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Miah, Gous; Rafii, Mohd Y.; Razi Ismail, Mohd; Bin Puteh, Adam; Abdul Rahim, Harun; Ashkani, Sadegh; Latif, Abdul

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Inheritance patterns and identification of microsatellite markers linked to the rice blast resistance in BC_2F_1 population of rice breeding

Gous Miah ([1]); Mohd Y. Rafii ([1,2]*); Mohd Razi Ismail ([1,2]); Adam Bin Puteh ([2]); Harun Abdul Rahim ([3]); Sadegh Ashkani ([1,4]); Abdul Latif ([2,5])

([1]) Universiti Putra Malaysia, Institute of Tropical Agriculture, Laboratory of Food Crops, 43400 UPM Serdang, Selangor, Malaysia.

([2]) Universiti Putra Malaysia, Faculty of Agriculture, Department of Crop Science, 43400 UPM Serdang, Selangor, Malaysia.

([3]) Malaysian Nuclear Agency, Agrotechnology and Bioscience Division, 43000 Kajang, Selangor, Malaysia.

([4]) Islamic Azad University, Shahr-e-Rey Branch, Department of Agronomy and Plant Breeding, Tehran, Iran.

([5]) Bangladesh Rice Research Institute, Gazipur 1701, Bangladesh.

(*) Corresponding author: mrafii@upm.edu.my

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Abstract

The BC_2F_1 population was derived from a cross between rice variety, MR219 (susceptible to blast) and Pongsu Seribu 1 (resistant to blast). The objectives of this research were to know the inheritance pattern of blast resistance and to identify the linked markers associated with blast resistance in BC_2F_1 population. Sixteen microsatellite markers were found as polymorphic between the parents related to blast resistant genes (*Pi*-genes). Among the selected blast resistant linked markers, two markers RM6836 and RM8225 showed expected testcross ratio (1:1) for single-gene model in the BC_2F_1 population with the association between resistant and susceptible progeny. A total of 333-BC_2F_1 plants were challenged with the most virulent pathotype P7.2 of *Magnaporthe oryzae*. Chi-square (x^2) analysis for phenotypic segregation in single-gene model showed goodness of fit (P = 0.4463) to the expected segregation ratio (1:1). In marker segregation analysis, two polymorphic markers (RM6836 and RM8225) clearly showed goodness of fit to the expected segregation testcross ratio (1:1) for the single-gene model. The marker RM8225 and RM6836 showed significant R^2 values higher than 10 for the trait of the blast lesions degree (BLD). The positions of RM6836 and RM8225 markers on rice chromosome 6 and the distance between these two markers is 0.2 cM. We conclude that single dominant gene control the blast resistance in Pongsu Seribu 1 located on chromosome 6, which is linked to RM8225 and RM6836 microsatellite markers. This information could be useful in marker-assisted selection for blast resistance in rice breeding involving Pongsu Seribu 1.

Key words: blast inheritance, microsatellite markers, BC_2F_1 population, rice variety.

1. INTRODUCTION

The evolution of new biotypes of pests and diseases, as well as the pressures of climate change, pose serious challenges to rice breeders, who would like to increase rice production by introducing resistance to multiple biotic and abiotic stresses (Miah et al., 2013). Among the biotic stresses, blast disease is the most harmful threat to high productivity of rice (Li et al., 2007). Rice blast caused by *Magnaporthe oryzae* (*M. oryzae*) is the most devastating diseases of rice worldwide (Khush & Jena, 2009; Liu et al., 2010). Rice blast severely reduces production in both irrigated and water stressed upland ecosystems of tropical and temperate countries (Suh et al., 2009). The incorporation of blast resistance genes into cultivars is the most preferential strategy in rice breeding program to prevent this disease. Most of the major resistance genes follow gene-for-gene interaction model (Kumbhar et al., 2013). Utilization of genetic resistance is the most effective and environmentally friendly strategy for control of the disease (Zhu et al., 2004).

