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Cloning and characterization of two major blast resistance genes *Pi-b* and *Pi-kh* from Malaysian rice variety Pongsu Seribu 2

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Abstract

Pongsu Seribu 2 (PS2) is a land race traditional rice variety grown in Malaysia having broad spectrum resistance to blast disease caused by *Magnaporthe oryzae*. The information on genetic basis of blast resistance in this cultivar is still lacking. In order to ensure the resistance of Pongsu Seribu 2, virulent pathotype of *M. oryzae* P7.2 was inoculated on Pongsu Seribu 2 and MR219 (susceptible used as control) where Pongsu Seribu 2 was found strongly resistance and MR219 susceptible. To determine the mechanism of resistance in PS2 cultivar, cloning and characterization of major blast resistance genes, *Pi-b* and *Pi-kh* were carried out. Primers covering the *Pi-b* and *Pi-kh* nucleotide sequence available in NCBI database were designed that successfully amplified the PCR PCR product. The amplified product was ligated with PGEM-T easy vector and cloned the blast resistance genes fragment into competent cell. The results revealed that nucleotide sequence contain an open reading frame (ORF) and the same is also highly conserved in nature. Deduced amino acid sequence indicates that *Pi-b* contains zinc finger-containing protein domain and *Pi-kh* have Leucine rich repeat domain. The translated nucleotide sequence into amino acid produces significant homology average 76.8% with *Pi-kh* and 93.4% with *Pi-b* blast resistance genes present in different cultivars of rice. The amino acid sequence of both genes also showed homology with NBS-LRR (Nucleotide binding site-Leucine rich repeat), proteins and BAC clones covering the chromosome 2 and 12 of rice with different cultivars. This study indicates that Pongsu Seribu 2 contain at least two dominant genes, *Pi-b* and *Pi-kh* involved for providing resistance against *M. oryzae* pathotype P7.2.

Keywords: *Magnaporthe oryzae, Pi-b, Pi-kh*, Leucine rich repeat, NBS-LRR.

Introduction

Rice is one of the most important staple food crops contributing a major role in the world food security. However, it is seriously affected by various biotic and abiotic stresses that reduce its maximum yield production. Among biotic stresses, blast disease caused by *Magnaporthe oryzae* is of major concern because it damages rice crop at every stage. The management of blast disease is quite difficult due to diversity in an isolate of *M.oryzae* and variability in pathogenicity (Sharma et al., 2002). Moreover, the blast resistance in rice cultivars remained for very short time in the field especially when resistance is based on a single gene. Therefore, development of blast resistant cultivars becomes the first priority on the demand of farmers and growers throughout the world. The utilization of resistant (*R*) genes is considered as very effective and environment friendly method to control the blast disease. Durable resistance is governed by combining multiple *R* genes into a single variety conferring resistance to different isolates of *M. oryzae* (Hittalmani et al., 2000). The continuous identification of new *R* genes is needed to utilize it as genetic resource. In Malaysia, blast disease caused severe yield lose in different rice growing areas including Kedah and Kelantan where 60% rice cultivated area was destroyed (Rahim 2010). Pongsu Seribu 2 is a blast resistant rice variety grown in Malaysia having broad spectrum resistance against different isolates of *M. oryzae* (Rahim et al., 2013). Several QTLs (Quantitative trait loci), associated with leaf blast resistance have been

