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SSR Marker-based Molecular Screening of Blast Resistant Genes in Selected Rice Genotypes

Israt Yasmin, Sourav Adhikary, G.H.M. Sagor✉

Department of Genetcis and Plant Breeding, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh

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Correspondence
G.H.M. Sagor
✉: sagorgpb@gmail.com

ABSTRACT

Rice blast caused by *Magnaporthe oryzae* is one of the most devastating disease, causing major yield losses worldwide every year. Use of resistant rice varieties is one of the most effective ways to control the disease and reduce yield loss. Molecular screening and allelic diversity of major rice blast resistant genes were determined in forty-eight genotypes of rice germplasms of Bangladesh with ten previously synthesized gene based SSR (Single Sequence Repeats) markers [RM 541, RM 224, RM21, RM527, RM 208, RM 247, RM 72, RM 259, RM 246 & RM 206]. The genetic frequencies of ten major blast resistance genes (*Pi-9, Pi-1, Pi-5(t), Piz-5, Pi-b, Pi-b, Pi-ta, Pi-33, Pi27(t), Pitp(t) and Pi-k^h*) were ranged from 2% to 93%. The blast resistance gene *Pi-k^h* was widely (93%) and Pi5 (2%) was sparsely distributed among the studied genotypes. Nine genotypes sonaanjana, jabedshail, karaja, pajon, IR-09, BAU 39, binni, katiabagdad and kataribhug, had maximum eight blast resistance genes and only one BRRIdhan 46 had minimum two blast resistance genes. Twelve genotypes possessed seven, twelve had six, seven had five and five had four resistant genes. Out of forty-eight genotypes, forty-one genotypes occupied at least five positive fragments of expected product size. The results are useful to identify and incorporate blast resistance genes from these germplasms into different elite cultivars grown in Bangladesh through marker assisted selection.

Introduction

Rice (*Oryza sativa)* is the second most important cereal crop of developing countries and the staple food for about 65% of the world's population (Kumar *et al.,* 2010). It is central to Bangladesh's economy, accounting for nearly 20 percent of gross domestic product (GDP) and providing about one-sixth of the national income of Bangladesh (Thomas *et al*., 2013). Day by day the world population is increasing and the demand for rice, is also increasing on that basis. So, to meet up that demand, rice production must be increased. But every year, several fungal and bacterial diseases affect the rice production worldwide which leads to significant yield losses annually. Among the fungal disease, rice blast is one of the most destructive diseases which is caused by single pathogen *Magnaporthe oryzae* (Tanksley *et al.,* 1997). This is considered as one of the most serious diseases of rice because of their pathogenic complexity related pathogen, host, and micro weather (Singh *et al.,* 2015; Kwon and Lee, 2002; Lee, 1994; Li *et al.*, 2007; Ou, 1985; Teng *et al*., 1991). Many races of the blast fungus are normally present in the field but, some predominate the others suggesting exclusive clonality of the population (Xia *et al*., 1993; Valent and Chumley, 1994; Yang et al. 2017; Yang *et al*., 2019 a, b). The identification and isolation of additional host *R* genes and pathogen avirulence gene are now required to deepen understanding of molecular mechanisms involved in the host-pathogen interaction (Valent, 1990). Recently, many rice varieties with complete resistance to *M. oryzae* have been developed, but in many cases this resistance has been breakdown within a few years of the initial cultivation owing to the emergence of stronger virulent isolates of rice blast fungus (Bonman *et al*., 1986; Han *et al*., 2001; Kiyosawa, 1981; Mackill and Bonman, 1992; Yaegashi, 1994). Partial or field resistance of rice blast has received much attention as a means of effective control of a parasite under natural field condition and conferring durable blast resistance when exposed to new races of that parasite (Hittalmani *et al*., 2000; Liu *et al*., 2005; Wang *et al*., 1994). Development of durable blast resistant variety is one of the best solutions to fight wheat blast and ensure food security. Molecular breeding approach has the potentiality to find out sources of resistance from various potential genotypes. Using of virulence analyses for detection of specific blast