Knowledge on the inheritance of disease resistance would facilitate the adoption of appropriate breeding strategies and improve the efficiency of selection procedures. Extensive studies have been conducted on inheritance of blast resistance using Japanese races and identified 13 dominant resistance genes at eight different loci (Kiyosawa, 1981). The inheritance of resistance in cultivars against two races of *M. oryzae* was studied and 11 dominant genes were identified (He & Shen, 1990). Expression of resistance is altered by modifiers or multiple alleles. There is very little information on the inheritance and nature of

resistance utilizing tropical isolates of *M. oryzae* (Filippi & Prabhu, 1996). The nature of resistance and susceptibility is influenced by inoculation techniques, environmental conditions, human mistakes in scoring and the virulence of *M. oryzae* (Srinivasachary et al., 2002).

Most of the blast R genes are dominant, and some of them are quantitative in nature (Xu et al., 2008). Furthermore, many R genes are located as gene clusters with focuses on chromosomes 6, 9, 11, and 12 (Ashkani et al., 2013; Ballini et al., 2008). Disease resistance is controlled by one or two (Padmavathi et al., 2005; Sharma et al., 2007), three (Mohanty & Gangopadhyay, 1982) or more pair of genes (Flores-Gaxiola et al., 1983). The traditional rice cultivars have one or two dominant resistance genes, which are effective against each fungal isolate (Mackill et al., 1985).

Normally, DNA markers are used to detect resistance genes (Ballini et al., 2008). Identification of resistance genes in genetically diversified rice material is important for identification of new sources of blast resistance. Pongsu Seribu 1 is a Malaysian traditional rice variety which is resistant to blast diseases, can be used as a blast resistant donor in rice breeding programs. It has mid-late maturity, tall plant stature with short grain type, developed by Malaysian Agricultural Research and Development Institute (MARDI). The commercial Malaysian *indica* rice variety MR219 has been classified as highly productive (Fasahat et al., 2012) but became susceptible to blast. Grain weight is as high as 28–30 mg, and 200 grains/panicle (Alias, 2002). A short maturation period of 105–111 days is the additional good feature of this variety. The aims of this study were to know the inheritance pattern of blast resistance in BC_2F_1 rice population against pathotype P7.2 isolate and to identify microsatellite markers linked to blast resistance. Inheritance of this highly effective source of blast resistance Pongsu Seribu 1 should determine for its efficient use in the rice-breeding programs targeted for improving blast resistance.

2. MATERIAL AND METHOD

MR219 is a high yielding, good eating quality and wide adaptability rice variety. Unfortunately, this cultivar is very susceptible to blast (Figure 1). The MR219-rice cultivar was used as the recurrent parent while the cultivar Pongsu Seribu 1(PS1) was used as donor for the blast resistance (Figure 2). Among the F_1 plants, two heterozygous F_1 plants were selected and backcrossed with "MR219" to generate the BC_1F_1s. In the BC_1F_1 plants, marker-assisted foreground selection was carried out and the markers RM6836 and RM8225 showed heterozygous plants. The heterozygous plants were used to estimate the recurrent genome recovery using background marker. Out of 70-polymorphic microsatellite background markers, at least four SSR background markers per rice chromosome were used for analysis of recurrent genome recovery in each generation. The highest recurrent genome recovery plants were subjected to phenotypic selection. The best four plants (i.e those that have phenotypically resemblance to the recurrent parent with maximum recurrent genome recovery) were backcrossed with MR219 to develop the BC_2F_1seeds. The BC_2F_1 plants were inoculated with the most virulent pathotype P7.2 and also subjected to foreground selection followed by phenotypic selection to identify best plants heterozygous for blast resistance with maximum recovery for recurrent parent genome (RPG).

One of the most virulent Malaysian rice blast pathotype P7.2 of *M. oryzae* was collected from the Malaysian Agricultural Research and Development Institute (MARDI). Currently, this pathotype is the most virulent pathogen in Malaysia (Rahim et al., 2013). Potato dextrose agar (PDA) was used as a media for growing the selected pathotype P7.2 of *M. oryzae.* PDA was prepared by mixing 39g of PDA in 500 ml of distilled water and boiled for 30 minutes in order to dissolve properly. After that, distilled water up to 1.0 L was added into the solution and was autoclaved at 121 °C for 20 minutes. Before plating PDA media, 10 mg of streptomycin was added for every 250 ml media to avoid bacterial contamination. The solution was then poured in Petri dish under the laminar flow cabinet and sealed with parafilm to avoid contamination. The blast conidial suspensions were filtered through nylon gauze mesh and