identified and mapped from Pongsu seribu 2 (Ashkani et al., 2013). Unfortunately, no single blast resistance gene have been cloned and characterized from Pongsu Seribu 2 cultivar. Another Malaysian rice variety MR219 is very high yielding, having slender grain and short maturation period but it is susceptible to blast disease (Fasahat et al., 2012). The genetic dissection of blast resistance genes revealed that blast is control by NBS-LRR (Nucleotide binding site-Leucine Rich repeat) protein, one of the largest families providing resistance against various viral, bacterial and fungal diseases (Leah et al., 2006). The main function of NBS domain is to control the cell death through nucleotide binding and hydrolysis whereas, LRR help to determine the resistance specificity (Takken et al., 2006). The molecular mechanism of resistance that how plant defense during host- plant interaction can be understood through cloning of blast resistance genes. More than 100 blast resistance genes have been identified and around 74 have been mapped on different rice chromosomes (Koide et al., 2009). About 23 blast resistance genes *Pi-km, Pi5, Pid3, pi21, Pit, Pb1, Pish, Pi-k, Pik-p, Pia, NLS1, Pi25, Pi54rh and Pid3-A4, Pib, Pita, Pi54, Pi-9, Pid2, Pi2, Piz-t, Pi-36, Pi-37,* have been cloned by different cloning strategies and molecularly characterized (Sharma et al., 2012). Among cloned blast resistance genes, *Pi-b* (Wang and Yamanouchi 1999), *Pi-kh* (Sharma et al., 2005) and *Pi-ta* (Baryan et al., 2000) have been molecularly characterized from different rice *indica* cultivars. The majority of blast resistance genes are found on chromosomes 6, 11 and 12 on rice chromosomes (Liu et al., 2010). The availability of sequence of these blast resistance genes publically provides an opportunity to clone and identify these genes within the local cultivars. The objective of current study was to identify the *Pi-b* and *Pi-kh* blast resistance genes from Pongsu Seirbu 2 variety which is highly resistant against different isolates of fungus pathogens in Malaysia.

Results

Disease reaction of M. oryzae pathotype P7.2 against Pongsu Seribu 2 and MR219

Pongsu Seribu 2 cultivar which is resistant to fungal isolate P7.2 was considered as positive control, while MR219 which is susceptible was taken as negative control. The evaluation of two cultivars was based on the disease assessment score. The disease was scored as resistant (R) and susceptible (S). The cultivar showing score greater than 3 were considered as susceptible and less than 3 were considered as resistant. For calculating the resistance and susceptibility, three plants were placed in one tray with at least 4 repeats as recommended by Valent (1997). Statistically the reaction scale in PS2 and MR219 deviated from the normal distribution (Shapiro-Wilk normality test, $p < 0.001$). According to Figure 1, blast disease reaction scale skewed towards left in P7.2 showing that PS is resistant against the pathotype P7.2 whereas in MR219 it deviated towards the right showing that MR219 is susceptibile. The disease reaction score showed that Pongsu Seribu 2 is strong resistant rice variety while MR219 cultivar is highly susceptible. The frequency of leaf blast lesion is shown in Fig 1.

Cloning of Pi-b and Pi-kh fragment from Pongsu Seribu 2

To determine the presence of *Pi-b* and *Pi-kh* blast resistance genes, we successfully cloned 1362 bp of *Pi-b* gene and 1583 bp of *Pi-kh* from genomic DNA of Pongsu Seribu 2 variety. *Pi-b* and *Pi-kh* are dominant blast resistance genes conferring resistance to different isolates of *M. oryzae* (Wang et al., 1999; Sharma et al., 2005). In order to get high quality sequence, cloned product was sequenced in both directions. The 1 kb ladder was used to confirm the size of our desired segment of *Pi-b* and *Pi-kh* blast resistance genes. The cloned product was separated on 1 % metaphor^{TM} agarose (Lonza) gel. The homology for sequence was search in BLASTn NCBI gene bank data base. Twenty six sequences showed high similarity with *Pi-b* blast resistance genes with different cultivars. In case of *Pi-kh*, twenty one sequences showed high similarities with different cultivars. *Pi-kh* blast resistance gene sequence also expressed similarity with partial coding sequence of *Pi54* gene. The amplified sequence analysis indicates that it contained open reading frame. Sequence was also blast in Gramene Gene bank data base (www.gramene.org). The sequence showed that *Pi-kh* is located on chromosome 11 from 12,852,908 bp to 13,352,958 bp in rice genome (previously isolated, mapped and cloned by Sharma et al. (2005) and *Pi-b* is located on chromosome 2 from 1 to 35,937,250 (previously cloned by Wang et al. (1999) in rice genome (Fig 2).

