ANALYSIS OF RECURRENT PARENT GENOME RECOVERY IN MR264 x PONGSU SERIBU 2 USING MARKER-ASSISTED BACKCROSSING

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ABSTRACT: *Marker-assisted backcross (MABC) is a common breeding technique used for introgression a blast resistance gene into a rice cultivar. It is considered as an advanced method to overcome the drawbacks of conventional backcross technique and fasten the recovery of the recurrent parent genome (RPG). The MABC technique was implemented to produce improve blast-resistant rice variety by introgressing a blast-resistant gene(s) from a traditional variety possess resistant genes (donor), Pongsu Seribu 2 into a local susceptible variety, MR264. The estimation of recurrent parent genome recovery in an earlier generation of backcrossing was analyzed using simple sequence repeat (SSR) markers. Parental polymorphism analysis with 375 SSR markers showed that only 70 of them were found to be polymorphic. In BC_1F_1 and BC_2F_1 generation, background analysis revealed 76.1 to 87.9% and 86.5 to 95.2% of recurrent parent genome recovery, respectively. In selected BC_2F_2 lines, the average proportion of recurrent genome recovery was 94.4% which showed a phenotypic similarity to MR264. In this study, seven improved lines associated with blast resistance genes and maximum genetic backgrounds of MR264 were identified as improved blast resistant variety. Findings in this study proved the efficiency of the MABC technique for the rapid recovery of a parental genome in a 2-3 generation of backcrossing population.*

Keywords: Blast; Background selection; Marker-assisted backcrossing breeding; SSR marker; Pongsu Seribu 2

1. INTRODUCTION

Rice is a primary source of nourishment that consists of carbohydrates, numerous vitamins, and minerals, which contributes over 30% of calorie intake for almost 50% of the global population [1]. However, *Magnaporthe oryzae*, *a* fungal pathogen for rice blast disease caused tremendous losses of yield production and plant growth due to the extensive range of biochemical, molecular, and physiological modification of the plants [2]. New improved blast resistant variety is the most significant method to fulfill the increasing feed-demand and challenge to food security. Generally, backcross breeding had been adopted to transfer a favorable gene from a poor agronomy donor genotype into an attractive recipient elite genotype [3]. This conventional breeding technique is aimed to modify the existing variety with introgression of a valued gene such as disease resistance. Phenotypic selection conducted at a certain growth stage which requires large samples and affected by environmental factors makes this breeding technique have multiple barriers. Numerous backcrosses required to be performed but generally between six to eight with approximately 3 to 4 years of breeding work. Despite that, numerous modern genotypes have been developing which were still used in this present day. The advancement technology of the molecular marker has revolutionized the varietal development by using genotyping tools for the selection process [4]. Marker-assisted backcrossing breeding (MABC) is a breeding approach that applies a selection technique through the advantage of a molecular marker associated with the desirable gene. Screening based on genotype could be done in an earlier generation, thus shorten the breeding time required for the development of ideal genotypes and reconstitute the original variety as rapidly as possible [5]. It has been considered as a highly powerful breeding technique that requires only a short period of time to develop new varieties and reduces the breeding cycle length by two important selection steps; (1) foreground selection, including screening of the donor parent at the desirable locus with marker allele and (2) background screening in which breeders screening in the entire genomic region excluding the target locus for recurrent parent marker alleles [6]. Currently, stringent phenotypic selection has generally been a couple with MABC in each backcrossed generation ensures screening of plants with targeted alleles with the highest RPG [7]. The efficiency of MABC influence by several factors including the availability of foreground markers, number of background markers, and size of the backcross population [8]. According to [9], well-placed markers (2 to 4 per chromosome of 100cM) could provide sufficient analysis of the genome in backcross programs and may contribute to the success of recovery RPG in the backcross generations. The first successful experiment reported by [10] who successfully introgress the bacterial blight resistance gene into Chinese hybrid rice. To date, the backcrossing method has been widely used to incorporate desirable genes by using a molecular marker for the development of new improved variety from wild type. MR264
is a local rice variety with high-yielding potential (7245 kg/ha), short maturation period (113 days), and semi-dwarf plant stature [11]. However, MR264 had not been released to cultivate by farmer due to high susceptibility to fungus, *M. oryzae*. With the consideration that MABC is a powerful breeding method, the purpose of this study was to convert the MR264 into an improved blast resistant variety with MR264 genetic background through MABC using Malaysian traditional variety, Pongsu Seribu 2. In addition, the acceleration of the recurrent parent genome was calculated in