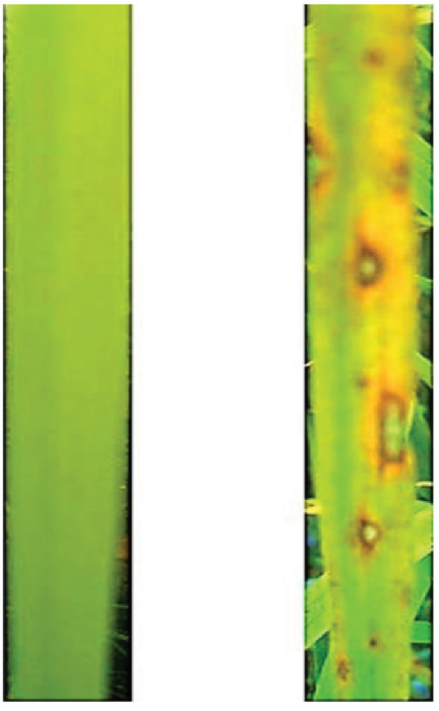

Figure 1. Rice cultivars Pongsu Seribu 1 (PS1) (resistant) and MR219 (susceptible) inoculated with pathotype 7.2 of *M. oryzae*. Severe blast lesions were observed in MR219 and no lesions in PS1.

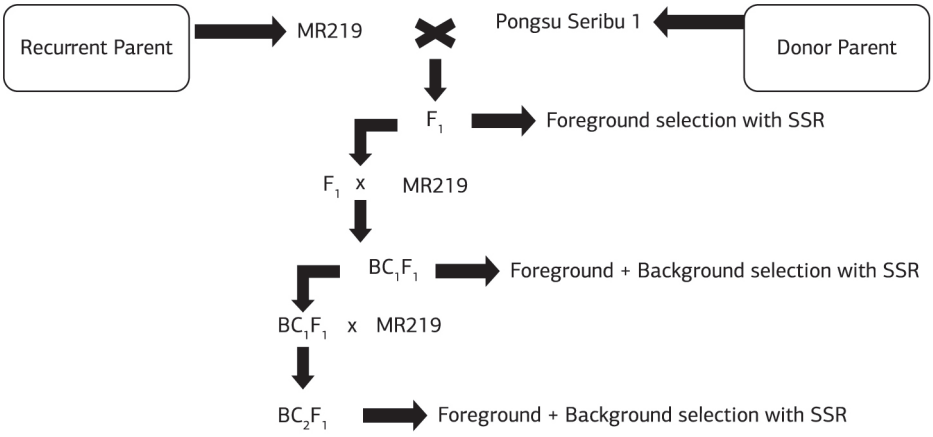

Figure 2. Crossing and selection scheme to produce blast resistant BC_2F_1 genotypes.

adjusted to a concentration of 1.5×10^5 conidia's mL^{-1} by haemocytometer using deionized water. Before inoculation, 0.05% Tween 20 was added to the suspension to increase the adhesion of the spores to the plant leaves.

