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Genetic analysis and identification of SSR markers associated with rice blast disease in a BC_2F_1 backcross population

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ABSTRACT. Rice (*Oryza sativa* L.) blast disease is one of the most destructive rice diseases in the world. The fungal pathogen, *Magnaporthe oryzae*, is the causal agent of rice blast disease. Development of

resistant cultivars is the most preferred method to achieve sustainable rice production. However, the effectiveness of resistant cultivars is hindered by the genetic plasticity of the pathogen genome. Therefore, information on genetic resistance and virulence stability are vital to increase our understanding of the molecular basis of blast disease resistance. The present study set out to elucidate the resistance pattern and identify potential simple sequence repeat markers linked with rice blast disease. A backcross population (BC_2F_1), derived from crossing MR264 and Pongsu Seribu 2 (PS2), was developed using marker-assisted backcross breeding. Twelve microsatellite markers carrying the blast resistance gene clearly demonstrated a polymorphic pattern between both parental lines. Among these, two markers, RM206 and RM5961, located on chromosome 11 exhibited the expected 1:1 testcross ratio in the BC_2F_1 population. The 195 BC_2F_1 plants inoculated against *M. oryzae* pathotype P7.2 showed a significantly different distribution in the backcrossed generation and followed Mendelian segregation based on a single-gene model. This indicates that blast resistance in PS2 is governed by a single dominant gene, which is linked to RM206 and RM5961 on chromosome 11. The findings presented in this study could be useful for future blast resistance studies in rice breeding programs.

Key words: Blast inheritance; SSR marker; Pongsu Seribu 2; MR264; Marker-assisted backcrossing

INTRODUCTION

Rice (*Oryza sativa*) is an essential food crop with 90% of its production and consumption coming from the Asian subcontinent. Unfortunately, biotic and abiotic stresses have become a limiting factor for rice production (Hasan et al., 2015). Blast disease caused by the hemi-biotroph fungus *Magnaporthe oryzae* (teleomorph) or *Pyricularia grisea* (anamorph) has been ranked the primary threat to rice productivity (Skamnioti and Gurr, 2009). Blast disease accounts for 50% of the yield losses and currently has been detected in 85 rice-producing countries (Jiang, 2013).

Introgression of major and minor blast resistance genes is the most favorable approach in rice breeding programs, to combat this disease. However, occurrence of the new races of fungus is a major problem for improving the resistance of any variety (Ashkani et al., 2013). Development of resistant varieties using conventional breeding is suboptimal due to heritable variation caused by environments as well as being tedious and time consuming (Wang et al., 2014). Utilization of DNA markers has greatly facilitated the tracking process of blast resistance genes in the rice genome. Identifying DNA markers that co-segregate with resistance genes is an important technique used to develop blast resistant rice cultivars. The availability of various DNA markers facilitates the identification of blast resistance genes and can be used in inheritance studies when there are interactions among *M. oryzae* avirulence genes (Naqvi and Chattoo, 1996). Microsatellite markers are popular for plant breeding applications, due to their high polymorphism, ubiquitousness in nature, and high allelic diversity.

Genetic analyses of disease resistance would improve the breeding approach and enhance the efficiency of the selection process. Several inheritance studies have been reported

that have used Japanese races (e.g., Kiyosawa, 1981; He and Shen, 1990). In Malaysia, inheritance studies have been performed using local isolates (Rahim et al., 2012), including BC_2F_3 and BC_2F_5 advanced backcrosses (*Oryza rufipogon* x MR219). Four dominant genes at different loci were identified against the most virulent pathotype of *M. oryzae* (Miah et al., 2015; Tanweer et al., 2015a). In addition, Ashkani et al. (2015) reported an epistatic effect in a study including two different races of fungus. Extensive information about host resistance as well as the pathogen are essential to develop new rice varieties with long-lasting blast resistance and a broad range of resistance consisting of both qualitative and quantitative genes (Padmavathi et al., 2005). Our objectives were to identify inheritance patterns of blast resistance against *M. oryzae* pathotype P7.2 in a BC_2F_1 rice population and to identify simple sequence repeat (SSR) markers associated with blast resistance.

