Genetic analysis of the resistance to rice blast in the BC$_2$F$_1$ population derived from MR263 × Pongsu Seribu 1

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ABSTRACT

Rice blast disease, caused by *Magnaporthe oryzae*, is the most important and most devastating rice disease globally. For genetic analysis of resistance to rice blast, the present study used a BC$_2$F$_1$ population developed through marker-assisted backcrossing from a cross between blast susceptible MR263 and blast resistant Pongsu Seribu 1 (PS1). We selected out of 450 markers, 65 polymorphic simple sequence repeat markers, including the Pi gene-based markers, and identified 16 markers associated with blast resistance that showed heterozygous bands in the BC$_2$F$_1$ population. Of the 16 polymorphic markers, only eight (RM5961, RM263, RM163, RM224, RM262, RM168, RM229 and RM169) showed a good fit to the expected segregation genotypic ratio (1:1) for the single dominance gene model ($\chi^2=1.0$, $P<0.05$), according to chi-square ($\chi^2$) analysis. An analysis of phenotypic data of the BC$_2$F$_1$ population also showed a good fit to the expected phenotypic ratio (1:1; R:S) for resistant and susceptible plants. The resistance to blast pathotype P7.2 in PS1 is most likely controlled by a single dominant gene that is linked to the eight markers we identified. These markers could be used in marker-assisted selection programmes to develop a durable blast resistant rice variety.

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Introduction

Currently, the severity of plant diseases and pests is increasing due to climate change. Blast, one of the most important diseases of rice, is caused by *Magnaporthe oryzae*; it has been recognized as the most important and devastating fungal disease worldwide [1,2]. Blast is responsible for yield losses as high as 50% throughout the world [2,3]. Although blast disease can be managed by fungicides, the inherent quality of disease resistance can be useful for appropriate breeding programmes [4,5].

Many studies have already been conducted on the inheritance of blast resistance. The first *Pi* gene in rice was named by Kiyosawa [6]. The approach used several cultivars that carried different, single resistance genes [6] to characterize the specific virulence of different isolates of the pathogen. Kiyosawa [7] used seven Japanese strains of blast fungus to investigate the inheritance of resistance and identified 13 resistant genes. Numerous studies using different races have demonstrated that resistance is controlled by one or two dominant genes [8,9]. He and Shen [10] identified 11 dominant genes while studying the inheritance of resistance in cultivars against two races of *M. oryzae*. Mackill and Bonman [11] noted that a single dominant resistance gene controls blast resistance in near isogenic rice lines.

Rice blast resistance can be classified into complete resistance and partial resistance types. The complete resistance type is race-specific and is controlled by a single dominant or recessive $r$ gene that can be recognized by a cognate avirulence ($Avr$) gene in the pathogen [12]. More than 85 major $R$ genes have already been mapped on all rice chromosomes except rice chromosome 3 [13,14]. Interestingly, a large group of $R$ genes are clustered in several genomic regions, such as on chromosome 6, 11 and 12.

Quantitative resistance is controlled by quantitative trait loci (QTLs) so it is usually considered non-race
specific and durable. The first set of blast QTLs was mapped by Wang et al. [15] using a recombinant inbred line (RIL) population which was developed by crossing ‘Moroberekan’ upland rice (durably resistant) and ‘CO39’ rice (susceptible to blast). In all 12 chromosomes of rice, a total of 362 QTLs have been identified. Most of the major resistance genes follow a gene-for-gene interaction model [16], in which any Pi gene in rice confers resistance to *M. oryzae* in a gene-for-gene manner [17,18]. When a single resistance gene controls the blast disease of a cultivar, it can be overcome due to natural selection of compatible races of the pathogen due to its high specificity [19]. Utilizing genetic resistance is the most effective and environmentally friendly approach for crop production. The method of artificial disease inoculation of the spores to the plant leaves.