The BC_2F_1 seeds were soaked in water for one day and germinated on moist Whatman filter paper in Petri dishes for 3 days in a 30 °C dark incubator. The germinated seeds were transferred in plastic trays (60 cm × 60 cm x 50 cm) containing 15 kg of soil with NPK (10 g of 15:15:15) as described by Prabhu et al. (2003). Plants were grown in a glasshouse at 25-30 °C for 3 weeks, until they were at the four-leaf stage. A total of 333 BC_2F_1 seedlings were inoculated with highly virulent pathotype P7.2 of *M. oryzae* to investigate the segregation patterns of blast resistance phenotypically. Twenty one-day-old plants were inoculated by spraying with aqueous spore suspension onto the leaves until run-off. The relative humidity (RH) was maintained at above 90% by covering them with black netting, as well as watering them two to three times during the daytime. Disease scoring was carried out nine (9) days after inoculation based on the Standard Evaluation System (SES) of the International Rice Research Institute (IRRI, 1996). The blast lesion degrees (BLD) were scored using a scale 0-9 as follows: 0= no evidence of infection; 1= brown specks(<0.5 mm in diameter); 2= brown lesions of 0.5–1 mm in diameter; 3= 1-3 mm in diameter with gray centers and brown margins; 4= typical spindle-shaped blast lesion 3 mm or longer, less than 4% of the leaf area infected; 5= typical blast lesion, 3 mm or longer in diameter, infected 4-10% leaf area; 6= typical blast lesions, 3 mm or longer in diameter, infected 11-25% leaf area; 7= typical blast lesions, 3 mm or longer in diameter, infected 26-50% leaf area; 8= typical blast lesions, 3 mm or longer, infected 51-75% leaf area; and 9= typical blast lesions, 3 mm or longer, infected more than 75% leaf area. In the case of single-gene model analysis, the rice plants showing lesion types 0 to 3 were considered as resistant and the plants showing lesions type 4 and above were considered to be susceptible to the selected pathotype P7.2 in BC_2F_1 population. Plant disease reaction was categorized according to Singh et al. (2012) with some modification.

Total genomic DNA was extracted from fresh leaves of 4-week-old individual plants using the modified CTAB (hexadecyltrimethylammonium bromide) method as described by Doyle & Doyle, (1990). DNA was quantified by using nano-drop spectrophotometry (ND1000 Spectrophotometer). The diluted DNA samples diluted with 1xTE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) to get a concentration of 70 ng/µl and kept in the refrigerator at –20 °C for PCR analysis.

Total sixteen microsatellites with known chromosomal positions distributed on rice chromosomes were selected from the Gramene database (www.gramene.org) related to blast resistance genes (McCouch et al., 2002; Temnykh et al., 2000). Parental lines were used to identify polymorphic markers related to rice blast resistance gene. PCR reactions with microsatellite markers were carried out in 15-µl reactions containing 1 µl (70 ng) of genomic DNA, 1.0 µM of each primer, 7.4 µl master mix and 4.6 µl nuclease-free water. PCR amplification was carried out in a thermocycler (T100™, Bio-Rad) using an initial denaturation at 94 °C for 5 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds, the final extension at 72 °C for 5 minutes, followed by rapid cooling to 4 °C prior to analysis. For electrophoresis, 3.0% metaphor™ agarose (Lonza) gel was prepared containing 1µl Midori green in 1X TBE buffer (0.05 M Tris, 0.05 M boric acid, 1 mM EDTA, pH 8.0). The gel was run at constant voltage of 80V for 80 minutes and visualized using Molecular Imager® (GelDoc™ XR, Bio-Rad).

For marker genotyping, the clearly SSR bands were scored manually. The plants that showed a pattern similar to the susceptible parent alleles were scored as "aa" and those with a banding pattern similar to the resistant parent alleles were scored as "AA", and the heterozygous plants were scored as "Aa".

Chi-square (χ^2) test was performed to test the goodness of fit of the BC_2F_1 population for the phenotypic and marker data by comparing the observed frequency distribution with an expected one. Chi-square analysis for goodness of fit to the backcross type of segregation was performed as mentioned by Tartarini (1996). The chi-square analysis for the genotypic and phenotypic ratio was calculated by using the formula, $\chi^2 = (O - E)^2 / E$, where O is the observed value, and E is the expected value. For the single-gene model, the chi-square value was considered as significant ($p \leq 0.05$) if its value was higher than 3.84. Frequency of disease reaction trait was analyzed using SPSS ver. 16.0. Single-marker analysis was carried out according to Divya et al. (2014) to know the association between the markers and trait of blast incidence.

