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# Inheritance Study of Blast Resistance with Pathotype P7.2 on Crosses of Pongsu Seribu 2 and MR264

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**Abstract:** Rice is an important agricultural crop particularly in Malaysia. The rice production was affected by many factors including rice blast disease. In attempt to overcome the rice blast disease, genetic analysis study on new crosses of cultivar from Pongsu Seribu 2 and MR264 was used. The objective of this study is to identify segregation pattern of $F_2$ and $F_3$ population resistance to *M. oryzae* at genotypic and phenotypic level with associated resistance genes linked with SSR markers. SSR markers were used to screen segregating pattern in genotypic level while phenotypic screening was done in greenhouse by observing the disease development to the rice plant. Chi-square results shows a good fit of 1:2:1 for a single gene model which proved the hypothesis of a single dominant gene and 3:1 for the segregation of resistance and susceptibility for *M. oryzae* but did not segregate with epistasis and two-gene model. SSR markers of RM101, RM206, RM413 and RM495 were significantly associated with blast resistance to *M. oryzae* in rice. These markers were highly accurate for resistant plant with resistance effect about 12% for phenotypic variation. This study also shows that there was a strong interrelationship between the selected phenotypic and the four markers in this study. Therefore, these studies can be utilized for future references to identify these resistant and susceptible varieties based on polymorphism. This research offered very useful information for local rice breeders in developing blast disease resistant rice cultivar.

**Key words:** Rice, blast disease, *Magnaporthe oryzae*, resistance genes, local rice breeders

## INTRODUCTION

Rice is not only a staple food that feeds for almost half of the world population; it is also a model plant for cereal crops with relative small genome size (Wang *et al*., 2011). Approximately 674, 250 ha planted area in Malaysia has shown the production of rice annually. In Malaysia, rice industry has always been given special treatment based on strategic important of rice as a staple food commodity (Fatimah *et al*., 2007). Although, the production of rice is increasing towards the increasing of the population, Malaysia still depends on the imported rice to fulfill consumer's demand. The rice production was affected by many abiotic and biotic stresses throughout the world like blast disease which had made the yield losses ranging about 50% a year. Rice blast disease is one of the most overwhelmed diseases intimidating the world of rice. *Magnaporthe oryzae* is the common fungus widespread in rice field. Broad spectrum resistance genes of *Pi* genes are the common linked genes found with rice blast genes. Therefore, there is a need to improve the rice yield through development of high yield potential rice variety in Malaysia that free from diseases. By studying this new cross variety will help increasing the demand of rice with its own benefits. In this study, the work done was focus on morphological and physiological components of the crosses variety from greenhouse trial against blast disease and even study on molecular marker that been used. Molecular markers are widely used in rice breeding for screening, selection and germplasm collection. The improvement and the use of DNA markers have changed the rice breeding. Simple sequence repeats,

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also known as microsatellites have been shown to be one of the most powerful genetic markers in biology. SSR have been proved to be most powerful genetic marker in biology (Jewell *et al*., 2006). The most important advantage of microsatellites as genetic markers is that they inherited in a Mendelian approach as codominant markers (Miah *et al*., 2013). The most useful genetic marker in plant breeding is has to be highly polymorphic, highly abundance and genomic specific for improving blast resistance. In the other way, the use of resistant cultivars is the most effective and economical way to control rice blast disease and therefore, breeding efforts to develop new resistant cultivars continue to be a priority for rice breeding programs. In attempt to overcome rice yield limitation, improvise high rice yield with resistance to blast disease has become the concern of rice field.

Selecting suitable parents is one of the crucial parts in breeding program. Therefore, it is important to choose good parents to gain functional segregation that follow Mendelian principle. By inclusion one of the local cultivar as parent would help to overcome weakness by the other parents. In this study, Pongsu Seribu 2 is resistance cultivar while MR264 is susceptible but have high yielding trait that can be used to complement each other. Moreover, crossing of both parents would give better recombinants against blast disease especially in early analysis involving segregating progenies. The objectives of this study is to identify and discuss on segregation pattern of resistance to *M. oryzae* pathotype P7.2 in Malaysian resistant local variety Pongsu Seribu 2, MR264, $F_2$ and $F_3$ populations at genotypic and phenotypic level with associated resistance genes linked with SSR markers.