MATERIAL AND METHODS

Plant materials and breeding strategy

A total of 195 BC_2F_1 seeds were developed using MR264 as the susceptible variety and Pongsu Seribu 2 (PS2) as the resistant variety. The breeding scheme (Figure 1) for the backcross population was developed to incorporate resistance genes from PS2. Seeds from the backcross population and both parental lines were soaked in fungicide before transfer to Petri dishes containing wet filter paper. Germinated seeds were then planted in a greenhouse at 25°C in Agency Nuclear Malaysia following Prabhu et al. (2003). Soil containing the recommended amount of NPK was supplied to plants sown in plastic trays (36 x 23 x 10 cm). In addition, 2 g ammonium sulfate was added to each tray before and 20 days after planting. A randomized complete-block design was used with three replications and ten seeds per variety.

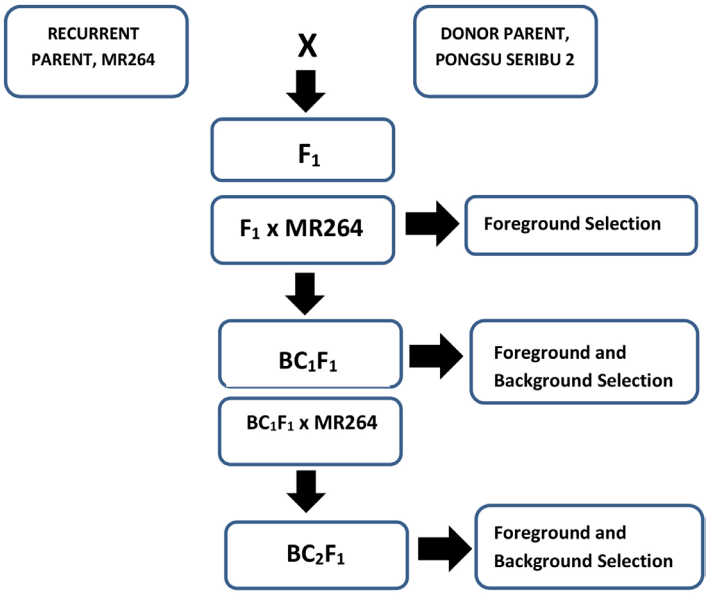

Figure 1. Breeding scheme to develop a blast resistant BC_2F_1 population.

Molecular marker analysis

Parental polymorphism analysis was carried out by screening 375 SSR markers distributed across 12 rice chromosomes. The information about the SSR primers associated with blast resistance was extracted from Gramene (http://www.gramene.org, McCouch et al., 2002; Ashkani et al., 2013). Twelve markers linked to blast resistance genes were subsequently used in the segregation analysis of BC_2F_1 individuals (Table 1).

Table 1. Summary of polymorphic microsatellite markers used in the segregation analysis.

Marker	Chromosome	Repeat motif	Linked gene
495	1	(CTG)7	*Pi-t*
RM1	1	(GA)26	*Pi*-24
RM85	3	(TGG)5(TCT)12	Qrbr 3.2
RM148	3	(TG)12	Qrbr 3.2
RM168	3	T15(GT)14	Candidate gene- Oxalate oxidase, 14-3-3 protein
RM303	4	[AC(AT)2-10]9(GT)7(ATGT)6	*Pi-kur*1
RM3	6	(GA)2GG(GA)25	*Pi-d2*
RM413	7	(AG)11	*Pi-26*
RM205	9	(CT)25	*Pi*-2(*t*)
RM5961	11	(CAG)8	*Pi-7(t)*, *Pi-k*^{h}
RM206	11	(CT)21	*Pi-kh*
RM101	12	(CT)37	*Pi-6(t)*

Genomic DNA extraction and amplification

Samples of the youngest leaves of 3-week old seedlings were collected at every generation. Leaves were cut into small pieces before rinsing in 70% ethanol to avoid potential contamination. A Tissue Lyser machine (Qiagen, Germany) was used to grind 2 g leaf samples with 5 mL extraction buffer. DNA precipitation was carried out using 70% ethanol and the dried pellet was re-suspended in 1X TE buffer. DNA was quantified using Nano-Drop spectrophotometry (Thermo Scientific, UK) and the DNA quality was further confirmed using 1.5% agarose gel electrophoresis at 90 V for 30 min. The polymerase chain reaction (PCR) analysis was carried out following Ashkani et al. (2013) with some modifications. The PCR program used included 30 cycles of 1 min denaturation at 94°C, 1 min at 55°C annealing, 2 min polymerization at 72°C, and fast cooling to 4°C. The PCR products were identified on 3.0% agarose in 1X TBE at 80 V for 80 min and were then visualized using Molecular Imager® (GelDocTM XR, Bio-Rad, USA).