3. RESULTS AND DISCUSSION

The frequency distribution of blast disease evaluation for the trait of the blast lesions degree (BLD) is shown in figure 3. The susceptible parent MR219 showed highly susceptible reaction with lesions type 5 to 7 score; whereas the parent Pongsu Seribu 1 was found to be resistant producing lesions type 0 to 2 under artificial inoculation in the glasshouse (Figures 1-3). Among the 333 BC_2F_1 plants, 159 plants showed resistant reaction and 174 plants showed susceptible reaction (Table 1). The observed frequencies, when tested for goodness of fit with chi-square (χ^2) test for single-gene model, showed goodness of fit (P = 0.4463) to the expected segregation testcross ratio (1:1) (Table 1). Therefore, resistance to blast pathotype P7.2 in Pongsu Seribu 1 is most likely controlled by a single dominant gene. The testcross progeny phenotypically segregated into a ratio of 1R:1S. Present studies are in agreement with findings of Bhatt et al. (1994), Mackill & Bonman (1992) for inheritance of blast resistance in rice and Beyer et al. (2011) for inheritance of Russian wheat aphid resistance in wheat. Several scientists reported that blast resistance is governed by dominant genes (Bhatt et al., 1994; Yamasak & Kiyosawa, 1966), but in a few cases the resistance was also reported due to recessive genes (Bhatt et al., 1994; Yu et al., 1987). This situation is in agreement with the statement that the ability of a plant to express resistance is also dependent on the genotype of the pathogen. A rice plant cannot be resistant to an isolate of *M. oryzae* unless the pathogen has a gene that makes it virulent to the rice plant. An isolate of *M. oryzae* cannot be avirulent on the rice plant unless the rice plant has genes that make it resistant to that isolate (Ellingboe & Chao, 1994). The findings of monogenic inheritance of a dominant nature resistance are in agreement with the results of several scientists (Orellana et al., 1980; Xue & Chen, 1987). The expression of the gene depends on the virulent gene(s) present in the fungus. Previous studies showed resistance to blast is governed either by a single gene or a polygenic system, depending on the genotypes or cultivars, as well as their specificity to *M. oryzae* isolates, where resistance to blast disease is host specific and effective against only specific strains of *M. oryzae* (Zhou et al., 2007). However, studies conducted in IRRI revealed that most of the traditional varieties have one or two dominant genes (Mackill et al., 1985). Our result is in agreement with a blast research done at IRRI in the Philippines, which indicated that one or two dominant genes present in the cultivars confer complete resistance against each fungal isolate (Yu et al., 1987).

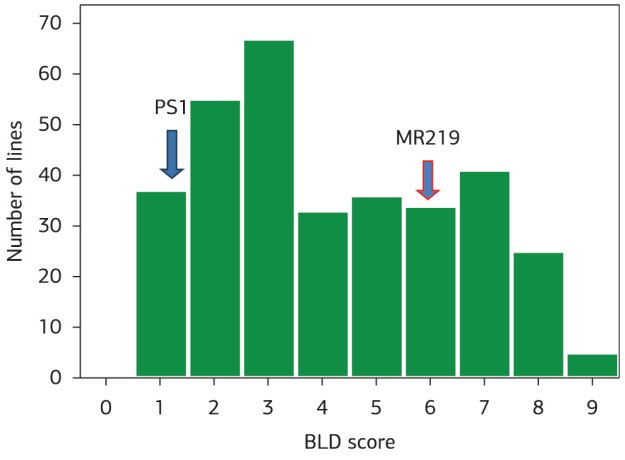

Figure 3. Frequency distribution of blast lesions degree (BLD) in BC_2F_1 population inoculated with rice blast pathotype P7.2. The mean scores of two parents are indicated by arrows.

A total of 16 polymorphic markers were found as the linked marker for blast resistance (Table 2). All linked markers were tested in F_1 population. In BC_1F_1 generation, two markers (RM6836 and RM8225 markers) showed heterozygous plants. Using other foreground markers none of plants were found as heterozygous condition which indicates that some blast resistant gene disappeared due to the backcrossed with

Table 1. Phenotypic segregation of blast resistance in BC_2F_1 population obtained from a cross between rice cultivars MR219 × Pongsu Seribu 1 inoculated with pathotype P7.2 of *M. oryzae*

Genotypes	Total seedlings	Resistant (R)	Susceptible (S)	Expected ratio	x^2 value	P-value
MR219 (P1)	128	0	128	-	-	-
PS1 (P2)	128	128	0	-	-	-
F_1	28	28	-	-	-	-
BC_2F_1	333	159	174	1:1	0.58	0.4463