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resistance genes is time consuming and it is also required strict control of environmental factors. But, when blast resistance of a cultivar is based on a single resistance gene, it can be rapidly overcome by the appearance of compatible races of the pathogen (Hittalmani *et al*., 2000). Conventional breeding program for introgression of a blast resistance gene into commercial cultivars is slow and less efficient because of multiplicity of races of the pathogen and the masking effect of *R* genes when they occur together (Valent *et al.,* 1991; Kang *et al*., 2001; Wang *et al*., 2007). Many PCR-based markers have been developed by fine mapping and cloning which can be used to screen and identify different effective and reliable blast resistance genes. The constraints of virulence analyses can also be overcome by these developed DNA markers because, they represent a significant advantage for increasing the precision of identification and incorporation of blast resistance genes in a breeding program (Jia *et al*., 2003; Wang *et al*., 2007; Liu *et al*., 2013). It facilitates the marker assisted selection (MAS) which is less time consuming than conventional breeding programs and also more effective. Molecular genetic markers are now widely used to characterize gene bank collections that contain untapped resources of distinct alleles which will remain hidden unless efforts are initiated to screen them for their potential use and function. Abundance of SSR markers have made it an attractive tool for marker assisted selection. SSRs can be detected using allele specific PCR primers and typed by the presence or absence of PCR amplified products on standard agarose gels. Considering the above facts, the present research work was undertaken to screen out and identify blast resistant genes using SSR markers.

Materials and Methods

Plant materials

List of plant materials used in the study are given in Table 1.

Genomic DNA extraction and quantification

Genomic DNA was extracted using CTAB method (Doyle and Doyle, 1987) with slight modification. Briefly, about 0.1g of young leaves was collected from each genotype grown in plant growth room, grinded using morter and pastle. Then CTAB extraction buffer was added to fine powder, and incubated at 65^{0}C for 10 minutes. Equal amount of chloroform was added to the mixture, votexed and centrifuged. Finally, elusion and washing were done using ethanol. The extracted DNA samples were quantified spectrophotometrically by measuring A260/A280 and quality was checked by electrophoresis using 2.5 % agarose gel.

Amplification of SSR Markers by PCR

Ten previously reported SSR markers (Singh *et al*. 2015) were used to identify the presence of blast resistance genes. The details of primers are shown in Table 2.

Table 1. List of plant materials used in the study

Sl.	Genotype	Sl.	Genotype	Sl.	Genotype
1	BAU-104-33-36-7	17	BRRI dhan40	33	BRRI dhan51
2	BAU-36-8	18	Shaheb shail	34	Mathamuta
3	BRRI dhan54	19	BRRI dhan49	35	Batraj
4	Suna onjona	20	Sorna	36	Ace 6106
5	BRRI dhan72	21	BRRI dhan57	37	Kataribhug
6	BR-11	22	BRRI dhan46	38	BRRi dhan34
7	Kadam shail	23	Dula aman	39	China atab
8	Jabed shail	24	BINA dhan-7	40	Narika
9	BAU-263-113-26	25	BRRI dhan62	41	BAU-280-80
10	Jinga shail	26	Sadalakkhi	42	BRRI dhan75
11	Bishmuri	27	BRRI dhan47	43	Lakkhidiga
12	Karaja	28	Binni	44	BINA dhan-17
13	Pajan	29	BINA dhan-7	45	Supahar
14	IR-09F534	30	BR-12	46	Khaiwa
15	BAU-263-113	31	Napeli sorna	47	BRRI dhan52
16	BUA-39-10	32	Kataibagdad	48	Lal sharna

Table 2. List of SSR markers, resistance genes and their details

Sl.	Gene name	Chromosome Locus	Marker	Product size
01	*Pi-9*	6	RM 541	158
02	*Pi-1*	11	RM 224	157
03	*Pi5-(t)*	11	RM 21	157
04	*Piz-5*	6	RM 527	233
05	*Pi-b*	2	RM 208	173
06	*Pi-ta*	12	RM 247	131
07	*Pi33*	8	RM 72	166
08	*Pi-27(t)*	1	RM 259	162
09	*Pitp(t)*	1	RM 246	116
10	*Pi-k^h*	11	RM 206	147

The reaction mixture or PCR cocktail was prepared separately for each genotype and each SSR marker. About 10 µl of reaction mixture was prepared in individual PCR tube, each of which contained 50ng genomic DNA, 1 µl forward primer, 1µl reverse primer, 5 µl PCR master mix and double distilled water. PCR Addbio® Taq Master Mix was used to prepare the PCR cocktail. This master mix contains DNA polymerase, dNTPs, MgCl2 and reaction buffers at optimal concentrations for efficient application of a wide range of DNA templates PCR. The master mix also contained two dyes (blue and yellow) that allow monitoring of progress during electrophoresis, so no additional dye was required for sample loading during electrophoresis.