Evaluation of disease resistance

Based on a virulence test against different varieties, the most virulent *M. oryzae* pathotype, P7.2, was used in this study. A fungus culture was obtained from Malaysian Agricultural Research Development Institute, Penang, and was maintained on potato dextrose agar. The solution was autoclaved at 121°C for 15 min before it was poured onto Petri dishes and sealed with parafilm. The experiment was conducted in a laminar flow cabinet, to minimize contamination. A master culture was prepared, using a single spore isolation technique. A master culture was prepared, using a single spore isolation technique. Conidia were harvested using a sterile brush by scraping fungal mycelia on the culture plates. The conidial suspension was then filtered through muslim cloth to remove mycelia and pieces of agar. A nylon gauze

mesh was used to filter the suspension before dilution with deionized water. The blast conidial suspension was measured using a hemocytometer (Merienfeld, Germany) for a 1.5 x 10^5 spores/mL standard spore concentration. To maximize adhesion of the spores to plant leaves, a few drops of 0.05% Tween 20 were added to the suspension.

Four leaf stage seedlings (10 plants per line) were inoculated with *M. oryzae* pathotype P7.2, to investigate the resistance pattern. Inoculation was performed by spraying 25 mL aqueous spore suspension (1 x 10^5 spores/mL) on the leaves. After inoculation, seedlings were kept in a moisture chamber for 24 h at 25°C. They were then transferred to a controlled greenhouse following the method by Filippi and Prahbu (2001). Relative humidity was kept at 90% by covering the seedlings with a black net. The plants were watered two to three times per day to aid disease development.

Nine days after inoculation, disease development was visually scored by estimating three phenotypic traits; blast lesion degree (BLD), blast lesion type (BLT), and percentage disease leaf area (%DLA). The %DLA and BLT scores were based on the method by Correa-Victoria et al. (2002) in which the %DLA score ranges from 0 to 100% and the BLT score ranges from 0 to 4 (highly resistant to susceptible). The BLD trait was scored based on the Standard Evaluation System developed by the International Rice Research Institute (IRRI, 2013).

Genotyping for marker segregation

Marker banding patterns were scored manually. Plants were scored AA if the SSR bands were identical to the resistant parent's alleles. Plants that showed similar patterns to the susceptible parent were scored aa, whereas heterozygous plants were scored Aa.

Statistical analysis

The scoring lesion data was analyzed using SPSS software v. 16.0. Distribution of data was analyzed using the Anderson-Darling method (Wardlaw, 2000). The goodness of fit for the phenotypic traits was obtained by comparing the observed and expected frequencies using chi-square (χ^2) tests, as described by Tartarini (1996). The phenotypic ratio was measured using the χ^2 formula: $\chi^2 = (O - E)^2 / E$, where O and E are the observed and expected data, respectively. The χ^2 was considered significant (P = 0.05) if its value was greater than 3.84 for the single gene model.

RESULTS

SSR markers associated with blast resistance genes

A total of 72 markers demonstrated clear polymorphism among both susceptible and resistant parents. These markers were selected based on clear amplification between parents. Among the 72 polymorphic markers, 12 were identified as being related to blast resistance, based on the expected allele size (Figure 2). Chromosome 3 contained the highest number of markers. The 12 identified markers were further used to observe the polymorphic pattern in the F_1 population. Two bands were amplified in F_1 plants that exhibited the heterozygous condition where both sequence motifs originated from the parents. The backcrossed generations, BC_1F_1 and BC_2F_1, were further tested for their association with blast resistance genes using the same

markers. In the backcross populations, only two polymorphic markers, RM206 and RM5961, demonstrated heterozygous plants (Figure 3). Another marker failed to exhibit a positive result by producing a binding pattern similar to MR264. Based on Gramene (www.gramene.org), the map position and associated genes for these SSR markers were identified. The two polymorphic markers, RM206 and RM5961, were both located on chromosome 11 and consisted of 147 and 129 bp, respectively. The markers were located 23 cM from each other on chromosome 11. Marker RM206 was linked to *Pi-kh*, whereas RM5961 was linked to *Pi-7(t)* (Figure 4). These results indicate that RM206 and RM5961 are two SSR markers that are linked to blast resistance in the BC_2F_1 generation.