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

Table 2. Information of polymorphic microsatellite blast resistant linked markers

Markers name	Chr.	Primer sequences (5'-3')		Product size (bp)	Linked genes
		Forward primer	Reverse primer		
RM168	3	TGCTGCTTGCCTGCTTCCTTT	GAAACGAATCAATCCACGGC	116	Candidate gene- Oxalate oxidase, 14-3-3 protein
RM413	5	GGCGATTCTTGGATGAAGAG	TCCCCACCAATCTTGTCTTC	79	*Pi-26*
RM5961	11	GTATGCTCCTCCTCACCTGC	ACATGCGACGTGATGTGAAC	129	*Pi-7(t)*
RM6836	6	TGTTGCATATGGTGCTATTTGA	GATACGGCTTCTAGGCCAAA	240	*Pi-z,Pi-2,Pi-9, Pi-8,Pi-3, PIi*
RM8225	6	ATGCGTGTTCAGAAATTAGG	TTGTTGTATACCTCATCGACAG	221	*Pi-z*
RM101	12	GTGAATGGTCAAGTGACTTAGGTGGC	ACACAACATGTTCCCTCCCATGC	324	*Pi-6(t)*
RM224	11	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG	157	*Pi-k,Pi-sh, Pilm2,Pi-18(t)*
RM140	1	TGCCTCTTCCCTGGCTCCCCTG	GGCATGCCGAATGAAATGCATG	261	*Pi-37(t),Pi-24(t)*
RM5	1	TGCAACTTCTAGCTGCTCGA	GCATCCGATCTTGATGGG	113	*qtl (qLs1)*
RM261	4	CTACTTCTCCCCTTGTGTCG	TGTACCATCGCCAAATCTCC	125	*Pi-21*
RM340	6	GGTAAATGGACAATCCTATGG	GACAAATATAAGGGCAGTGTGC	163	*Pi-tq1*
RM547	8	TAGGTTGGCAGACCTTTTCG	GTCAAGATCATCCTCGTAGCG	235	*Pi-11(t)*
RM495	1	AATCCAAGGTGCAGAGATGG	CAACGATGACGAACACAACC	159	*Pi-t*
RM251	3	GAATGGCAATGGCGCTAG	ATGCGGTTCAAGATTCGATC	147	*qtl*
RM229	11	CACTCACACGAACGACTGAC	CGCAGGTTCTTGTGAAATGT	116	*Pi-7(t)*
RM247	12	TAGTGCCGATCGATGTAACG	CATATGGTTTTGACAAAGCG	131	*Pi-20(t),Pi-ta*

MR219. This statement is more coincide with the findings of Suh et al. (2009) who found that some genes were lost during the recombination process in segregating generations of advanced backcross lines. The two polymorphic (RM6836 -238bp and RM8225 -212bp) linked markers were used to evaluate BC_2F_1 progenies. These two markers located on chromosome 6 of rice showed linkage with resistance and susceptibility in BC_2F_1 progeny. The banding patterns of two polymorphic markers RM6836 and RM8225 linked with *Pi* genes in F_1 and BC_2F_1 population for 14 samples along with two parents are shown in figure 4 and figure 5, respectively. The position of RM6836 (54.3 cM) and RM8225 (54.1 cM) markers are shown in figure 6. These two markers are 0.2 cM apart from each other on chromosome 6 of rice. Fjellstrom et al. (2006) and Rathour et al. (2008) mentioned that markers RM8225 and RM6836 are tightly linked with *Piz* gene located on chromosome 6, whereas Rathour et al. (2008) found that these two markers located at distance of 1.2-4.5 cM from the gene. Results indicate that individuals of the BC_2F_1 population (derived from Pongsu Seribu 1) had the alleles linked with these two microsatellite markers resistant against pathotype P7.2 of *M. oryzae*. This finding has potential use in marker-assisted selection to develop rice cultivars with blast resistance genes in rice breeding programs. Because these markers had high-selection accuracy for resistant plant sources, they can be used in MAS for the resistant gene.