The PCR tubes containing the PCR reaction mixture in them were sealed and placed in a thermocycler for amplification and the PCR reaction was started immediately. Thermal cycling program involved an initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 2°C below Tm of respective primers for 30 sec, primer extension at 72°C for 30 sec, followed by a final extension at 72°C for 8 min. The amplified PCR products were separated using 2.5% agarose gel prepared in TAE buffer and visualized using ethidium bromide in a gel documentation system. The fragment amplified were scored (1) for presence and (0) for absence of specific resistant gene (Singh *et al.,* 2015).

Results

Amplicon-differentiation from Agarose Gel Electrophoresis

Forty-eight rice genotypes were amplified using ten SSR markers. The results of molecular screening of these forty-eight genotypes, for the presence or absence of 10 major rice blast resistance genes using SSR markers are given in Table 3.

Genetic frequencies of rice blast resistance genes

The electrophoresis pattern of each SSR marker linked to blast resistant gene with selected genotypes are given in the following figures (Fig. 1). Determination of PCR results for 10 blast resistance genes viz *Pi-9, Pi-1, Pi-5(t), Piz-5, Pi-b, Pi-b, Pi-ta, Pi-33, Pi27(t), Pitp(t) and Pi-k*^{h} were determined by visualization of amplicons on near 158 bp, 157 bp, 157 bp, 233 bp, 173 bp, 131 bp, 166 bp, 162 bp, 116 bp and 147 bp of positive fragments, respectively. From these PCR results, the genetic frequencies of 10 blast resistance genes were estimated. The estimated genetic frequencies were ranged from 2% to 93%. Among forty-one (41) genotypes, forty-five (45) genotypes: BAU-104-33-36-7, BAU-36-8, BRRI dhan54, Suna onjona, BRRI dhan72, BR-11, Kadam shail, Jabed shail, BAU-263-113-26-23, Jinga shail, Bishmuri, Karaja, Pajan, IR-09F534, BAU-263-113, BAU-39-10, BRRI dhan40, Shaheb shail, BRRI dhan49, Sorna, BRRI dhan57, BINA dhan-8, BRRI dhan62, Sadalakkhi, BRRI dhan47, Binni, BINA dhan-7, BR-12, Kataibagdad, BRRI dhan51, Mathamuta, Batraj, Ace 6106, Kataribhug, BRRI dhan34, Narika, BAU-280-80-28, BRRI dhan75, Lakkhidiga, BINA dhan-17 containing at least five positive bands of the ten (10) rice blast resistance markers.

The blast resistance gene *Pi-kh* was widely distributed in 93% genotypes followed by *Pi-b* in 92%, *Pita* in 83%, *Pi-9* in 81.3%, *Piz5* in 73%, *Pi-27(t)* in 66%, *pitp(t)* in 25%, *pi33* in 63%, *pi-1* in 25% and *pi-5* in only 2% genotypes. Nine (9) genotypes: Suna onjona, Jabed shail, Karaja, Pajan, IR-09F534, BAU-39-10, Binni, Kataibagdad kataribhug had maximum eight (8) blast resistance genes, while twelve (12) genotypes: BAU-104-33-36-7, BAU-36-8, BRRI dhan54, BRRI dhan72, Kadam shail, BAU-263-113-26-23, Jinga shail, Bishmuri, Shaheb shail, BRRI dhan51, Mathamuta had twelve (12) blast resistance genes, twelve (12) genotypes: BR-11, BRRI dhan40, BRRI dhan49, sorna, BRRI dhan57, BINA dhan-8, Shadalakkhi, BRRI dhan47, BINA dhan-7, BR-12, BRRI dhan34, Nerica had six (6) genes and only one (1) genotype: BRRI dhan46 had minimum two (2) blast resistant genes.