## MATERIALS AND METHODS

Genotyping in $F_2$ population were used plant materials of $F_2$ individuals were used for extraction and genotyping materials. The leaves were extracted by using DNA extraction method according to CTAB method (Doyle and Doyle, 1990; McCouch, 1998) with slightly modification. PCR reaction was carried out in 25 μL reaction mixtures containing 50 ng of template (Genomic DNA), 1.8 mM $MgCl_2$, 0.2 mM of dNTPs, 1 unit of Taq polymerase and 1 μM of each primer in 1x reaction buffer. The amplification was performed with initial denaturation for 3 min 94°C at followed by 35 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 s and a final extension at 72°C for 5 mins, followed by rapid cooling to 4°C prior to analysis. The amplification was done using Mycycler Thermal Cycler (Bio-rad, USA). The electropherograms was documented under UV transluminator using

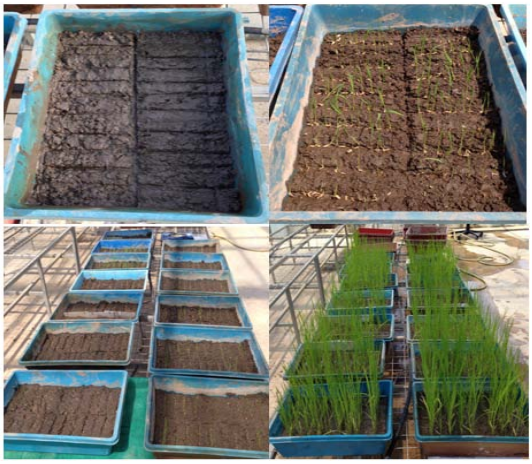

Fig. 1: Rice $F_3$ seedlings growing on plastic trays (36×23×10 cm) for blast screening containing 3 kg of soil with NPK (5 g of 15:15:15)

Molecular Imager Gel Doc XR System (Bio-rad, USA). The molecular weight of PCR products was estimated relative to a 100 bp ladder that served as the size standard. In the present study, the inoculation of the disease with $F_3$ population was used the most virulent *M. oryzae* pathotype P7.2 which were obtained from the University Putra Malaysia (UPM), Serdang. The pathotype used in this experiment was selected based on their virulence towards MR 264 and Pongsu Seribu 2 where MR 264 was susceptible and Pongsu Seribu 2 was resistant to the selected pathotype. The $F_3$ seeds collected from 293 $F_2$ individual were used to evaluate for reactions against *M. oryzae*. Plants were grown in a greenhouse at 25-30°C for 3 weeks until they were at the four-leaf stage (Filippi and Prahbu, 2001). $F_3$ seedlings were developed in greenhouse of Malaysian Nuclear Agency for 3 weeks before transferred to greenhouse in University Technology of MARA at Kuala Pilah branch. Details management practices was followed with standard practices (Fig. 1).

The *M. oryzae* were cultured at room temperature and incubated at 28-30°C for 2 weeks. Potato Dextrose Agar (PDA) was used as a media for growing *M. oryzae*. After two weeks old of *M. oryzae,* spores for inoculation were prepared (Chen, 2001)ith slightly modification as shown in Fig. 2. The entire inoculation experiment was replicated three times for *M. oryzae* P7.2 isolates. The 21 days old plants (10 plants per line) with three or four fully expanded leaves were inoculated by spraying with 25 mL aqueous spore suspension ($1\times10^5$ spores/mL) onto the leaves until run-off using hand sprayer. Inoculated plants were incubated in the greenhouse (controlled environment) at

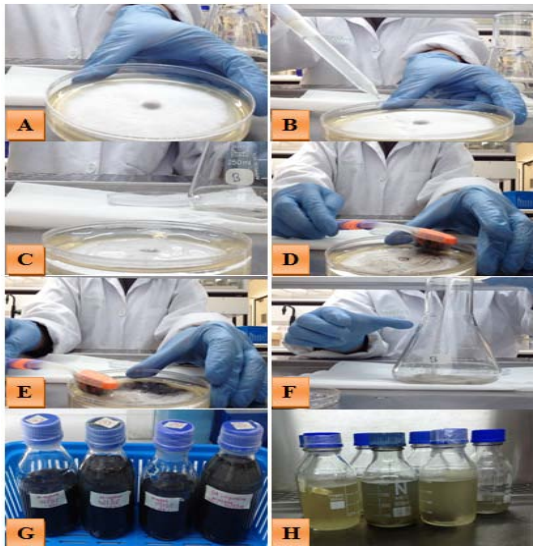


Fig. 2: Inoculum preparation procedures for P7.2 pathotype of (*Magnaporthe oryzae*: a) Non-contaminated fungus was selected; b) 10 mL of sterile RO water was added on the fungus growth; c) The spore was soaked with sterile water; d) Sterile toothbrush was used to taken out the spore; e) Remaining spore was discarded gently with toothbrush until leave the black mark; f) The suspension of fungus was transfer to conical flask; g) The spore suspension was ready to filter and h) The clear spore suspensions were filtered through nylon gauze. Before inoculation 0.05% Tween 20 was added to the suspension to increase the adhesion of the spores to the rice leaves