A total of 333 plants of BC_2F_1 population were evaluated with the linked markers. The observed segregation ratio for resistance and susceptibility in BC_2F_1 lines for 16 polymorphic microsatellite markers is shown in table 3. The chi-square (χ^2) analysis for RM6836 and RM8225 showed a good fit to the expected testcross ratio (1:1) for a single-gene model (d.f. = 1.0, $p>0.05$) in BC_2F_1 population (Table 3). The rest of the markers did not fit the expected segregating Mendelian ratios. Results indicate that RM6836 and RM8225 have an association with blast resistance gene against to pathotype P7.2 of *M. oryzae* in rice.

The segregation ratio was not in agreement with the expected Mendelian ratio for other polymorphic markers in BC_2F_1 population. This is due to the fact that, none of the plants found as heterozygous condition using other foreground markers. In chi-square analyses, two microsatellite markers showed an expected testcross segregation ratio of 1:1, inherited in simple Mendelian fashion. Phenotypic data for disease reaction of resistance and susceptibility to blast pathotype P7.2 also segregated in 1R:1S ratio in the BC_2F_1 population. So, we can conclude that resistance to blast pathotype P7.2 in Pongsu Seribu 1 is controlled by a single dominant gene. The plants resistant to blast pathotype P7.2 from BC_2F_1 lines had genotypes with microsatellite markers RM8225 and RM6836, these markers could be used for marker-assisted selection. The existence of Pongsu Seribu 1 with individual blast resistance genes provides a powerful tool for future studies on the rice blast disease. The virulence patterns of blast races (pathotype P7.2) will be easier to study with lines possessing known resistance genes. Moreover, it should be easier to identify additional resistance genes. This blast resistant Pongsu Seribu 1 is currently being used to tag the resistance genes using microsatellite markers. This information can be useful in practical breeding programs as well as in the eventual cloning of the resistance genes.

Marker trait association was performed using SPSS ver. 16.0 software to identify the association of resistance component trait with linked polymorphic markers of the blast resistance gene. The genotypic segregation data set of the linked microsatellite markers generated from the BC_2F_1

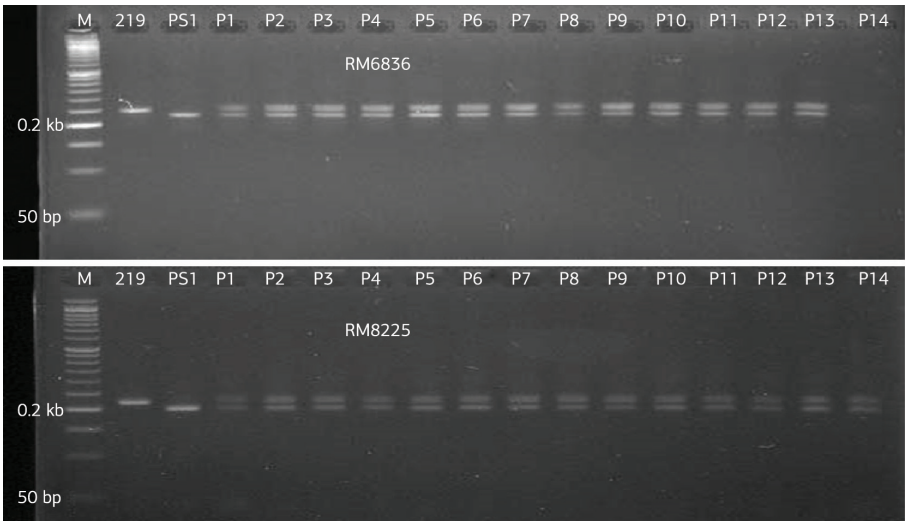

Figure 4. Genotyping with markers RM6836 and RM8225 linked to blast resistance genes in F_1 population of rice derived from MR219 × Pongsu Seribu 1 (PS1). Running on 3% metaphor agarose gel stained with midori green, only 14 samples plus the two parents for each marker are shown (M=50 bp ladder).