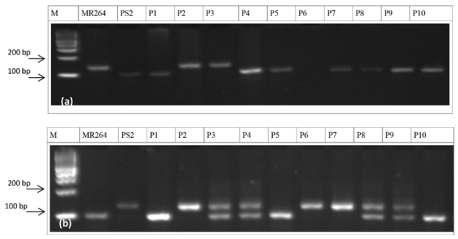

Figure 2. Marker banding pattern in the BC_1F_1 population for SSR markers RM5961 (**a**) and RM206 (**b**) linked to blast resistance genes. M = 100-bp ladder; M = marker; MR264 = recurrent parent; PS2 = Donor parent; P1-P10 = plant number.

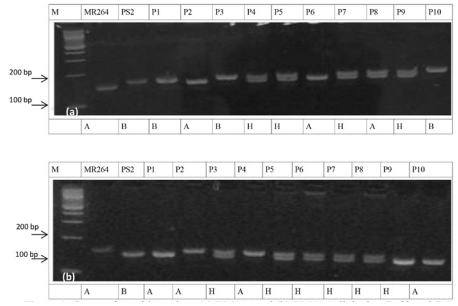

Figure 3. Genotyping with markers RM206 (**a**) and RM5961 (**b**) linked to *Pi-kh* and *Pi7(t)* resistance genes in the BC_2F_1 population. M = 100-bp ladder; A = MR264; B = PS2; H = heterozygous; P1-10 = progenies.

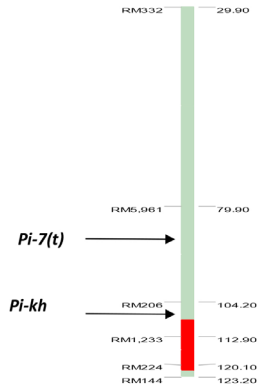

Figure 4. Position of RM206 and RM5961 markers on rice (*Oryza sativa*) chromosome 11.

Marker segregation analysis of the BC_2F_1 population

BC_2F_1 individuals were scored based on the parental bands. Bands from amplified PCR products were indicated as alleles at the respective loci. Table 2 shows the observed segregation proportion between resistance and susceptibility in the BC_2F_1 population for the 12 polymorphic markers. Based on the single gene model, only markers RM206 and RM5961 followed the expected Mendelian ratio (1:1) in the BC_2F_1 population (d.f. = 1.0, $P < 0.05$). By contrast, all other markers failed to fit the expected Mendelian ratio.

Table 2. Genotype analysis of the BC_2F_1 population (MR264 x PS2).

Marker	Chromosome	Marker analysis		χ^2 (1:1)	P
		AA = R	Aa = S		
RM1	1	35	160	40.06	<0.0001
RM495	1	2	193	93.5	<0.0001
RM168	3	12	183	74.9	<0.0001
RM148	3	15	175	87.7	<0.0001
RM85	3	17	195	85.86	<0.0001
RM303	4	6	189	85.8	<0.0001
RM3	6	8	187	91.59	<0.0001
RM413	7	7	188	84.0	<0.0001
RM205	9	3	192	91.5	<0.0001
RM206	11	89	106	0.741	0.3893
RM5961	11	103	92	0.310	0.5775
RM101	12	14	181	71.51	<0.0001

d.f. = 1.0; $\chi^2(0.05, 1) = 3.84$.

Inheritance of blast resistance

A total of 195 BC_2F_1 plants were selected for screening with the fungal pathogen *M. oryzae* pathotype P7.2. The evaluation of partial resistance in the BC_2F_1 lines was conducted by estimating three phenotypic traits. Under artificial inoculation in a controlled greenhouse environment, the susceptible parent, MR264 showed high susceptibility towards blast disease with BLD scores of 5-7. In contrast, PS2 produced BLD scores of 0 to 2, suggesting resistance to pathotype P7.2. In the single-gene model analysis, inoculation of pathotype P7.2 against the BC_2F_1 population resulted in a segregation ratio of 1:1 between resistance and susceptibility ($\chi^2 = 0.62$, $P = 0.43$) (Table 3).

Table 3. Phenotypic segregation in the BC_2F_1 population (MR264 x PS2) inoculated with *Magnaporthe oryzae* pathotype P7.2.