temperatures ranging from 25-30°C (Filippi and Prahbu, 2001). After inoculation, the trays were covered with a transparent plastic sheet for 36 h to maintain high humidity and high moisture. The plastic sheet was then open daily before 8 a.m. and covered back after 5 p.m.

Disease assessment of genotyping for segregation analysis was by scoring based on the parental bands. A total of 293 $F_2$ progenies were genotyped for SSR marker alleles. The plants that show a pattern similar to the resistant parent alleles was scored as "RR" and those with a banding pattern similar to the susceptible parent alleles was scores as "SS" and the heterozygous plants was scored as "RS". For phenotypic assessment, three components of partial resistance, Blast Lesion Degree (BLD), Blast Lesion Type (BLT) and percentage Disease Leaf Area (% DLA) were estimated and considered as separate traits. The scoring of traits, percentage DLA and BLT was based on selected methods (Correa-Victoria and

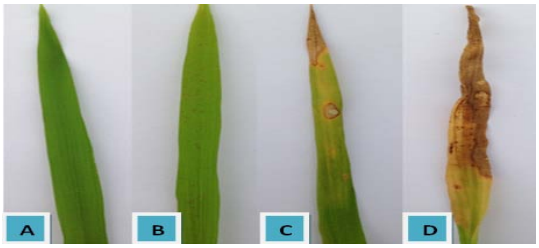


Fig. 3: Blast symptoms evolution: a) Control-uninfected leave from blast disease; b) Visible symptoms appear on the leaves as small brown or grayish dots on the leaves; c) The most common symptom is diamond shaped lesions occur on the leaves after 2-3 with a grey or white centre and d) The infected leave started to die

Zeigler, 1993) oring for BLD trait was carried out based on the Standard Evaluation System (SES) of International Rice Research Institute (IRRI, 1980, 1996) and standardized protocol (Mackill and Bonman, 1992). Figure 3 shows simple blast symptom evolution. The observed segregation ratio of R (resistant) and S (susceptible) was tested for goodness of fit to the expected Mendelian ratio using a $\chi^2$-test (Samuels and Witmer, 1999). Homogeneity among the replicated goodness of fit tests was tested using homogeneity Chi-square analysis (Jayawardana *et al*., 2015). Data from blast test were subjected to an Analysis of Variance (ANOVA) to determine the significant of treatment and experiment repeats by SPSS. Chi-square was tested for each of the SSR bands generated to determine whether the segregation ratio in $F_2$ was significantly different from the expected Mendelian ratio of 1RR: 2RS: 1SS. A data matrix was constructed from polymorphic bands for all $F_2$ individuals. Chi-square analysis for the genotypic ratio was calculated by using the below formula where O is the observed value and E is the expected value:

$$\chi^2 = \frac{\sum O\text{-}E}{E}$$

For single gene model and epistasis, each Chi-square value is considered significant ($p \leq 0.05$) if its value is greater than 3.84 while for two independent genes if it is greater than 7.84. This test applied for analysis goodness of fit for 3:1, 15:1 and 9:3:3:1 ratios. Simple regression analysis was conducted using SPSS for test of association between markers and phenotypes with a threshold significance level of $p = 0.05$ and 0.01. Individual markers explained the variation percentages of phenotypic trait for Blast Lesion Degree (BLD).

Correlation study was tested between the four markers and Blast Lesion Degree (BLD) trait to study the interrelationship between them (Zar, 1999).

## RESULTS AND DISCUSSION

Polymorphism in $F_2$ progenies were tested with twelve SSR markers were used in segregation analysis of $F_2$ population. The markers used were tested for polymorphism before proceed with segregating analysis. The banding patterns of all the markers varied in segregating population (Fig. 4).