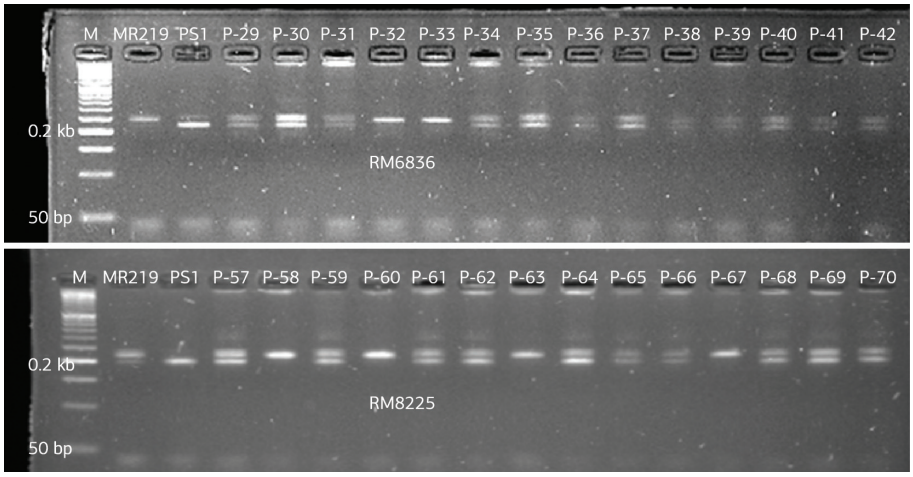

Figure 5. Genotyping with markers RM6836 and RM8225 linked to blast resistance genes in BC_2F_1 population of rice derived from MR219 × Pongsu Seribu 1 (PS1). Running on 3% metaphor agarose gel stained with midori green, only 14 samples plus the two parents for each marker are shown (M=50 bp ladder).

population combined with phenotypic segregation data for blast resistance trait. This data was subjected to linear model regression analysis and significance of R^2 was identified using F value comparison. The marker RM8225 and RM6836 showed significant R^2 values higher than 10 for the trait of the blast lesions degree (BLD) (Table 4). We conclude that RM6836 and RM8225 are two linked microsatellite markers to blast resistance locus in BC_2F_1 generation. The deployment of major gene resistance will minimize selection pressure and thereby prevent evolution of resistance in the pathogen population (Bonman et al., 1992). This approach will help breeders to expedite breeding research in crops by enabling a selection based on the genotype rather than on the phenotype. The markers reported here provide rice breeders and geneticists a valuable tool for marker-aided selection of a disease resistance gene.

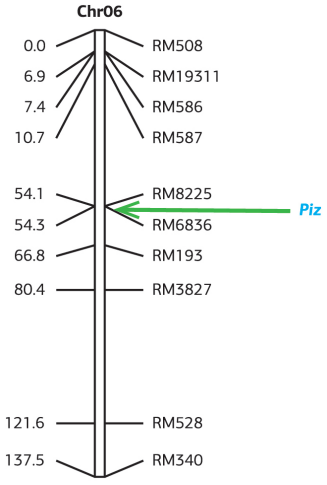

Figure 6. The position of RM6836 and RM8225 markers on rice chromosome 6.

Table 3. Marker segregation analysis in BC_2F_1 population derived from a cross between rice varieties MR219 × Pongsu Seribu 1

Markers	Chromosome	Marker analysis		x^2 (1:1)	Probability
		Aa	aa		
RM168	3	0	333	331	<.0001
RM413	5	0	333	331	<.0001
RM5961	11	0	333	331	<.0001
RM6836	6	162	171	0.20	0.6547
RM8225	6	156	177	1.20	0.2733
RM101	12	0	333	311	<.0001
RM224	11	0	333	331	<.0001
RM140	1	0	333	331	<.0001
RM5	1	0	333	331	<.0001
RM261	4	0	333	331	<.0001
RM340	6	0	333	331	<.0001
RM547	8	0	333	331	<.0001
RM495	1	0	333	331	<.0001
RM251	3	0	333	331	<.0001
RM229	11	0	333	331	<.0001
RM247	12	0	333	331	<.0001

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

Table 4. Marker trait association in the BC_2F_1 population by regression analysis

Traits	Marker	Chromosome	Position	R^2 value (%)
BLD	RM8225	6	54.1	11.42*
	RM6836	6	54.3	10.82*

*R^2 values are significant at $p<0.05$ level of significance.

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