backcross populations derived from MR264 and Pongsu Seribu 2

2. EXPERIMENTAL DETAILS

2.1 Planting materials and breeding strategy

BC_1F_1 seeds were produced from a backcross method between MR264 and four F_1 plants carrying *Pi*-gene(s). BC_1F_1 plants were screened with foreground selection using tightly linked markers for blast resistance genes. Plants linked with resistance genes and maximum background recovery of MR264 were backcrossed with MR264 to produce BC_2F_1 seeds (Figure 1). Foreground, background, and phenotypic screening were performed in all backcrossed generation to select elite plants.

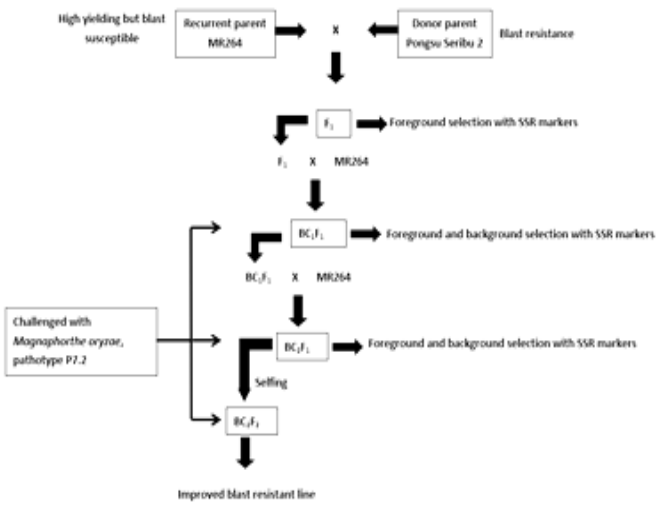


Fig (1) Development of improved blast resistant lines using marker-assisted backcrossing scheme

2.2 Analysis of molecular marker

Gramene website (http://www.gramene.org) and [12, 13] were applied to extract the information on the SSR primer-linked with blast resistance. F1 and the backcrossed generation were identified by using twelve markers associated with blast resistance. Polymorphism surveys between MR264 and PS2, recurrent and donor parent, respectively were screened with 375 SSR markers across the 12-rice chromosome. A minimum of four markers per chromosome was considered to perform background screening to recover the recurrent genome.

2.3 DNA Extraction and PCR Amplification

Tissue Lyser machine (Qiagen, Germany) were used to extract the genomic DNA at 30Hz for 4 minutes. Nano-drop spectrophotometry (ND1000 Spectrophotometer) was used to quantify DNA. PCR amplification was performed following a program of 30 cycles of 94°C for 1 min at denaturation, 1 minute at 55°C for annealing, 72°C for 2 min for polymerization, 72°C for 7 min for final elongation and allow to fast cooling at 4°C prior to analysis. Molecular Imager® (GelDocTM XR, Bio-Rad) was used to visualize the PCR product.

2.4 Phenotypic screening

The plant linked with blast resistance gene(s) with maximum phenotypic similarity to MR264 were screened at the vegetative and flowering stage. The phenotypic screening was conducted in BC_1F_1 and BC_2F_1 and BC_2F_2 generation after foreground screening. Data were recorded for agronomic traits. Individuals with the maximum phenotypic ranking with MR264 were used selected to produce backcrossed seeds. A similar procedure was used to screen individual plants with a blast resistance gene.