Detection and fine mapping of multiple genes of rice through DNA makers is now possible due to the advancement in genomics research and complete sequencing of the rice genome. Molecular markers can be used to detect the presence of a desired gene (e.g., one that is economically important). Marker-assisted selection (MAS) can be utilized to incorporate different resistance genes into rice cultivars. Therefore, blast can be managed by providing rice cultivars with multi-genetic resistance against a wide spectrum of blast races [21]. Adhikari et al. [22] suggested that the use of a disease resistant variety offers an inexpensive and environmentally friendly approach for crop protection. Pongsu Seribu 1 (PS1), a local Malaysian variety with lower yield, has been used as a source of broad-spectrum blast resistance genes and as a donor parent in a marker-assisted backcrossing programme, whereas MR263 is a high-yielding variety (>6 t/ha) with fewer days to maturity (97–104 days) but blast susceptible [2,5]. The objective of this study was to select suitable simple sequence repeat (SSR) markers linked to the rice blast resistance gene (*Pi*) in the BC$_2$F$_1$ population derived from a cross between the resistant variety PS 1 and the susceptible rice cultivar MR263.

**Materials and methods**

**Plant materials**

The donor PS1, a local Malaysian variety from the Malaysian Rice Research Centre, Malaysian Agricultural Research Development Institute (MARDI), was used as the source of blast resistance genes. PS1 was discovered to be resistant to many Malaysian blast pathotypes, similar to other donor resistant varieties, including Tadukan from the Philippines and Tetep from Vietnam [23]. MR263, a popular and high-yielding rice variety, was used as a recurrent parent with elite agronomic traits that is susceptible to blast. The BC$_2$F$_1$ population was produced from a cross between MR263 and PS1 and genotyped with suitable SSR markers that were linked to blast resistance genes [2,5].

**Fungal culture and inoculum preparation of *M. oryzae*, pathotype7.2**

*M. oryzae* pathotype 7.2 is the most devastating and virulent blast pathogen in Malaysia as reported by Rahim et al. [24]. This pathogen was collected from the Malaysian Agricultural Research and Development Institute (MARDI). Pathotype 7.2 was cultured using potato dextrose agar medium. Then, the spores were harvested from pure culture plates by scraping the fungal mycelia with a sterile slide and filtering through a nylon gauze mesh. The concentration of conidial suspension of $1.5 \times 10^5$ spores/mL was measured by a haemocytometer with deionized water, and 0.05% Tween-20 was mixed properly to increase the adhesion of the spores to the plant leaves.

**Artificial inoculation and disease evaluation in BC$_2$F$_1$**

Twenty-one-day-old seedlings of 300 BC$_2$F$_1$ plants were inoculated by spraying with a conidial suspension of $1.5 \times 10^5$ spores/mL containing 0.05% Tween-20, similar to what has been described previously [25]. At this stage, the leaves of the plants are very soft, succulent and appropriate for disease infection and development. The method of artificial disease inoculation was previously described by Ashkani et al. [25]. Approximately 3 mL of conidia and hyphal suspension from an agar slant were seeded in the medium in a Petri plate. The inoculated plates were incubated at 25–28 °C for 5–7 days, until the entire agar surface was covered with mycelial growth. The growth was scraped with a sterilized rubber spatula, and the plate was exposed to fluorescent light for 3 days to induce heavy sporulation. The culture was flooded with distilled water mixed with 0.05% Tween-20, and the conidia were dislodged by scraping. The suspension was filtered through cheese cloth, and the concentration of conidia was estimated with a haemocytometer (standardized to $1.5 \times 10^5$ conidia per mL). Plants were inoculated 18–20 days after sowing, using a hand sprayer to apply 30 mL of the conidial suspension per tray of seedlings. The trays were rotated slowly during
inoculation to ensure uniform distribution of inoculum. Inoculated seedlings were incubated in a dew chamber for 24 h at 25°C, and then transferred to a greenhouse.

In this study, the inoculated seedlings were kept in a humid chamber maintained at 25–30°C. The seedlings were sprinkled with distilled water three to four times a day to maintain high humidity. The disease reaction of each rice line was recorded 7 days after inoculation. Scoring was carried out based on the standard evaluation system of the International Rice Research Institute [26] and the protocol standardized by Mackill and Bonman [11].

**Genomic DNA extraction**

To extract genomic DNA, fresh young leaves were collected from individual 4-week-old plants using the cetyltrimethylammoniumbromide method which was modified from the method of Doyle and Doyle [27].