Genetic diversity of Pi-9 and pi-1 genes

The blast resistance gene *pi-9* on chromosome locus 6 and *pi-1* on chromosome locus 11 are linked to the SSR markers: RM 541 and RM 224 respectively. The PCR results for the genes *pi-9* and *pi-1* were estimated by the electrophoresis patterns or positive bands near 158 bp and 157 bp respectively. Among forty-eight (48) genotypes, *Pi-9* gene was scored on forty-one (41) genotypes while, *Pi-1* gene was scored in twelve (12) genotypes only. *Pi-1* gene fragment was the fourth most prevalent (82%) among the genotypes studied (Fig 1). Eleven (11) genotypes amplify both SSR markers (RM 541 and RM 224) corresponding to the resistance check BAU dhan-3 while four (4) genotypes did not amplify either of the two markers and that's why, negative for these two genes.

Genetic diversity of Pi-5(t) and Piz-5 genes

The blast resistance gene *pi-5(t)* on chromosome locus 11 and *piz-5* on chromosome locus 6, are linked to the SSR markers: RM 21 and RM 527 respectively. The PCR results for the genes *pi-5(t)* and *piz-5* were estimated by the electrophoresis patterns or positive bands near 157 bp and 233 bp respectively. Among forty-eight (48) genotypes, *Pi-5(t)* gene was scored on only six (6) genotypes while, *Piz-5* gene was scored in thirty-five (35) genotypes. *Pi-5(t)* gene fragment was the least prevalent (2%) among the genotypes (48) studied. Three (3) were found that amplify both SSR markers (RM 21 and RM 527). On the other hand, nine (9) genotypes were found that did not amplify either of the two markers and that's why, negative for these two genes.