2.5 Data Analysis

Marker data were scored manually based on the high intensity of microsatellite bands. For foreground selection, plants were marked as R if the banding patterns were similar to the resistant parents' alleles; those exhibiting a similar pattern to susceptible plants were marked as S. For background analysis, Graphical Genotyper software was used to analyze the proportion of markers by scoring %A for homozygous recipient parents and %B for donor alleles and % H for the heterozygous plant. Independent t-test was used to compare the mean difference of agro-morphological data between the parent MR264 and blast-resistant improved using SPSS 16.0 software. 2.

3. RESULTS AND DISCUSSION

3.1 Foreground and background polymorphic SSR markers

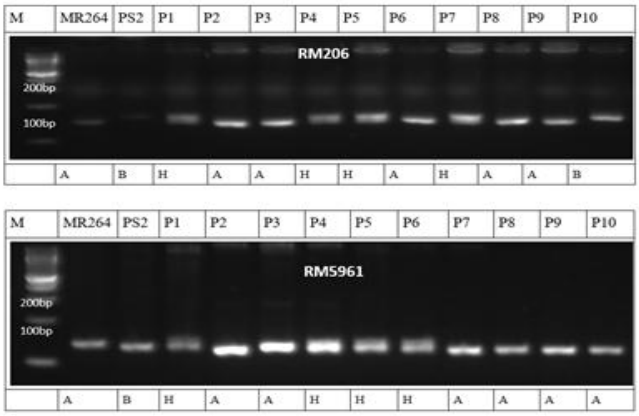
Seventy-two polymorphic markers distribute across 12 rice chromosomes were identified which 11 of them were polymorphic markers that associated with blast resistance genes. Targeted gene together with the recovery of the recurrent parent in backcrossed generations, BC_1F_1 and BC_2F_1 were analyzed using all polymorphic markers. Polymorphic markers are essential because it can differentiate between two parental genotypes [14]. Monomorphic markers were discarded in the screening process because it is not useful. This present study demonstrated that the ratio of polymorphic markers (19.2%) was in agreement with the study of [15] screened 435 SSR markers and found 72 (16.55%) markers polymorphic throughout 12-rice chromosomes. The ability to find many polymorphic markers distributed across a 12-rice chromosome could give a vast potential to restore recurrent parent segments rapidly by reducing the number of backcross generation required with the targeted genes.

3.2 Foreground and background polymorphic SSR markers

3.2.1 Foreground selection of blast resistance gene

By using a foreground marker, selection can be made at the reproductive stage and seedling stage, which allow only the best plant to proceed to the backcrossing. Two DNA markers were used in this study; RM206 closely associated with *Pi-kh* and RM5961 tightly linked with *Pi*-7(*t*)) positioned at the long arm chromosome 11 on 79.90cM and 102.90cM respectively [16], [17], [18], [19] and [20]. Four best F_1 plants that carried the targeted blast resistance genes were backcrossed with MR264 to produce 136 BC_1F_1 plants.

Based on two foreground markers, RM206 and RM5961 which were associated with *Pi-kh* and *Pi*-7(*t*) blast resistance gene, 23 heterozygous plants were selected (Figure 2). Another ten foreground markers exhibited negative results indicating that a few blast resistance genes were deleted in the backcrossing program with MR264.



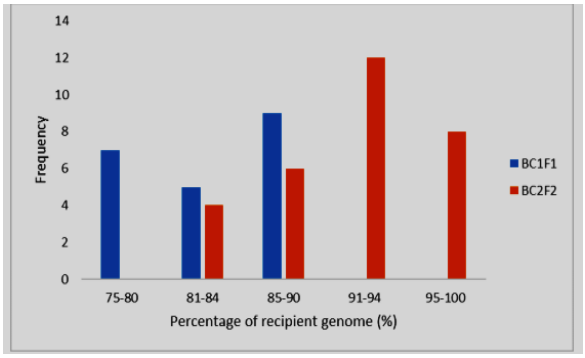
Fig(2) Banding pattern in the BC_1F_1 population using foreground markers; RM206 and RM5961 (M=100 bp DNA ladder)