Characterization of transcript product of Pi-b and Pi-kh blast resistance genes

The sequence of nucleotide was converted into protein by Expasy.org online translating tool. Comparison of amino acid sequence of *Pi-kh* and *Pi-b* blast resistance gene demonstrated a homology with known blast resistance NBS-LRR family protein of *O. sativa*. Several putative conserved domains found for *Pi-b* and *Pi-kh* blast resistance gene and sequence for both genes produced significant alignment. Further, in case of *Pi-kh* gene analysis, sequence revealed that it contained LRR domain. Previous studies also reported that *Pi-kh* contain NBS-LRR domain (Sharma et al., 2005) and this LRR domain play an important role in protein-to-protein interaction. The sequence analysis of *Pi-b* gene protein indicated that it contains zinc finger-containing protein domain and the same is in agreement with findings of Wang et al. (1999) who also cloned and characterize *Pi-b* gene previously and reported that *Pi-b* contain unique zinc finger domain. (Fig 3 and 4).

Multiple sequence alignment with known R-genes

The amino acid sequence of *Pi-b* and *Pi-kh* blast resistance genes were compared with other known cloned blast resistance genes. The various motifs of *Pi-b* and *Pi-kh* blast resistance genes found conserved with other blast resistance genes (Fig 5 and 6).

Searching for identical protein sequence by using BLASTp tool in NCBI database

The translated nucleotide sequence into polypeptide molecule was analyzed into NCBI data base for searching of identity with the BLASTp algorithm. Both blast resistance *Pi-b* and *Pi-kh* protein expressed desire similarity with the previously identified proteins. The deduced amino acid sequence of *Pi-kh* showed various similarities with different NBS-LRR proteins of *O. sativa* (*Indica group*) with lower E value (See table 1). The maximum and minimum identity observed was 100 % and 66 % respectively for deduced amino acid sequence of *Pi-kh* blast resistance gene. However, deduced amino acid sequence of *Pi-b* blast resistance gene also

Table 1. Result of searching similarity between *Pi-kh* and *Pi-b* blast resistance gene deduced amino acid sequence with other identified sequences by using BLASTp algorithm.

Gene	Gene Bank protein accession showing the maximum identity	Gene Bank ID	Amino acid Identity (%)	E VALUE
Pi-kh	NBS-LRR [*Oryza sativa* Indica Group]	CCD32373.1	84	0.0
Pi-kh	Mutant resistance prtien (*oryza austerliensis*)	AFE56227.1	86	1e-122
Pi-kh	PREDICTED: putative disease resistance protein RGA3-like isoform X3 (Seteria italic)	XP_004983449.1	48	6e-99
Pi-kh	Hypothetical protein OsI-36804 (*Oryza sativa* indica group)	EEC68521.1	100	2e-85
Pi-kh	Os11g0640600 [*Oryza sativa* Japonica Group]	NP_001068349.1	66	2e-80
Pi-b	*Oryza sativa* japonica group gene for *Pib*-complete cds	AB013448.1	100	0.0
Pi-b	*Oryza sativa* japonica group, Genomic DNA chromosome 2, PAC clone p0689B12	AP005056.2	93	0.0
Pi-b	Oryza sativa chromosome 12, BAC OSJNBb0092007 of library OSJNBb from chromosome 12 of cultivar Nipponbare of ssp. Japonica of *oryza sativa* (rice)	AL928748.4	86	3e-113
Pi-b	Oryza sativa indica group cultivar Sercher *Pib* protein (*Pib*) gene, complete cds	JN564624.1	99	2e-80
Pi-b	*Oryza sativa* japonica group Genomic DNA, chromosome 2, BAC clone:OJ2056 H01	AP004098.3	89	2E-25