**DNA identification and quantification**

Each DNA sample (1 μL) was measured by NanoDrop spectrophotometry (ND-1000, NanoDrop Technologies Inc., Wilmington, DE, USA) to estimate the relative purity and concentration of the extracted DNA. The final concentration of each DNA sample was diluted to the required level with 1× TE buffer (10 mmol/L Tris–HCl, pH 8.0, 1 mmol/L EDTA, pH 8.0) and kept at −20°C for further polymerase chain reaction (PCR) analysis.

**Analysis of SSR markers**

A total of 450 SSR markers, including blast resistant Pi gene-based markers, were selected and used from the Gramene database (www.gramene.org) that had been mapped by Wu and Tanksley [28], Akagi et al. [29], Temnykh et al. [30] and McCouch et al. [31]. Primer pairs were optimized for PCR to amplify microsatellite loci. Parental varieties were used to identify SSR polymorphisms associated with the rice blast resistance gene.

**Polymerase chain reaction (PCR)**

Genotype data were obtained by analyzing the DNA with SSR markers using 15-μL PCR reactions containing 1 μL of DNA, 2 μL of forward and reverse primer, 7.4 μL of master mix and 4.6 μL of water, using an Eppendorf single or dual 96-well thermal cycler. After initial denaturation for 5 min at 94°C, 1 min of annealing at 55°C for all primers and 2 min of extension at 72°C were performed with a final 5 min extension at 72°C occurring at the end of 35th cycle. The PCR products were loaded into a 3% metaphor agarose gel and analyzed by electrophoresis with a horizontal Thermo Scientific Gel Electrophoresis tank (Waltham, MA, USA). The gels were documented using an Alpha Imager 1220 (Alpha Innotech, CA, USA). SSR markers were used for selection [31–33].

**Genotyping for marker segregation**

A total of 300 progenies for different SSR marker alleles were scored based on their parental bands. ‘r’ was designated as the recipient parent allele at the homozygous state; ‘R’ was designated as the donor parent allele at the homozygous state, and Rr was designated as the heterozygous plant alleles. The size (in nucleotide base pairs) of the most intensely amplified band for each microsatellite marker was determined based on its migration, relative to molecular-weight size marker (50Xbp Ladder) using computer software Alpha Ease 4.0.

**Statistical analysis**

To analyze the segregation data, we performed a chi-square (χ²) test. Chi-square analysis for the genotypic and phenotypic ratio was carried out using the formula, χ² = (O−E)²/E, where O is the observed value and E is the expected value. For the single gene model and epistasis, each chi-square value was considered significant (P ≤ .05) if its value was greater than 3.84, while for the two independent genes, it was considered significant if it was greater than 7.84.

**Results and discussion**

**Survey of SSR markers of the parental and BC₂F₁ populations**

In this study, 65 polymorphic SSR markers were selected (out of 450 markers), including Pi gene-based markers, and identified 16 markers associated with blast resistance which showed heterozygous band in the BC₂F₁ population (Table 1). Therefore, 16 polymorphic markers were used for segregation analysis. The patterns of all of the markers varied in the segregating population. For the banding patterns of the microsatellite primer pairs for the eight polymorphic markers linked to blast resistance along with their two
parents, among which here only one marker has been shown in Figure 1.

### Analysis of marker segregation in the BC$_2$F$_1$ population

The banding patterns of the BC$_2$F$_1$ lines derived from MR263 × PS1 for RM5961 linked to blast resistance genes (MR263, recurrent parent; PS1, Pongsu Seribu 1 donor resistant parent; Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, BC2F1 population and M; 50 bp ladder).