3.2.2 Recovery of recurrent parent genome

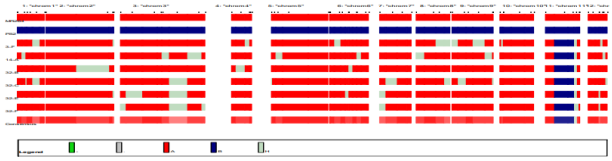
With the advent of genotyping technology, graphical genotyping can be used to helps breeders to visualize the genotype of individuals and populations [21]. It will display the specific chromosome for the parental origin and allele composition according to the arrangement and coded allele in map order across the entire genome. It is considered as an available software package that assists in the screening process and assessment of plant material with the illustration of genotyping data [22].

The number of markers varied from the lowest number, 4 (chromosomes 2, 7, and 8) to the highest number of markers per chromosome, 10 (chromosome 6). The percentage of recurrent genome segment restores varied from 76.1% to 87.9% in BC_1F_1 generation. The recurrent parent segment recoveries of plants in the BC_1F_1 generation are shown in Figure 3. The average RPG recovery in the BC_1F_1 generation was 81.6%. [23] found a similar result by using Vietnam elite variety with 80% to 89.01%. The majority of the residual segments were distributed over chromosome 11 which indicates the introgression of the donor segment associated with blast resistance genes. This result supported by the finding of [24] who reported the average recovery of the recurrent parent genome in BC_1F_1 in Swarna and Samba Mahsuri with 83% and 85% respectively.

All the chromosomes in the BC_1F_1 improved line consisted of heterozygous segments (Figure 4). Background screening demonstrated that the residual segment of the donor parent resembles on the chromosome no. 11. Six selected the BC_1F_1 plants were selected based on the foreground and background analysis to produce BC_2F_1 generation.



Fig(3) Recovery of recipient parent segment in the BC_1F_1 and BC_2F_1 population derived from MR264 x Pongsu Seribu

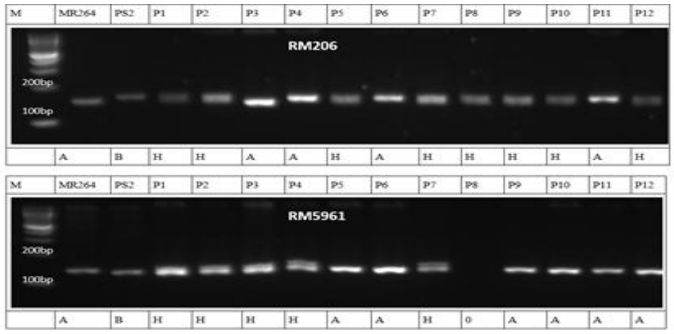


Fig(4) Distribution of recipient genomes of the six best individuals in the BC_1F_1 generation.

3.3 Genotyping the BC_2F_1 generation

3.3.1 Foreground screening of blast resistance gene

In BC_2F_1 generation, 103 plants confirmed the incorporation of, *Pi-kh* and *Pi7(t)*, blast resistant genes with two foreground markers, RM206 and RM5961 respectively (Figure 5). Six plants were selected to develop the BC_2F_2 population based on the targeted genes and maximum agro-morphological similarity to MR264.



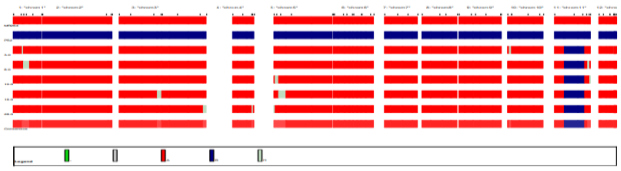
Fig(5) Banding pattern in the BC_2F_1 population using foreground markers; RM206 and RM5961 (M=100 bp DNA ladder)