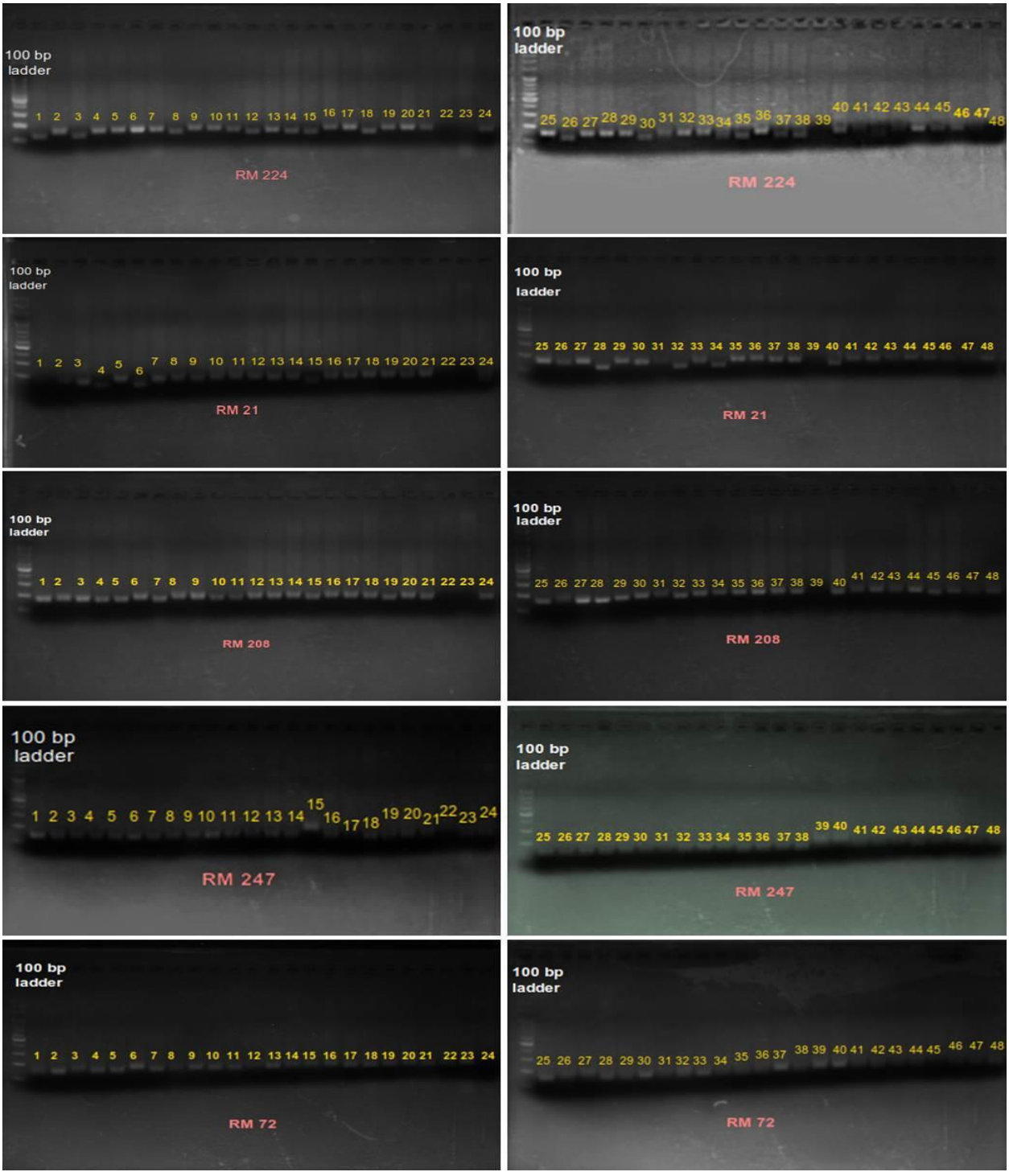

Figure 1. Agarose gel electrophoresis pattern of forty-eight rice germplasms using ten SSR (RM224, RM21, RM208, RM247, RM72, RM259, RM246, RM206 RM527 and RM541) markers. 1-48 stand for the name of different germplasm according to table 1. Left side lane; 100 bp ladder was used to identify specific DNA fragment. Names of different markers were written on individual gel photograph.