Markers segregation data analysis were tested with parental bands were amplified and used as a control to score the alleles in the gel along with the $F_2$ individuals. All markers were used for genotyping assays and evaluated on 293 $F_2$ families developed from crosses of Pongsu Seribu 2 and MR264. A ladder added was used to identify the allele size observed in the parental and progeny survey. The observed segregation ratio for the resistance and susceptibility in $F_2$ lines for 12 polymorphic SSR markers are shown in Table 1.

The Chi-square analysis for 4 SSR markers RM101, RM206, RM413 and RM495 showed a good fit to the expected segregation ratio (1:2:1) for single gene model (df = 2.0, p = 0.05) in Table 1. Table 2 shows in details the marker size and related genes that this experiment used. Evaluation of blast resistance in $F_3$ Population was evaluated with the reaction pattern of the pathotype P7.2 is given in Table 3. Resistant and susceptible reactions were based on a disease reaction scale where reaction scale >3 was considered as susceptible. The resistance and susceptibility of each parental cultivar and $F_3$ families were determined based on the disease reactions using 5 plants randomly choose in each tray with three replicates.

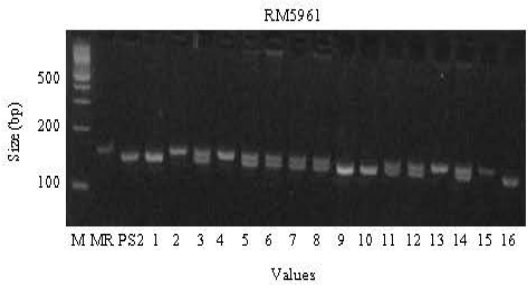


Fig. 4: Marker banding patterns in $F_2$ population of rice derived from Pongsu Seribu 2 (PS2)×MR264 (MR) for SSR marker RM5961 linked to blast resistance genes. Running on 4% agarose gel stained with ethidium bromide only 16 samples plus the two parents (their corresponding parents) for each marker are shown (M = 100 bp ladder)

Pathotype P7.2 was virulent on MR264 while Pongsu Seribu 2 showed strong resistance towards pathotype P7.2. Pongsu Seribu 2 was discovered to be resistance to many Malaysian blast pathotype (Soleiman and Nasser, 2014; Rahim *et al*., 2013).

Resistance traits segregation in $F_3$ Population were evaluated by testing the single gene model that assumed by 25% of the $F_2$ population present as susceptible parent while 75% was present like resistant parent plant (Ashkani *et al*., 2011). Segregation ratios for 3:1 in the $F_3$ population inoculated with Pathotype P7.2 is shown in Table 4. From data analysis of Chi-square goodness of fit, $F_3$ families was segregated in a 3:1 (R:S) ratio ($\chi^2_{3:1} = 1.239$, df = 1 at p = 0.266). This result may suggest that there may be a single resistance gene segregating against pathotype P7.2. Effects of markers on disease severity and correlation between markers and BLD trait were tested.

Table 1: Segregation in $F_2$ generation derived from cross between Pongsu Seribu 2 and MR264

| | | Marker analyzed | | | | |
|---|---|---|---|---|---|---|
| Markers | Chr | RR | RS | SS | $\chi^2$ (1: 2: 1) | p-values |
| RM101 | 12 | 76 | 131 | 86 | 3.962 | 0.138 |
| RM168 | 3 | 92 | 124 | 77 | 8.447** | 0.015 |
| RM206 | 11 | 80 | 137 | 76 | 1.341 | 0.511 |
| RM413 | 5 | 87 | 144 | 62 | 4.352 | 0.114 |
| RM495 | 1 | 86 | 141 | 66 | 3.143 | 0.208 |
| RM5961 | 11 | 47 | 143 | 103 | 21.573** | 0.000 |
| RM18 | 7 | 160 | 91 | 42 | 137.096** | 0.000 |
| RM259 | 1 | 90 | 127 | 76 | 6.529* | 0.038 |
| RM261 | 4 | 120 | 126 | 47 | 42.113** | 0.000 |
| RM3 | 6 | 86 | 149 | 58 | 5.437* | 0.066 |
| RM333 | 10 | 126 | 123 | 44 | 53.437** | 0.000 |
| RM85 | 3 | 123 | 115 | 55 | 45.109** | 0.000 |

Table 2: SSR markers size and genes linked related

| Markers | Exp. size (bp) | Obs. size PS2 (bp) | Obs. size MR264 (bp) | Linked genes |
|---|---|---|---|---|
| RM101 | 324bp | 280 | 300 | Pi-157(t), Pi-6(t) |
| RM206 | 147 | 150 | 148 | Pi-kh, Pi-44 |
| RM413 | 79 | 80 | 70 | Pi-26 |
| RM495 | 159 | 170 | 157 | Pi-t |