3.3.2 Recovery of recurrent parent genome

Thirty plants were analyzed from the result of foreground selection. A greater number of RPG was observed in BC_2F_1 plants ranged from 86.5% to 95.2%. These results also in correlation with findings of [25] who found 97% RPG in Swarna and 95% of RPG in Samba Mahsuri using BC_2F_1 generation. Great acceleration of RPG recovery was achieved in two backcrossed generations compared to the conventional

methods. Compared to the conventional backcross, the result of this study showed the maximum percentage of recurrent genome recovery with only 2 generations. The success of this study is influence by the number of polymorphic markers used (at least 4 markers per chromosome) and evenly distributed across 12 rice chromosomes. A well-placed marker (2 to 4 per chromosome of 100cM) could provide sufficient analysis of the genome in backcross programs and may contribute to the success of selection making. If the number of polymorphic markers is more, marker-assisted selection could be very effective. However, with 19.20% of polymorphic markers between PS2 and MR264 used in this study, the background recovery was still adequate. The present finding proves that the estimation of recurrent parent genome in each backcross strongly enhances the reduction of linkage drag which distributed across genome carried by donor parent.

In this study, the six best plants 3-F-3, 3-F-8, 3-F9, 3-F-10, 3-F24, and 3-F-28 were chose based on foreground, background, and phenotypic selection. The entire chromosome segments of the recurrent alleles' recoveries of the best six plants are presented in Figure 6. Among the six plants, chromosomes 2, 8, and 9 were fully recurrent types. Plant 3-F (BC_1F_1) and 3-F-10 (BC_2F_1) were the best plant and the recovery of this plant is shown in Figure 7(a)(b).



Fig(6) Six best plants recurrent genome restoration in BC_2F_1 generation.

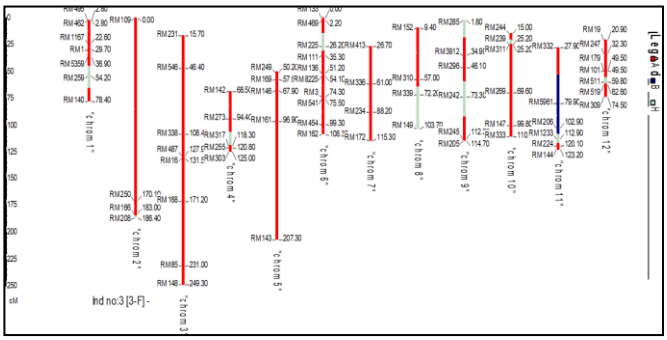
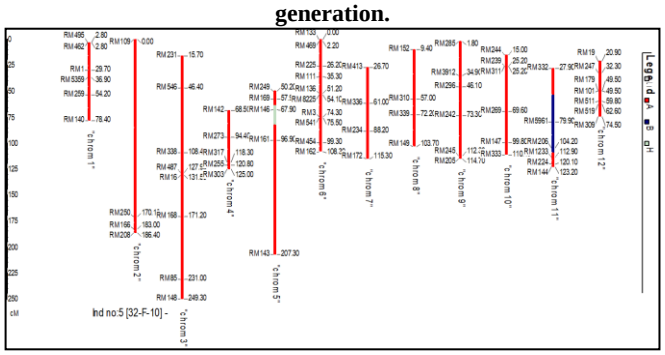


Fig (7a) Highest recurrent segment of plant 3-F in BC_1F_1 generation.



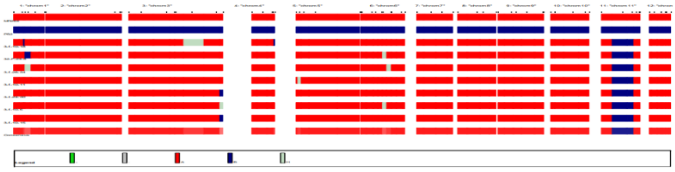
Fig(7b). A highest recurrent segment of plant 32-F-10 in the BC_2F_1 generation. (Red color = homozygous segments, MR264, blue color= homozygous segments, Pongsu Seribu 2 and light grey color= heterozygous segment)

3.4 Foreground selection of blast resistance gene in BC_2F_2

Plants appearing phenotypically similar to the MR264 background with homozygous resistant alleles were selected using RM206 and RM5961 markers. Finally, 7 lines were selected as BC_2F_2 improved blast resistant lines among 36 plants.