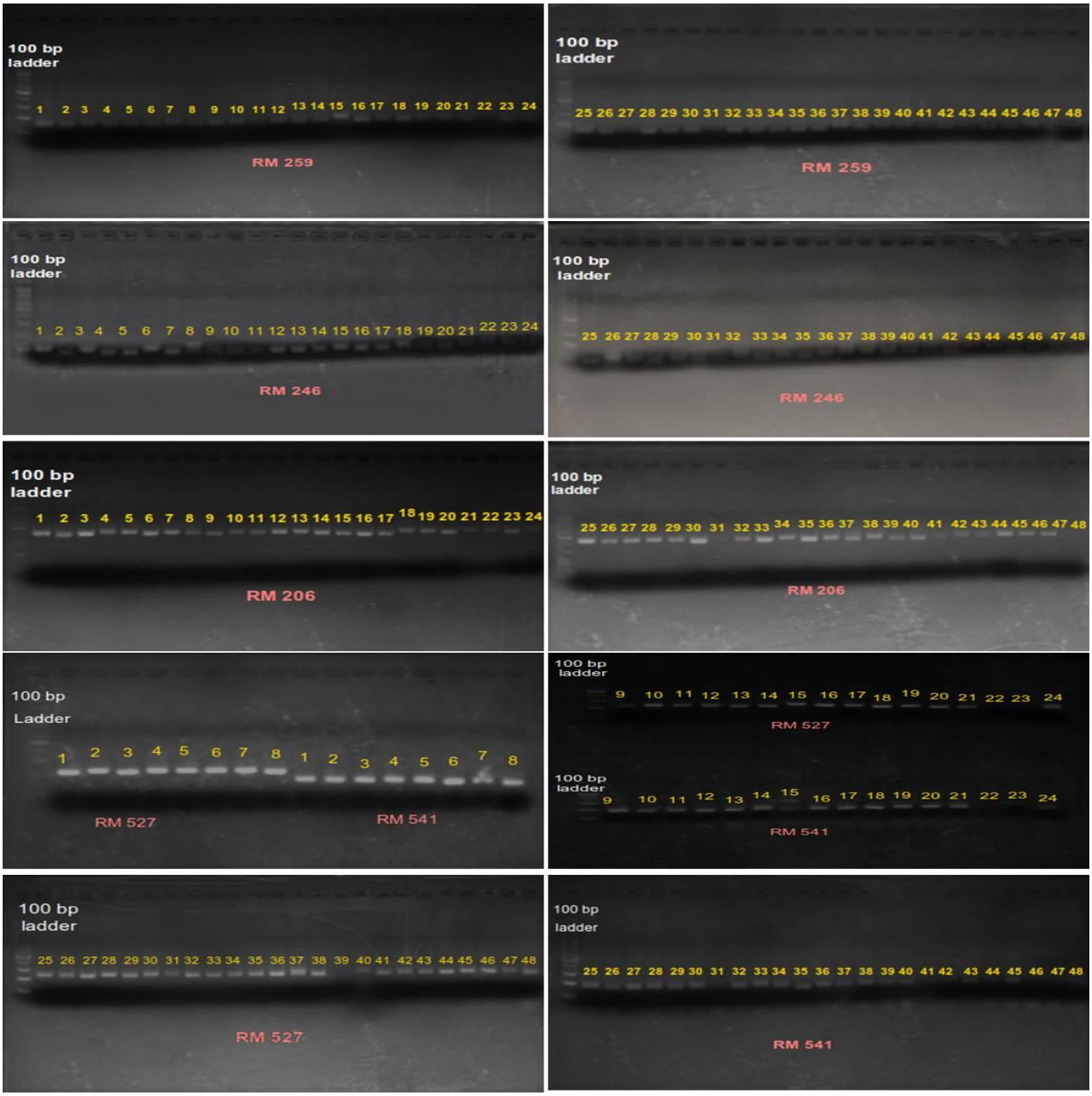

Figure 1. (Continue) Agarose gel electrophoresis pattern of forty-eight rice germplasms using ten SSR (RM224, RM21, RM208, RM247, RM72, RM259, RM246, RM206 RM527 and RM541) markers. 1-48 stand for the name of different germplasm according to table 1. Left side lane; 100 bp ladder was used to identify specific DNA fragment. Names of different markers were written on individual gel photograph.

Table 3: List of rice germplasm and screening for blast resistance gene with SSR markers