**Table 1.** The sequence and size of polymorphic microsatellite markers used in the BC$_2$F$_1$.

| SSR markers | Primer sequences (5' → 3') | Chromosome | Marker analyzed | R = SG | r = S | $\chi^2$ (1:1) | Probability |
|-------------|-----------------------------|------------|-----------------|--------|------|---------------|-------------|
| RM5529      | AGCCGAAACTACATTCGGTG TTGTGTAGTTGGCACGCTTC 2 (TG)12 55 167 | 2          | RM5529          | 175    | 125  | 8.00**        | 0.0047      |
| RM10022     | CCTCCATAGAGTAAGGTTTGCATGG CCTCCTCCTCTGTCTTTCTCTGC 1 (AAAG)5 55 176 | 1          | RM10022         | 172    | 128  | 6.16*         | 0.0131      |
| RM5         | TGCAACTTCTAGCTGCTCGA GCATCCGATCTTGATGGG 1 (GA)14 55 112 | 1          | RM5             | 127    | 173  | 6.76**        | 0.0093      |
| RM262       | CATTCCGTCTCGGCTCAACT CAGAGCAAGGTGGCTTGC 2 (CT)16 55 148 | 2          | RM262           | 146    | 154  | 0.16          | 0.6892      |
| RM224       | ATCGATCGATCTTCACGAGG TGCTATAAAAGGCATTCGGG 11 (AAG)8(AG)12 55 157 | 11         | RM224           | 115    | 143  | 1.48          | 0.2238      |
| RM168       | TGCTGCTTGCCTGCTTCCTTT GAAACGAATCAATCCACGGC 3 T15(GT)14 55 116 | 3          | RM168           | 160    | 140  | 1.20          | 0.2733      |
| RM510       | AACCGGATTAGTTTCTCGCC TGAGGACGACGAGCAGATTC 6 (GA)15 55 122 | 6          | RM510           | 178    | 122  | 10.08**       | 0.0015      |
| RM1089      | CAGAAGGATTATCTCGATACC AATAGGGCTTGAAATAAATTG 5 (AC)33 55 239 | 5          | RM1089          | 121    | 179  | 10.84**       | 0.0010      |
| RM242       | GGCCAACGTGTGTATGTCTC TATATGCCAAGACGGATGGG 9 (CT)26 55 225 | 9          | RM242           | 117    | 145  | 0.56          | 0.4543      |
| RM1132      | ATCACCTGAGAAACATCCGG CTCCTCCCACGTCAAGGTC 7 (AG)12 55 93  | 7          | RM1132          | 132    | 168  | 4.08**        | 0.0434      |
| RM163       | TGGCTGGCTCCGTGGGTAGCTG TCCCGTTGCCGTTCATCCCTCC 5 (GA)12 55 167 | 5          | RM163           | 158    | 142  | 0.76          | 0.3833      |
| RM589       | ATCATGGTCGGTGGCTTAAC CAGGTTCCAACCAGACACTG 6 (GT)24 55 186 | 6          | RM589           | 174    | 126  | 7.36**        | 0.0067      |
| RM169       | TGGCTGGCTCCGTGGGTAGCTG TCCCGTTGCCGTTCATCCCTCC 5 (GA)12 55 167 | 5          | RM169           | 145    | 155  | 0.28          | 0.5967      |
| RM263       | CCCAGGCTAGCTCATGAACC GCTACGTTTGAGCTACCACG 2 (CT)34 55 199 | 2          | RM263           | 159    | 141  | 0.96          | 0.3272      |

Notes: S: susceptible; SG: segregant.
$\chi^2$, the actual value of the chi-square test for resistant/susceptible ratio. Bold markers are nonsignificant for Mendelian ratio (1:1).

*0.05 significance level; **0.01 significance level.

Blast resistance against the pathotype 7.2 was segregated
in the BC2F1 generation according to chi-square tests, but was not segregated into 1:1:1:1 or 15:1 ratios. Therefore, the eight SSR markers (RM5961, RM263, RM163, RM224, RM262, RM168, RM229 and RM169) for blast resistance in PS1, specifically against pathotype P7.2 are controlled by a single dominant gene. This implies that the plants’ ability to express resistance depends on the genotype of the pathogen. These findings are in agreement with those of Hasan et al. [2] and Wu-ming et al. [18] in rice blast, Latif et al. [34] in tungro and Latif et al. [35] in brown plant hopper. In the segregation analysis, eight polymorphic markers clearly showed goodness of fit to the expected segregation ratio for the single gene model. The segregation ratio was not in agreement with the expected Mendelian ratio for remaining polymorphic markers. This is in agreement with the statement that the ability of a plant to express resistance is also dependent on the genotype of the pathogen [18].