3.5 Recipient parent genome (RPG) in improved lines

Background selection of advanced blast resistant lines covering 1357.6 cM was constructed with an average of 18.85 cM regions per marker of the *O. sativa* genome. The distribution of the donor segment was observed to be dominant on chromosome eleven. Complete recovery of the recurrent parent chromosome was observed on most rice chromosomes except on chromosome three, four, eleven, and twelve (Figure 8). The average proportion of the recurrent genome in selected improved lines was 94.4%, indicating the maximum similarity at the phenotypic level with a recurrent parent, MR264.



Fig(8) Distribution of recurrent genome of the six-best individual in the BC_2F_2 generation

3.6 Performance of agro-morphological traits between improved lines and parent, MR264

To certify the presence of a targeted gene and reducing genotyping error, phenotypic selection must be coupled with marker-assisted selection [26]. Agronomic characteristics normally play a part in backcross breeding and have been used to develop a new variety. Agronomic characteristics were first introduced by [27] for the selection of superior yield plants.

The agro-morphological trait between improved lines carrying *Pi-kh* and *Pi7*(*t*) blast resistance gene was compared and measured with a recurrent parent, MR264 (Table 1). The results in this study demonstrated significant differences between agro-morphological traits. Mean values of blast resistant lines introgressed with *Pikh* and *Pi7*(*t*) genes for all agronomic traits excluding 50% flowering days were likely identical with the recurrent parent; in which MR264 revealed that the capability of improved lines is identical to MR264 for tested traits. However, the 50% flowering day showed a minor variation between improved lines and MR264. Furthermore, it would be a tremendous achievement to decrease yield losses in blast disease endemic region and increase yield productivity with the cultivation of improved blast resistant lines developed in this study.

Table 1. Agro-morphological trait between improved lines and the recipient parent, MR264

Traits	MR264 (n=7)	BC_2F_2 lines (n=7)
Days of 50% flowering (day)	85.71 [a] ±0.95	84.42 [b] ±0.78
Plant height (cm)	83.57 [a] ±2.64	83.14 [a] ±0.89
Days to maturity (day)	111.57 [a] ±1.51	114.0 [a] ±1.0
Total tiller /hill (no)	23.85 [a] ±1.67	24.85 [a] ±2.11
Effective tiller/hill (no)	22.71 [a] ±1.11	23.28 [a] ±.1.25
Panicle length (cm)	24.57 [a] ±0.97	24.42 [a] ±0.53
Total grain/panicle (no)	176.42 [a] ±14.78	185.85 [a] ±14.06
No. of filled grains/panicle (no)	158.85 [a] ±13.03	168.14 [a] ±15.86
Seed setting rate (%)	90.04 [a] ±0.5	90.4 [a] ± 0.34
1000 grain weight(gm)	23.35 [a] ±0.80	24.0 [a] ±0.76
Yield/plant (g)	39.71 [a] ±0.26	39.9 [a] ±0.28
Grain length (mm)	9.77 [a] ±0.21	9.57 [a] ±0.53
Grain width (mm)	1.92 [a] ±0.075	1.97 [a] ±0.04
Grain length/width	5.06 [a] ±0.17	4.86 [a] ±0.35
Flag leaf length (cm)	44.21 [a] ±1.14	44.5 [a] ±0.64
Flag leaf width (cm)	1.35 [a] ±0.09	1.42 [a] ±0.05

Mean± SE with different letter indicates the significant differences and independent t-test with a 5% level of significance: n=7

4. CONCLUSIONS

The improved BC_2F_1 lines carrying blast resistance genes (putative *Pi-kh* and *Pi*7(*t*)) with the maximum genetic background of MR264 were demonstrated similar agronomic performance than a recurrent parent, MR264. Therefore, the MABC is a powerful breeding technique to restore the genetic background of a recurrent parent in a smaller number of backcross generation and the improved lines could be used by future breeders and genetics in developing durable resistant variety in Malaysia.

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