Genotype	Total No. of seedlings	Pathogenicity assay				χ^2 (1:1)	P
		Resistant	Susceptible	Observed ratio	Expected ratio		
MR264	20	0	20	-	-	-	-
PS2	20	20	0	-	-	-	-
F_1	32	32	-	-	-	-	-
BC_1F_1	136	76	60	68:68	1:1	1.88	0.17
BC_2F_1	195	103	92	97.5:97.5	1:1	0.62	0.43

$\chi^2(0.05, 1) = 3.84$.

Based on this analysis, the BC_2F_1 population did not fit the 1:1:1:1 ratio ($\chi^2 = 55.46$, $P < 0.0001$) for pathotype P7.2. These findings suggest that blast resistance, as induced by pathotype P7.2 in the BC_2F_1 population, was not controlled by two-gene model. An epistasis

test showed that the BC_2F_1 population did not fit the segregation ratio for pathotype P7.2 (χ^2 = 5.319, P = 0.0219), which indicates that no locus interaction of blast resistance was found in the BC_2F_1 population derived from MR264 and PS2 (Table 4).

Table 4. Segregation analysis for two independent genes (1:1:1:1) and epistatic effect (15:1) for blast resistance in the backcrossed population (BC_2F_1).

Gene model	Observed ratio				Expected ratio	χ^2	P value
	R	MR	MS	S			
Two independent genes	68	35	72	20	1:1:1:1	55.46	<0.0001
Epistatic effect	175	-	-	20	15:1	5.319	0.0219

$\chi^2(0.05, 3) = 7.81$, $\chi^2(0.05, 1) = 3.84$; R = resistant; MR = moderately resistant; MS = moderately susceptible; S = susceptible.

Trait Frequency Distribution

The phenotypic traits for *M. oryzae* pathotype P7.2 in the backcrossed population BC_2F_1 showed a broad range of variation for the quantitative leaf blast disease traits. The %DLA showed the highest variation, whereas BLT exhibited the lowest variation. An Anderson-Darling normality test showed that none of the three phenotypic traits were normally distributed (P < 0.01) (Figure 5). As shown in Figure 6, PS2 produced lesion degree 2, whereas MR264 produced lesion type 5, which is considered as susceptible to the tested pathotype. We found positive correlations among all three traits with %DLA and BLT showing the strongest correlation with r = 0.984 (Table 5).

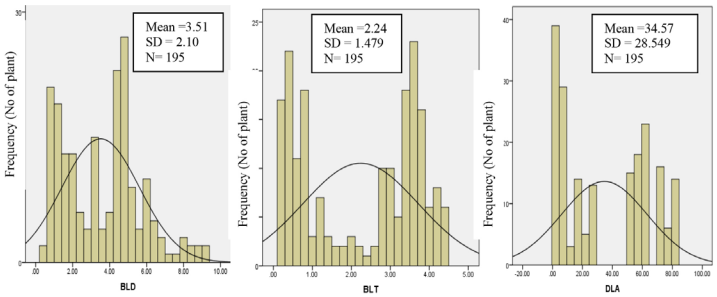

Figure 5. Trait frequency distribution of the leaf blast disease reaction scales for the P7.2 pathotype in the BC_2F_1 population. BLD = blast lesion degree, BLT = blast lesion type, and %DLA = percentage disease leaf area. In each histogram, a normal curve is included.

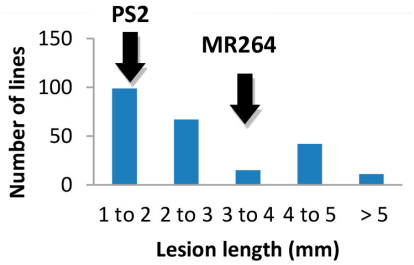

Figure 6. Blast lesion length distribution after inoculation of *Magnaporthe oryzae* pathotype P7.2 in both parents and seven blast improved lines of the BC_2F_1 population. Average lesion length: PS2 = 1.76 mm and MR264 = 3.4 mm.

Table 5. Correlation coefficients between phenotypic traits for fungus pathogen *Magnaporthe oryzae* pathotype P7.2.

Phenotypic trait	BLD	BLT	%DLA
BLD	0	0.932**	0.920**
BLT	0.932**	0	0.984**
%DLA	0.920**	0.984**	0

**Significant correlation at $P \leq 0.01$ (two-tailed). BLD = blast lesion degree; BLT = blast lesion type; and %DLA = percentage disease leaf area.