PS2 = Pongsu Seribu 2, Exp. = Expected, Obs. = Observed

Table 3: Trait variation of pathotype P7.2 in challenged in $F_3$ plants

| Pathotype | Trait | Mean | SE of mean | SD | N |
|---|---|---|---|---|---|
| P7.2 | BLD | 2.360 | 0.129 | 2.2020 | 293 |
| | BLT | 2.660 | 0.078 | 1.3350 | 293 |
| | DLA (%) | 24.15 | 1.372 | 23.491 | 293 |

BLD = Blast Lesion Degree; BLT = Blast Lesion Type; % DLA = percentage disease leaf

Table 4: Segregation ratios for 3:1 in the $F_3$ population inoculated with Pathotype P7.2

| | Pathogenicity assay | | | | |
|---|---|---|---|---|---|
| Population | Reaction | Exp | Obs. | $\chi^2$ (3:1) | p-value |
| $F_3$ lines | Resistant (R) | 219.75 | 228 | 0.301 | - |
| | Susceptible (S) | 73.25 | 65 | 0.928 | 0.266 |
| | Total | 293.00 | 293 | 1.239 | - |

Table 5: Molecular markers statistics associated with blast resistance for Blast Lesion Degree (BLD) trait in the $F_3$ families

| Trait | Marker | SMR ($R^2$) | SMR (F) | $R^2$ (%) | p-values |
|---|---|---|---|---|---|
| BLD | RM101 | 0.000 | 0.96000 | 0 | 0.328 |
| | RM206 | 0.042 | 13.9370 | 4 | 0.000 |
| | RM413 | 0.027 | 9.15800 | 3 | 0.003 |
| | RM495 | 0.534 | 335.279 | 5 | 0.000 |

SMR = Single Marker Regression; % $R^2$ = proportion of the total phenotypic variance accounted for the markers; p-value = the probability of an association

Table 6: Correlation between SSR markers and blast Lesion degree

| Markers | RM101 | RM206 | RM413 | RM495 | BLD |
|---|---|---|---|---|---|
| RM101 | 1 | -0.062$^{NS}$ | 0.069$^{NS}$ | -0.010NS | -0.057$^{NS}$ |
| RM206 | | 1 | 0.085$^{NS}$ | 0.136* | 0.214** |
| RM413 | | | 1 | 0.119* | 0.175** |
| RM495 | | | | 1 | 0.732** |
| BLD | | | | | 1 |

* = significant at ($p<0.05$); ** = highly significant at ($p<0.01$); NS = Not Significant

Marker trait association analysis showed there is good association between genotypes of four SSR markers RM101, RM206, RM413 and RM495 with blast resistance in resistant plant in segregated $F_2$ population. These markers have resistance effects about 12% for phenotypic variation to a given pathotype. Single point analysis of variance or single marker regression analysis revealed the significant ($p<0.05$) associations between markers and blast resistance (Table 5). Table 6 shows relationship between four markers with phenotypic trait, Blast Lesion Degree (BLD). SSR marker of RM495 was highly correlated with BLD which is significant at 0.01 ($p = 0.732$), followed by RM206 ($p = 0.214$ at 0.01) and RM413 ($p = 0.175$ at 0.01) while RM101 was not correlated with any of the markers and BLD. This shows that there was a strong interrelationship between the selected phenotypic and the four markers in this study.

Molecular marker can facilitate breeding program and made selection of parents is more efficient and accurate. This study was to be used in segregating $F_2$ populations and evaluates the relationship between SSR marker and important trait of rice disease. Parental cultivars demonstrated a high polymorphism and there is no crossing incompatibility. Parental combinations of the crossing plant were choosing base on their differences of morphological traits that fit this study. Segregation data were analyzed by the $\chi^2$-test. For the single gene model, Chi-square value was considered to be significant ($p = 0.05$) if its value was greater than 3.84. Genetic analysis with $\chi^2$-test indicated goodness of fit to the expected ratio of 1:2:1 for single gene model representing the association of RM101, RM206, RM413 and RM495 with blast resistant gene in the $F_2$ population. Another 8 markers were not in agreement with the expected Mendelian ratio. The Chi-square method would allow measure goodness of fit between observed and expected values obtained within the study of population. This study with an agreement from others studies agreed that RM206 was polymorphic and linked to Pi-kh blast resistance genes in 17 varieties (Strickberger, 1976) while other study using RM206 linked to Pi-kh blast resistance genes as check variety in Tetep (Sharma *et al*., 2005). RM413 was related with blast resistant gene clearly differentiated some of the resistant and susceptible varieties. This study also supported by other researcher using similar SSR markers in segregating analysis. Another recorded, 3 polymorphic SSR markers of RM413, RM101 and RM206 were showed a good fit to the expected segregation ratio and linked to the above genes (Rahim *et al*., 2013). This confirmed that the resistance for blast in present survey was control by single dominant gene. Analysis of selected SSRs markers in $F_2$ segregating populations indicated that these markers correlated to significant resistance for phenotypic variation to *M. oryzae*. These SSR markers had high accuracy for resistant plant and can be used for further study on this cultivar.

