Insights on Diversity of Leucine-Rich Repeat (LRR) Domain among Major Blast Resistance Genes of Rice

S.J.S. Rama Devi*, B. Umakanth*, B. Vishalakshi*, Supriya B. Aglawe, V. Ravindra Babu and Maganti S. Madhav*

Indian Institute of Rice Research, Rajendranagar, Hyderabad, India. *These authors equally contributed to this study.

ABSTRACT: Rice blast is a continuous threat in rice ecosystems across the globe; its dynamics is becoming complex in the changing climatic conditions. Host resistance is still a viable option; hence, the exploration of resistance genes and their novel alleles is indispensable. The majority of the blast resistance genes belong to nucleotide-binding site and leucine-rich repeat (LRR) domain. In the present study, diverse LRR alleles of five major blast resistance genes (*Pi2*, *Pi9*, *Pib*, *Pita*, and *Pi37*) were cloned from 13 different *Oryza* species to determine the nucleotide diversity as well as to identify the single nucleotide polymorphisms, InDels, conserved domains, and protein functional sites. Although *Pi9* and *Pi2* are homologous genes, significant nucleotide variations and variants in the motifs distribution were observed. Among the five genes, *Pi37* showed the highest nucleotide diversity and *Pita* showed the least diversity. The phylogenetic groups of alleles were correlated with the identified haplotypes. The motif (xxLxLxx) was present among all the alleles of blast R gene sequences across various *Oryza* species, indicating its importance. The appearance of post-translational modification sites in the protein sequences of these alleles also indicates its nature of involvement in host–pathogen interactions. The present study offers clues in further understanding the molecular evolution of the LRR domain of resistance genes, which is a key determinant of host–pathogen interactions.

KEYWORDS: leucine-rich repeats, blast resistance genes, nucleotide diversity, allele mining

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CORRESPONDENCE: sheshu24@gmail.com

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Introduction

Rice is the staple food crop for more than half of the population across the globe. However, it has been challenged by various biotic and abiotic stresses every year, leading to major yield losses. Among the various biotic stresses, rice blast caused by *Magnaporthe oryzae* leads to significant yield loss ranging from 10% to 30% annually.[1] This pathogen not only infects rice but also causes damages in other important crops like wheat and barley. Because of the importance of this disease, it has become a good model system to the researchers for the study of plant–pathogen interactions.

Plants have diverse defense mechanisms to overcome the disease-causing pathogens; among these, R gene-mediated resistance is prominent.[2] Among the cloned and well-characterized rice blast R genes, majority of them are nucleotide-binding site (NBS) and leucine-rich repeat (LRR) proteins that are characterized by NBS and LRR domains, as well as variable amino- and carboxyl-terminal domains.[3] Among the four domains, NBS domain involves in signal transduction[4] and LRR domain involves in pathogen recognition.[5] Thus, LRR domain plays a key role in recognizing pathogen-specific effectors. Several studies conducted across different genera revealed that NBS–LRR genes play an important role in plants.[6] Of all the domains, LRRs are highly variable and known to be under diversifying selection.[7,8] This suggests that the LRR region would be always under selection pressures, which promote the evolution of new pathogen specificities, thus contributing to the recognition of different pathogen *Avr* proteins.[9]

Although there are many R genes identified, cloned, and characterized, the highly variable pathogen plasticity demands for the identification of new blast R genes to overcome the disease severity. In this context, understanding the origin, evolution, and diversification of LRR domains from various wild species comprising different *Oryza* genomes may certainly give some possible clues regarding the co-evolution of LRR domain under selection pressure. This understanding would be highly advantageous for incorporating these novel alleles into the cultivated varieties through classical breeding approaches. In our present study, an attempt has been made to understand the identification of variations among various R genes in the LRR domain. For this, we had chosen 13 *Oryza* species for five important blast R genes, ie, *Pi2*, *Pi9*, *Pib*, *Pita*, and *Pi37*.[10–14] The obtained sequences were analyzed using various bioinformatics tools. The study revealed the nucleotide sequence diversity and the evolutionary relationship among

the *Oryza* species. In addition, the distribution of conserved domains and the protein functional sites of five genes are also discussed.