DISCUSSION

Selection strategies depend on the identification of markers that segregate following a Mendelian fashion. In the BC_2F_1 population, 12 polymorphic markers were identified as being closely associated with blast resistance. These were chosen among 72 polymorphic SSR markers for further segregation analysis. The highest number of polymorphic markers was found on chromosome 3. A higher number of parental polymorphic markers, i.e., approximately four polymorphic markers per chromosome, may provide a better coverage per chromosome for genetic background selection (Basavaraj et al., 2010). Ragimekula et al. (2013) stated that selection of primers is most effective when based on multiple primers located on different chromosomes. Of 12 polymorphic markers that distinguished the two parents clearly in F_1 plants, only two markers (RM206 and RM5961) demonstrated heterozygous plants in the backcross populations BC_1F_1 and BC_2F_1. This indicates that the blast resistance gene was associated with both markers in backcrossed plants. The blast resistant genes linked to the other ten polymorphic markers disappeared during the backcross breeding program. Suh et al. (2009) and Miah et al. (2015) suggested that some genes in the segregating generation of advanced backcross lines might had disappeared during the crossing over event.

The two polymorphic markers, RM206 and RM5961, were found to be closely linked to blast resistance genes [putative *Pi-kh* and *Pi-7(t)*] in the current study. They were both located on chromosome 11 with 149 and 129 bp, respectively. These results agree with the findings by Tanweer et al. (2015a), who identified the *Pi-kh* gene using RM206. Likewise, Hasan et al. (2015) and Ashkani et al. (2013) identified *Pi-7(t)* using RM5961 and PS2 as the donor parent. Our results are further supported by Tanweer et al. (2015b) who suggested that PS2 consisted to 23% of the *Pi-kh* resistance gene on different chromosome locations. Our genotype analysis indicated that the backcrossed population followed Mendel segregation (1:1) at 0.05-probability level, which was also supported by Mondal et al. (2012) and Iftekharuddaula et al. (2012). Based on these findings, we suggest that RM206 and RM5961 are two linked SSR markers that carry the blast resistance locus in the BC_2F_1 population against pathotype P7.2 of *M. oryzae*. However, additional molecular genetic analyses, such as cloning of the major resistant genes, are required for further confirmation of the potential resistance genes identified in this study.

During artificial inoculation, BC_2F_1 plants demonstrated linkage with resistance and a susceptibility pattern against *M. oryzae* pathotype P7.2. Backcrossed plants demonstrated the expected Mendelian segregation ratio. This association suggests that a single dominant gene likely governs resistance in PS2. These results are in line with Ashkani et al. (2013) and Tanweer et al. (2015b) who also used the donor parent PS2 combined with Mahsuri and MR219, respectively. Our findings were also in agreement with blast research carried out at IRRI in the Philippines, which revealed a major gene present in most of the traditional varieties (Mackill et

al., 1985). Backcrossed plants in our study deviated from epistasis and the independent model. However, Ashkani et al. (2015) reported that a dominant epistatic interaction exists in PS2, using pathotype P5.0 with an F_3 population. This suggests the presence of two independent dominant genes that result in epistatic effects of multiple loci with major and minor resistance genes. The best explanation for this pattern may be the high phenotypic variation observed in the selected pathotype. According to Bonman et al. (1992) and Talukder et al. (2004), blast resistance depends on the pathogen genotype and the presence of the avirulent gene in the host plant. The present findings may advance disease resistance research to field blast on rice crop by utilizing the identified markers in marker-assisted backcross breeding. Furthermore, our findings could initiate potential rice breeding programs in Malaysia to develop rice varieties with blast resistance using marker-assisted selection. The aim is to develop a durable blast resistant variety to increase the yield and rice self-sufficiency percentage in Malaysia.

To conclude, the markers RM206 and RM5961 follow a Mendelian ratio based on the single gene model. BC_2F_1 population demonstrated the Mendelian ratio based on the phenotypic data. Our findings suggest that resistance in PS2 against the most virulent blast pathotype in Malaysia is likely governed by a single dominant gene. The selected BC_2F_1 lines that were resistant to blast pathotype P7.2 could be used by future breeders and geneticist in developing a durable and resistant variety in Malaysia. The identification of closely linked markers will facilitate the mapping of genes and quantitative trait loci underlying the blast resistance trait.

Conflicts of interest

The authors declare no conflict of interest.

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