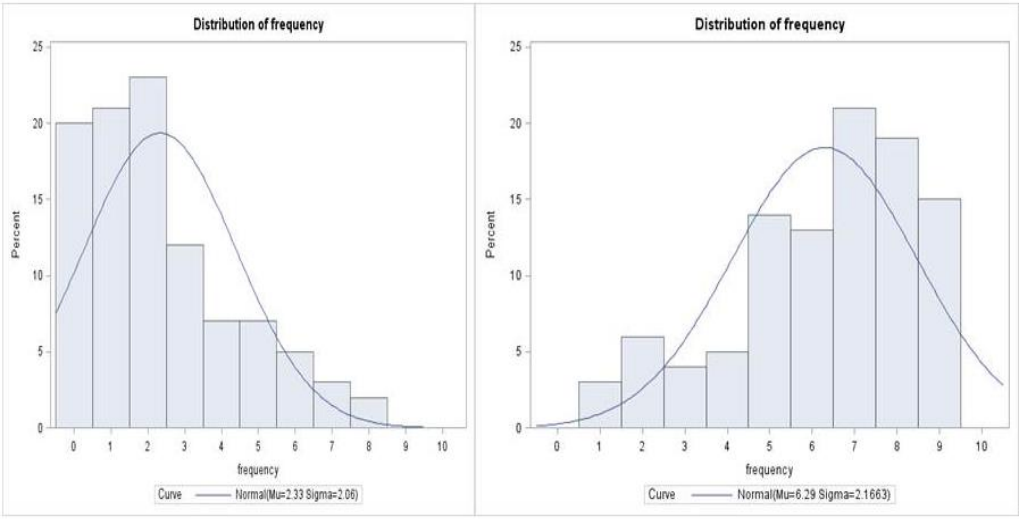

Fig 1. Frequency of leaf blast disease reaction between PS2 and MR219 cultivar.

showed 100 % maximum and 89 % minimum similarities with different *O. sativa* BAC (bacterial artificial chromosome) clones covering chromosome 2 and 12 along with genomic DNA.

Phylogenetic analysis

Phylogenetic analysis of *Pi-b* and *Pi-kh* blast resistance genes with previously cloned blast resistance genes was done by constructing average distance tree in the CLUSTALX software. The cluster showing similarity between *Pi-b* and *Pi-kh* blast resistance genes with other cloned blast resistance genes have been shown in Fig 7.

Discussion

One of the major challenges for plant breeders is to select the appropriate donor parent for introgression of the desirable gene into recipient parents. The selection of true plant with known character can reduce the pressure of selecting individual plant carrying blast resistance gene of interest (Miah et al., 2013). The resistance of Pongsu Seribu 2 variety against highly virulent pathotype P7.2 confirmed its resistivity. The results also revealed that pathotype P7.2 was virulent in case of MR219 while Pongsu seribu 2 showed resistance against it. Our results are in agreement with statement described previously by Wang et al. (2008) that the *R* genes express specific resistance to particular pathogen race having particular avirulence (*AVR*) gene. The resistance of plant also depends upon the pathogen genotype (Ellingboe et al., 1994). By correlating the above statement we can say that Pongsu seribu 2 contain resistance gene having compatibility with avirulence gene exist in pathotype P7.2. The Pongsu seribu 2 showed resistance reaction to *M. oryzae* pathotype P7.2. It is confirmed that Pongsu Seribu 2 is blast resistant rice variety (Rahim et al., 2013). Several studies

Table 2. List of primers used for amplification designed from flanking sequence of *Pi-b* and *Pi-kh* blast genes.

