic diversity and cluster analysis provide

valuable insights for selecting genetically diverse parent lines and understanding the genetic foundation of the rice germplasm.

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