The goodness of fit for 3:1, 9:3:3:1 and 15:1 segregation ratios between observed and expected distributions were tested using Chi-square analysis. The result showed from the expected number of resistant and susceptible plants to pathotype P7.2 in the segregation ratio for a single gene model was significant and fitted as 3:1 segregation ratio ($\chi^2 = 1.239$, $p = 0.266$). This study shows that the observed segregation ratios in $F_3$ in the greenhouse screening for pathotype P7.2 were mostly consistent with a single dominant gene model. Analysis on the Chi-square good of fitness shows 9:3:3:1 and 15:1 segregation ratios did not segregate in a ratio for plants with resistant to susceptible, respectively. This result suggests that there may be a single specific resistance gene segregating against pathotype P7.2 in the $F_3$ families. Segregation pattern signifying did not influence by mild effect however the blast resistance in Pongsu Seribu 2 is solely due to nuclear gene. These results imply that the resistance disease was controlled by the same locus found in both resistance parent and in $F_3$ families. The complement of race specific of resistant genes may cause by the durability of the resistant in Pongsu Seribu 2 cultivar. The resistance may not be giving any specific effect due to the small buildup of the genes within the genome. The complement of the resistant genes could be due to the donor genes from Pongsu Seribu 2 that is carried highly resistant genes. Study from previous researcher also agreed that resistance genes is depending on the donor cultivar and the specificity of *Magnaporthe oryzae* isolates and the effectiveness to the specific host on specific strains that governed by single

gene or polygene (Sharma *et al*., 2005). Even earlier study done (Zhou *et al*., 2007) for IRRI revealed that most of the traditional varieties generally have one or two dominant gene, this study particularly found that Pongsu Seribu 2 was resistance to blast disease and controlled by a single gene. Another study also proved that the resistance to blast disease is governed either by a single gene or moderate and polygenic resistance, depending on the genotypes or cultivars (Rahim *et al*., 2013; Mackill *et al*., 1985; Latif *et al*., 2008). These findings showed that the observed segregation ratios in $F_3$ were probable work as a single dominant gene. These results were in good agreement with the earlier work where the inheritance of the blast disease was studied by crossing resistant variety Pongsu Seribu 2 with susceptible variety Mahsuri (Rahim *et al*., 2013; Latif *et al*., 2013). Another studies agreed that the segregation ratio (3:1) suggested a single specific resistant gene segregating against BPH (Mackill *et al*., 1985; Latif *et al*., 2008; Correa-Victoria and Zeigler, 1993).

## CONCLUSION

In conclusion, the genetic analysis showed a good fit to the expected segregation ratio of 1:2:1 for a single gene model which proved the hypothesis of a single dominant gene for Pi-157(t), Pi-6(t), Pi-kh, Pi-26 and Pi-t loci. Chi-square analysis showed a good fit to a ratio of 3:1 for the segregation of resistance and susceptibility for *M. oryzae* of blast disease resistance. This study implies that single specific resistance gene segregating against pathotype P7.2 in the $F_3$ families is governed either by a single gene or moderate and polygenic resistance. SSR markers of RM101, RM206, RM413 and RM495 were significantly associated with blast resistance to *M. oryzae* in rice. These markers were highly accurate for resistant plant with resistance effect about 12% for phenotypic variation. This study also shows that there was a strong interrelationship between the selected phenotypic and the four markers in this study. Therefore, this research offered very useful information for local rice breeders in developing blast disease resistant rice cultivar. By expanding this study, categorizing of SSR markers has facilitated in identification of the markers closely related to the specific genes and help assess germplasm and utilize the breeding resources. This information would give prospect for the rice world against blast resistance disease that responsible for the losses of the rice production yield.

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