Sl no.	Accession / Variety	Blast resistance genes (R)										
		Pi-9 (541)	Pi-1 (224)	Pi5 (21)	Piz5 (527)	Pi-b (208)	Pita (247)	Pi33 (72)	Pi27 (259)	Pitp(t) (246)	PiKh (206)	Total
1	BAU-36-7	1	1	0	1	1	1	0	1	0	1	7
2	BAU-36-8	1	0	0	0	1	1	1	1	1	1	7
3	BRRIdhan 54	1	1	0	1	1	1	0	1	0	1	7
4	Sona anjana	1	0	1	0	1	1	1	1	1	1	8
5	BRRIdhan 72	1	0	0	0	1	1	1	1	1	1	7
6	BR 11	1	0	1	0	1	1	0	1	0	1	6
7	Kadam shail	1	0	0	0	1	1	1	1	1	1	7
8	Jabed shail	1	1	0	1	1	1	1	1	0	1	8
9	BAU-26-23	1	0	0	1	1	1	1	1	0	1	7
10	Jingashail	1	0	0	1	1	1	1	1	0	1	7
11	Bishmuri	1	0	0	1	1	1	1	1	0	1	7
12	Karaja	1	1	0	1	1	1	0	1	1	1	8
13	Pajon	1	0	0	1	1	1	1	1	1	1	8
14	IR-09F534	1	0	0	1	1	1	1	1	1	1	8
15	BRRIdhan 72	0	1	0	0	1	0	1	0	1	1	5
16	BAU 39-10	1	0	0	1	1	1	1	1	1	1	8
17	BRRIdhan40	1	0	0	0	1	0	1	1	1	1	6
18	Shaheb shail	1	1	0	1	1	1	1	1	0	1	7
19	BRRIdhan 49	1	0	0	0	1	1	1	1	0	1	6
20	Sorna	1	0	0	1	1	1	1	1	1	1	6
21	BRRIdhan57	1	0	0	1	1	1	1	1	0	1	6
22	BRRIdhan 46	0	0	0	0	0	0	1	0	0	1	2
23	Dula aman	0	0	0	0	0	1	1	1	0	1	4
24	BINAdhan 8	1	0	0	0	1	1	1	1	0	1	6
25	BRRIdhan 62	1	0	0	1	1	1	1	1	0	1	7
26	Sadalakkhi	1	1	0	1	0	1	0	0	1	1	6
27	BRRIdhan 47	1	0	0	1	1	1	0	0	0	1	6
28	Binni	1	0	1	1	1	1	1	1	0	1	8
29	BINAdhan 7	1	0	0	1	1	1	1	0	0	1	6
30	BR 12	1	1	0	1	1	1	1	1	0	1	6
31	Nepali sorna	0	0	0	1	1	1	0	0	0	0	4
32	Katiabagdad	1	0	1	1	1	1	1	1	0	1	8
33	BRRIdhan 51	1	0	0	1	1	1	1	1	0	1	7
34	Mathamuta	1	1	1	1	1	1	0	0	0	1	7
35	Batiraj	1	1	0	1	1	1	0	0	0	1	5
36	Ace-6106	1	0	0	1	1	1	0	0	0	1	5
37	Kataribhug	1	1	0	1	1	1	1	1	0	1	8
38	BRRIdhan 34	1	1	0	1	1	1	0	0	0	1	6
39	China atab	1	0	0	0	0	0	1	1	0	1	4
40	Nerica	1	0	1	0	1	0	1	1	0	1	6
41	BAU-80-28	0	0	0	1	1	1	0	1	0	1	5
42	BRRIdhan 75	0	0	0	1	1	1	0	1	0	1	5
43	Lakkhidag	1	0	0	1	1	1	0	0	0	1	5
44	BINAdhan 17	1	0	0	1	1	1	0	0	0	1	5
45	Supahar	1	0	0	1	1	0	0	0	0	1	4
46	Khaiwa	0	0	0	1	1	1	0	0	0	1	4
47	BRRIdhan 52	0	0	0	1	1	0	0	0	0	0	3
48	Lal saran	0	0	0	1	1	0	1	0	0	0	4
	Frequency (%)	81.3	25	2	73	92	83	63	66	25	93	

Genetic diversity of Pi-b and Pi-ta genes

The blast resistance gene *pi-b* on chromosome locus 2 and *pi-ta* on chromosome locus 12, are linked to the SSR markers: RM 208 and RM 247 respectively. The PCR results for the genes *pi-b* and *pi-ta* were estimated by the electrophoresis patterns or positive bands near 173 bp and 131 bp respectively. Among forty-eight (48) genotypes, *Pi-b* gene was scored on forty-four (44) genotypes while, *Pi-ta* gene was scored in forty (40) genotypes. *Pi-ta* gene fragment was the second most prevalent (92%) and *pi-ta* was the third most prevalent (83%) among the genotypes (48) studied. Thirty-eight (38) genotypes amplify both SSR markers (RM 208 and RM 247) while one (1) genotype did not amplify either of the two markers and that's why, negative for these two genes.

Genetic diversity of Pi33 and Pi-27(t) genes

The blast resistance gene *pi33* on chromosome locus 8 and *pi-27(t*) on chromosome locus 1, are linked to the SSR markers: RM 72 and RM 259 respectively. The PCR results for the genes *pi33* and *pi-27(t)* were estimated by the electrophoresis patterns or positive bands near 166 bp and 162 bp respectively. Among forty-eight (48) genotypes, *Pi33* gene was scored on thirty (30) genotypes while, *Pi-27(t)* gene was scored only in thirty-two (32) genotypes. *pi-27(t)* was the fifth most prevalent (77%) among the genotypes (48) studied. Twenty-five (25) genotypes amplify both SSR markers (RM 72 and RM 259) while twelve (12) genotypes did not amplify either of the two markers and that's why, negative for these two genes.