		Primers Sequence	
Locus	Primers name	Sequenced Forward 5'-3'	Sequence Reverse 5'-3'
Pi-kh	PikhP1	CTATTTTGCTCTGGCCCATC	TCTTCAGGCTGTGAGGGTCT
Pi-b	PBF1R1	CGGCCGCATAATACGACT	CAGAATAAGCAACATATAAGCCCTGA
Pi-b	PBF2R2	TCATCCACCCTCTTCTCCAC	TCAAGAATTCACGTGGCTGA
Pib	PBF3R3	TCCGAAGATCAGCTCCAATC	TCTTCGCGTCTGATGTTCAC

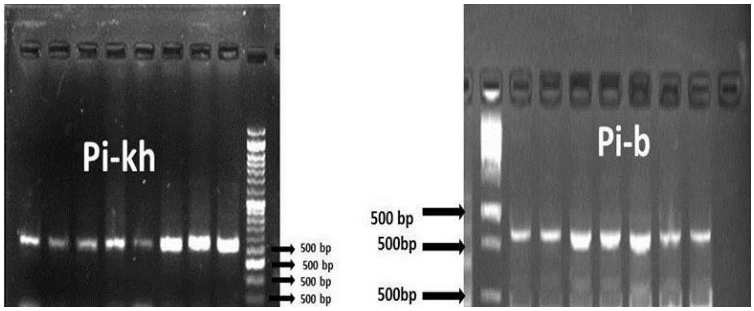

Fig2. Amplification of cloned segment of *Pi-b* and *Pi-kh* blast resistance gene after transformation into competent cell and then separated through restriction enzyme. bp= base pairs

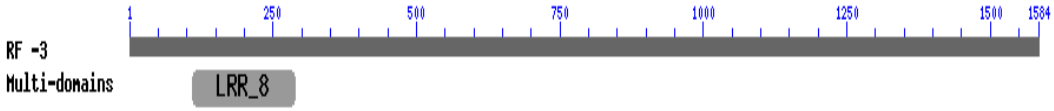

Fig 3. Figure showing the presence of LRR domain in the cloned fragment of *Pi-kh* blast resistance gene.

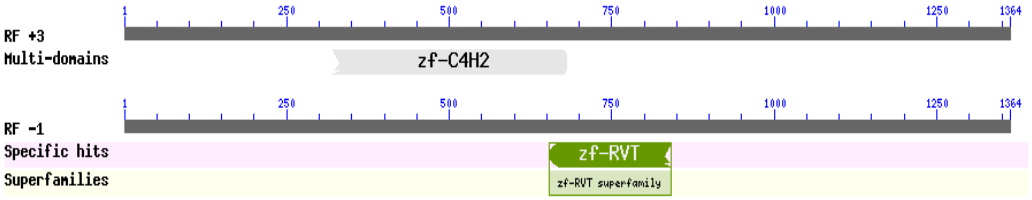

Fig 4. Figure showing the presence of zinc-finger containing protien LRR domain in the cloned fragment of *Pi-b* blast resistance gene.

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      475 GSLEILILSSC     NLLSIDQQAFHGLR [1].VN     HLDLS.[1].NSL     TGDSM.[1].ALSHL.[1]. 523
979    50 ERLQVLDLGGQ.[1].VPLVIRKEAFSRLG.[1].LR     RLVLG     SNL.[1].LRLEP.[1].AFAGL.[1]. 99
949   130 SHIDHLWFRNS     RIDVIATEAFHYLT.[1].ID     YIYFH.[1].TKI     GRIER.[1].AFSKM.[1]. 178
4985  251 IKTDELILDNN     ALTDIHGAAFFGSQ.[1].AK.[1].SLKLN     FKL     KHIHP.[1].AFVGM.[1]. 299
159    97 SSLEILNICRN     SIYVIQQGAFLGLN.[1].LK     QLYLC.[1].NKI     EQLNA.[1].VFVPL.[1]. 145
0952  109 SNVTYLSVGDN     ELDEIPKHVLNHMP.[1].LA     TLDIG.[1].CNI     RAVQQ.[1].DLKGI.[1]. 157
5785  640 TRLYVLDLENN     LLQVVEPAWLFGLK.[1].TM     LMNLG.[1].NEI     NSISP.[1].SFQQI      687
0301   52 SGVTDLDLRNN     DIAIIETNSFHDIS     AG     YLYLN.[1].GSV     AVIES.[1].AFYTV.[1]. 99
6765  157 EAAGKIHVRDA     TLGLVDSGAFAGLN.[1].AR     SVIFE.[1].CRI     DVVRA.[1].AFAGM.[1]. 205
1181  141 RRLSKMSIGDS     SVDVLRKGWFDGLS.[1].LR     TFSIS.[1].SKL     GHVED.[1].ALSGL.[1]. 189