A rice plant cannot be resistant to an isolate of *M. oryzae* unless the pathogen has the gene that makes it avirulent to the rice plant. This finding has potential for use in MAS programmes and confirmation of blast resistance genes to develop varieties of Malaysia rice with blast resistance.

**Phenotypic screening for blast resistance in the BC2F1 population**

According to the IRRI [26], plants with scores of 0–3 are considered resistant, whereas those showing reactions that scored 4–9 are considered susceptible. For the donor parent (PS1; which possesses the blast resistant *Pi* gene), the plants with lesion scores of 0 and 1 were considered resistant (R), and a score of 3 was considered moderately resistant (MR) [5]. For the recurrent parent MR263, the plants with lesion scores of 5 were considered moderately susceptible (MS), and a score of 7 was considered susceptible (S) [5]. The frequency distribution of the blast disease evaluation for the trait of blast lesion degree (BLD) is shown in Figure 2. In MR263, the average BLD score was 6.2 and in PS1, the average BLD score was 1.34 against the virulent pathotype 7.2. The blast disease reactions in BC2F1 families are shown in Figure 3. An isolate of *M. oryzae* cannot be avirulent to the rice plant, unless the rice plant has genes that make it resistant to that isolate [24,36]. Partial resistance can be race specific [1,2,18]. The findings of this research support the research output reported by IRRI, Philippines; that is, that one or two dominant genes are present and the ‘Mashuri’ cultivar was susceptible (S). The complete resistance against each fungal isolate has been proved by the 3R:1S segregation ratio of the F2 population [8]. The segregation patterns of F3 and F4 generations confirmed that blast resistance is governed by a single dominant gene in ‘Laxmi’ cultivar (resistant; R) [36]. Pan et al. [37] opined that a native Indian rice cultivar Aus373 was governed by dominant alleles at two loci, from the resistance ratios of the subsequent F2.
progenies. Additional genetic studies are needed to understand the molecular mechanism of broad-spectrum resistance to rice blast through the investigation of whether qualitative and quantitative genes affect the level of resistance in rice.

Among the 300 BC$_{2}$F$_{1}$ plants, 165 plants showed resistance, and 135 plants showed susceptibility. The test cross progeny phenotypically segregated into a ratio of 1R:1S (Table 3). The observed frequencies, when analyzed with the chi-square ($\chi^2$) test for a single-gene model, showed goodness of fit ($P = 0.0943$) to the expected segregation ratio (1:1; Table 4). Therefore, resistance to the blast pathotype P7.2 in PS1 is most likely controlled by a single nuclear gene.

The individuals of the BC$_{2}$F$_{1}$ population (derived from PS1/C2 MR263) that had the alleles RM5961 (129 bp), RM263 (199 bp), RM163 (124 bp), RM224 (157 bp), RM262 (148 bp), RM168 (116 bp), RM229 (116 bp) and RM169 (167 bp) were resistant to pathotype P7.2. The blast resistant plants had the alleles of these eight SSR markers. An analysis of the selected eight SSR markers in the BC$_{2}$F$_{1}$ segregating population indicated that these markers were linked to blast resistance. These markers had high selection accuracy in resistant plants; therefore, these markers can be used for MAS.

**Conclusions**

Sixteen polymorphic markers were used to identify the segregation ratios in the BC$_{2}$F$_{1}$ population. Chi-square analyses of eight SSR markers showed an expected segregation ratio of 1:1, which was inherited in a simple Mendelian fashion. The phenotypic data based on resistance and susceptibility reactions to the disease caused by the blast pathotype P7.2, segregated in a 1:1 (R:S) ratio in the BC$_{2}$F$_{1}$ population. Therefore, the resistance to blast pathotype P7.2 in PS1 is likely controlled by a single gene. The plants resistant to the blast pathotype P7.2 in the BC$_{2}$F$_{1}$ lines had genotypes with the SSR markers RM5961 (129 bp), RM263 (199 bp), RM163 (124 bp), RM224 (157 bp), RM262 (148 bp), RM168 (116 bp), RM229 (116 bp) and RM169 (167 bp). These markers could be used for MAS.

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**Disclosure statement**

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

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