Genetic diversity of Pitp(t) and Pi-k^h genes

The blast resistance gene *pitp(t*) on chromosome locus 1 and *Pi-k*^{h} on chromosome locus 11, are linked to the SSR markers: RM 246 and RM 206 respectively. The PCR results for the genes pitp(t) and *Pi-k*^{h} were estimated by the electrophoresis patterns or positive bands near 116 bp and 147 bp respectively. Among forty-eight (48) genotypes, *Pitp(t)* gene was scored on twelve (12) genotypes while, *Pi-k*^{h} gene was scored in forty-five (45) genotypes. *Pi-k*^{h} gene fragment was the most prevalent (93%) among the genotypes (48) studied. Eleven (11) genotypes amplify both SSR markers (RM 246 and RM 206) while only two (2) genotypes did not amplify either of the two markers and that's why, negative for these two genes.

Discussion

In this study, molecular screening or genotyping of the selected rice genotypes with allelic related markers (SSR markers) were conducted to help in identifying of ten (10 major rice blast resistance genes: *Pi-9, Pi-1, Pi-5(t), Piz-5, Pi-b, Pi-b, Pi-ta, Pi-33, Pi27(t), Pitp(t) and Pi-k*^{h} where genetic frequencies of these ten (10) blasts resistance genes were ranged from 2% to 93%. Almost similar results were reported by Singh *et al*. (2015) in 192 accessions of rice germplasm, where seventy-three (73) accessions possessed at least five (5) positive bands of ten (10) rice blast resistance markers and the genetic frequencies of that ten (10) major rice blast resistance genes: *Pi-k*^{h} *, Pi-b, Pi-ta, Pi-33, Pi27(t), Pitp(t), Pi-9, Pi-1, Pi-5(t), Piz-5* were ranged from 19.79% to 54.69% (Singh *et al*., 2012) and Imam *et al*. (2014) reported the genetic frequencies of nine (9) blast resistance genes*: Piz, Piz-t, Pik, Pik-p, Pi-k*^{h}*, Pita/Pita-2, pita, Pi9* and *Pi-b* were ranged from 6% to 97% 32 accessions of rice germplasm. On the other hand, Kim *et al*. (2010) reported the genetic frequencies of six (6) major blast resistance genes: *Piz, Piz-t, Pik, Pik-m, Pik-p* and *Pit* were ranged from 30% to 99% in eighty-six (86) accessions of aromatic rice germplasm where all that eighty-six (86) accessions possessed at least more than three (3) positive bands of six (6) major blast resistance genes and Yan *et al*., (2017) reported in 32 rice germplasms where genetic frequencies of eleven (11) major blast resistant genes: *Pi-d2, Pi-z, Piz-t, Pi-9, Pi-36, Pi-37, Pi5, Pi-b, Pik-p, Pik-h and Pi-ta2* were ranged from 9.4% to 100.0%.

This present study also showed allelic diversity among the blast resistance genes that can be suggested that, in ancient populations of landraces can direct the rice blast resistance breeding program and rice blast control by genetic diversity. Many rice varieties have been developed as completely resistant to *M. oryzae* strains, but soon breakdown of rice blast resistant genes occurred because of the emergence of stronger virulent isolates of rice blast fungus (Mackill and Bonman, 1992). Molecular screening of the selected genotypes with allele specific SSR markers helped to identify 10 major blast resistance genes. In this study certain genes such as, *Pi-k*^{h}*, Pi-b, Pi-ta* and *pi-9* were more diverse than others [*pi-5(t), pi-1, pi33*)] and these effective were identified in 45, 44, 40 and 39 accessions on chromosome 11, 2, 12 and 6, respectively. Similar results were reported by Singh *et al.* (2015) where, certain genes [*Piz5, Pi9, Pitp (t) and Pi-1*] were more diverse than others and these were identified in 105, 101, 92 and 85 accessions on chromosome 6, 6, 1 and 11, respectively and Imam *et al*. (2014) reported in his study that the genes (*Pi-9, Pita2, Piz-t*) were more effective than others in thwarting infection. *Pi-k*^{h} and *Pi-b* were highly distributed genes in the present study but neither the germplasm possessing them nor the isogenic lines in the previous evaluations had exhibited resistance (Variar *et al*., 2009).

Conclusion

This study illustrated the utility of SSR markers to identify rice varieties likely carried the same *R* genes with potentially novel resistance.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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