      524 GLY     LNMASN     NI 534
979   100 RLQ.[1].LHLDHC     SL 111
949   179 QID.[1].LYFKDS.[1].EI 191
4985  300 NIR.[1].LDLSGT     AL 311
159   146 SLK.[1].LNLQGN     LI 157
0952  158 VVT.[1].LILPSN     NI 169
5785  688 QLN.[1].LDLTAN     DM 699
0301  100 LSG.[1].ILMYSC     AF 111
6765  206 AVV.[1].LRLRNN     RI 217
1181  190 NVD.[1].IELRSC     HL 201
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Fig 5. Alignment of *Pi-kh* putative conserved domain with other known blast resistance gene.

Fig 6. Alignment of *Pi-b* putative conserved domain with other known blast resistance genes.

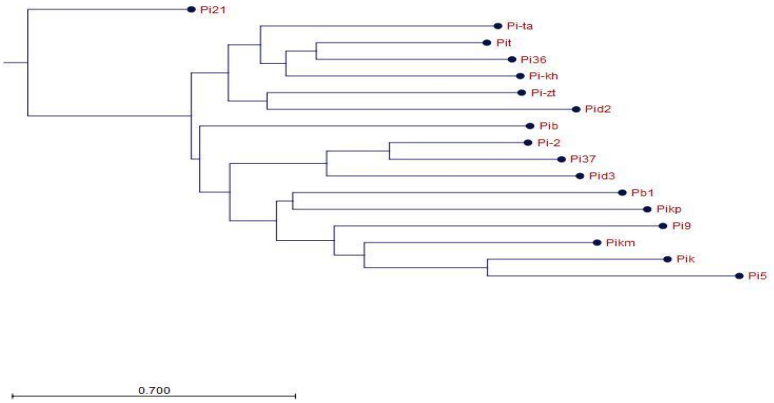

Fig 7. Average distance tree using % identity showing relationship of *Pib and Pikh blast resistance genes with other cloned blast resistance genes*NBS analogs with NBS-LRR class of R-genes.

have been conducted on genetic dissection of blast resistance and QTL mapping in Pongu Seribu 2 (Ashkani et al., 2012). From QTL mapping study and disease reaction against *M. oryzae* pathotype P7.2, it has been shown that QTL covering the regions contain *R* genes. The mechanism of resistance in cultivar will remain unknown until the resistance gene is not identified from it. Currently major focus of molecular breeding is to study the *R* genes. The only way to understand the function and structure of genes are facilitated by cloning and host-plant interaction (Wang et al., 2003; 2004). Rice genome is sequenced already and whole genome sequence is available publicly. The blast resistance genes *Pi-b*, *Pi-ta*, *Pi-kh* are considered as major blast resistance genes because these genes are already cloned from different cultivars. Different techniques such as map-based cloning, transposon technology, expressed sequence technique were used from many varieties for isolation of blast resistance genes (Liu et al., 2007). We designed the primers covering the *Pi-b* and *Pi-kh* locus and cloned successfully 1362bp of *Pi-b* and 1583bp of *Pi-kh* from Pongsu Seribu 2. This is the first time identification and cloning of blast resistance gene from Pongsu Seribu 2 variety. The different primer combinations were designed to amplify the maximum target gene sequence. The degenerate primers pair was efficient to isolate and characterize the *Pi-b* and *Pi-kh* locus from Pongsu Seribu 2 in this study. The transcript product of nucleotide sequence produces several conserved domains for *Pi-b* and *Pi-kh* blast resistance genes. The whole sequence of *Pi-b* gene contains more than 10 kb nucleotide and *Pi-kh* more than 3.5 kb nucleotide containing coding and non-coding regions and forms full protein of NBS-LRR. Our product was based on 1362bp of *Pi-b* and 1583bp of *Pi-kh* gene. From our transcript product, *Pi-kh* produce LRR domain which helps in recognition of pathotype as earlier described by Sharma et al. (2005). The *Pi-b* produces zinc binding domain which is unique character of this blast resistance gene (Wang et al., 1999). From this finding, it is strong evidence that *Pi-b* and *Pi-kh* genes involve for providing resistance against pathotype P7.2 in Pongsu Seribu 2. The nucleotide sequence obtained was translated into polypeptides and similarity was searched by using BLASTp algorithm. This was done because of two main reasons; the comparison at protein level search is more advance and reliable because it shows more homology with NBS-LRR regions of RGAs (resistance gene analogue) as compare to nucleotide level. The chances of degeneration of genetic code are more widely seen at nucleotide level. The second main reason was that amino acid found more conserved near the structural motif, so greater chance to find the function of that particular resistance gene. Thus, the comparison at amino acid level is more accurate, precise and authentic than at nucleotide level. Different comparisons have been made previously to find the identity of RGAs from different species (Totad et al., 2005). The comparison of deduced amino acid sequence of *Pi-b* and *Pi-kh* gene showed strong homology with NBS-LRR protein of *O. sativa* (indica group) and with others BAC clone covering different chromosomes in rice deposited in the gene bank (Table 2). The exploration of resistance genes and resistance

gene analogues in rice local cultivars is necessary to confirm the resistance pattern within them. The information collected from resistance genes helps to determine the structural domain that is a basic step for searching RGAs in any crop plant (Totad et al., 2005). The present study proved that degenerated primers based on the identified sequence of rice blast resistance genes can be used to isolate the resistance genes from local cultivars. Pongsu Seribu 2 variety can be used as a donor parent for blast resistance in any breeding programme. Thus, from this study we conclude that Pongsu Seribu 2 contain at least two dominant *Pi-b* and *Pi-kh* blast resistance gene conferring resistance to *M. oryzae* isolate P7.2 The Pongsu Seribu 2 will be useful for genetic improvement of blast susceptible varieties through introgression of blast resistance genes.

Material and Methods

Plant materials

Rice cultivar Pongsu Seribu 2 (resistant to blast) and blast susceptible cultivar MR219 were used in this study. The seed of both cultivars were soaked in water for at least 24 hours. The floating seeds were skimming off from the petri dishes to get maximum number of germinated seed. On next day seed were placed on moist whatman filter paper. When radicle and plumule came out from seed, it was transplanted into green house. After 21 days, young fresh leaves were harvested and kept in freezer at -80°C.

Disease reaction and pathotype used

The Malaysian rice blast isolate P7.2 is highly virulent pathogen collected from Malaysian Agriculture Research and Development Institute (MARDI). The virulence of this pathogen is already confirmed against rice different cultivars (Rahim et al., 2013). The 21 old days young plants of PS2 (resistant) and MR219 (Susceptible) were transferred to glass house for confirmation of resistivity against the blast disease. The inoculation procedure was followed as earlier described by Chen et al. (2001). The plants were covered with black net for maintaining relative humidity above 90 % also water was applied 3,4 times during the day time. Disease reaction was scored after 9 days based on the standard evaluation system of the International Rice Research Institute (IRRI 1996) and protocol described by Mackill and Bonman (1992). To determine the blast lesion type (BLT) and percentage of disease leaf area method of Correa-victoria and Zeigler (1993) was followed. The plants with 0, 1, 3 score for disease rating were considered as resistant plants whereas 5, 7, 9 were considered as susceptible.

Genomic DNA extraction

Genomic DNA was extracted from 3-4 week old plant of Pongsu Seribu 2 cultivar by following the protocol of Doyle and Doyle (1990) with minor changes. Fresh leaf of about 1 g was ground into liquid nitrogen to make it into fine powder with pestle and mortar. After grinding the extract leaf material was transferred into 1000 µl CTAB (cetyltrimethylammonium bromide) buffer and 3µl β-mercaptoethanol. The samples were shifted to micro centrifuge for incubation for 1 hour by gently shaking at the interval of 5 minutes. The centrifugation was carried out at 13000 rpm for 10 minutes. After centrifugation, supertenant layer was transferred into new falcon tube and isopropanol was added with the concentration of 600µl. Again, centrifugation was done at 13000 rpm for 10 minutes to get DNA pellet. The pellet of DNA was washed with 70% alcohol and 1 µL of RNAs added to remove RNA. For dilution, 50 ml of Tris-EDTA ((Ethylenediamine Tetraacetic Acid; buffered solution) was added. The nano-drop spectrophotometer was used for testing the quality and measurement of DNA concentration.

Designing of primers and PCR amplification

The Public data base NCBI http://www.ncbi.nlm.nih.gov/, was used to search the complete sequence of *Pi-b* and *Pi-kh* blast resistance genes. Different accession in NCBI database such as GenBank: AB013448.1 for *Pi-b* blast resistance gene and GenBank: AB013448.1 for *Pi-kh* gene is available publically. We designed primers with different combinations in order to get maximum nucleotide base pairs of both genes. Forward and reverse primers were designed from the flanking sequence of both genes. After designing primers, PCR amplification was carried out as described by McCouch et al. (2002). The total volume of 15 µL containing template DNA, dNTP, MgCl2, primers (forward and reverse), 1X PCR buffer and Taq polymerase were settled down. Thermocycler machine was used to perform the PCR reaction (GeneAmp System 9700 - Applied Biosystems, Foster City, CA). The initial denaturation was done at 95 °C for 5 min followed by 35 cycles then 94 °C for 30 s, 59°C for 30 s, 72°C for 30 s and final extension was carried out at 72°C for 5 min, then 4°C for rapid cooling. The amplified product was visualized under UV light and analyzed by using Bio imaging system. The gel staining was done by using 0.2 µg of ethiduim bromide at 85 volts for 2 hours to detect the amplified fragment.

Cloning and sequence of PCR amplified products
Preparation of competent cells

DH5α cells were used as competent cells by multiplying them for overnight at 37°C. The cells were incubated in antibiotic free Lysogeny medium (LB) medium. After replication of competent cells, single colonies were selected from grown cells. The single colonies were cultured into SOB medium. The culture was further incubated in orbital shaker for 2 hours until value of OD600 reach to 0.6. The culture was finally centrifuged at 3000 rpm for 10 min to get pellet of bacteria's. Cells were flash-frozen in liquid nitrogen at the temperature of 80°C.

Ligation and Transformation

The PCR product was purified by using the gel extraction kit. The PGEM-T easy vector system was used for the ligation. The ligated mixture was added to competent cells and thaw on ice for 30 min before heat shocking. After heat shocking, the transformed cells were incubated on ice and LB medium were added. The cells were then incubated on 37 °C in an orbital shaker. Finally the transformed cells were plated on LB medium and incubated for overnight.

Analysis of DNA sequence

Competent cell which contains the desired cloned DNA fragments were digested with restriction enzyme to separate the targeted *Pi-b* and *Pi-kh* fragments. To carry out the separation of desired segments, 1 µL of plasmid DNA was diluted with 9 µL of double distilled water for the confirmation of the true clones containing *Pi-kh* and *Pi-b*

fragments. After selection of the clones, the Gel documentation was carried to visualize the image. The true clones were sequenced commercially from the NHK Bioscience limited. Further, according to instruction of the AB13700 capillary sequencing system, M13 Forward and M13 Reverse primers used.

Conclusion

Two dominant blast resistance genes, *Pi-b* and *Pi-kh* on chromosome 2 and 11 were cloned and characterize from Pongsu Seribu 2 respectively. From this research, it is concluded that Pongsu Seribu 2 variety can be used as a genetic resource of blast resistance in rice breeding programme.

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