Materials and Methods

Plant materials. Thirteen *Oryza* species were chosen for the current study to understand the LRR diversity among five blast R genes, namely, *Pita*, *Pib*, *Pi9*, *Pi2*, and *Pi37* (Table 1).

Amplification and cloning of LRR domains of selected R genes. The genomic DNA of 13 *Oryza* species was isolated from the leaves of 20-day-old seedlings by modified potassium acetate method.[15] Based on the reported allele sequence of R genes, the LRR domain was determined and the primers were designed specifically for the LRR domain using the Primer3 software (Supplementary Table 1). In order to identify the gene specificity, the primers were designed such that they cover the entire LRR region and also have slight overlap (~100 bp) with the NBS domain of the gene. The selected regions were amplified from 13 *Oryza* species by using high-fidelity Taq DNA polymerase (Fermentas). The PCR amplification was performed in 20 µL reaction volume with a profile of initial denaturation at 94°C for five minutes, followed by 35 cycles of denaturation at 94°C for one minute, annealing at 57°C for one minute, extension at 72°C for two minutes, and final extension of 10 minutes at 72°C. The targeted alleles were amplified and cloned into ZeBaTA cloning vector from the selected plant materials (Table 1).[16] *Escherichia coli* DH5α cells were used for the transformation and multiplication of recombinant DNA, and the plasmid was isolated using DNA purification kit (Promega). Each amplicon was sequenced in both directions with four clones per gene, which was carried out at the central facility of Ohio State University (OSU; USA). Only high-quality sequences (Phred score >20 per base) were selected for further sequence analysis.

Table 1. List of *Oryza* species used in the study.

S.NO.	SPECIES	GENOME COMPOSITION	ACCESSION NO.
1	*O. sativa*	AA	
2	*O. rufipogon*	AA	106424
3	*O. longistaminata*	AA	110404
4	*O. glaberrima*	AA	96717 (CG14)
5	*O. officinalis*	CC	100896
6	*O. punctata*	BBCC	105690
7	*O. minuta*	BBCC	101141
8	*O. alata*	CCDD	105143
9	*O. australiensis*	EE	100882
10	*O. brachyantha*	FF	101232
11	*O. granulata*	GG	102118
12	*O. ridleyi*	HHJJ	100821
13	*O. coartata*	HHKK	104502

Nucleotide sequence analysis. The sequences obtained from various *Oryza* species for different genes were analyzed separately for their nucleotide polymorphisms. For each gene LRR sequence, single nucleotide polymorphisms (SNPs), InDels and Ka/Ks values were identified by comparing these sequences with the reported allele using DnaSP version 5.10 (http://www.ub.edu/dnasp/).[17] In addition, nucleotide diversity (π), Tajima's D test, and haplotype number were determined using DnaSP version 5.10. Pairwise alignment, evolutionary distances was calculated through comparing with the reference allele and phylogenic analysis of the sequences using the neighbor-joining method with a bootstrap value of 1000 replicates; all these analysis were done using MEGA version 4.1.[18]

Protein sequence analysis. All the nucleotide sequences were converted to protein sequences using web-based ExPASy—translate tool (http://web.expasy.org/translate/). The obtained protein sequences were analyzed for conserved domain search using National Center for Biotechnology Information's (NCBI's) conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) with a standard result mode. Protein functional sites were determined using Prosite (http://prosite.expasy.org/scanprosite/) and MyHits motif scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) with default parameters.

Results

The 13 alleles of each blast resistance gene were amplified, cloned, and sequenced from different wild species of *Oryza* as discussed in Materials and methods. The cloned sequences were ready to submit in NCBI (Supplementary Table 2).

Nucleotide variations among the five blast R genes. Nucleotide diversity among all alleles varied from 0.01 to 0.58; further to know this diversity, SNPs and InDels were detected. Among all the alleles, *Pi37* allele of *Oryza punctata* followed by *Pi9* allele of *Oryza australiensis* and *Pi2* allele of *Oryza rufipogon* showed the highest number of SNPs. The least number of SNPs were observed in alleles such as *Pita* allele of *O. australiensis*, *Pi9* alleles of *Oryza glaberrima* and *O. rufipogon*, and *Pi2 allele of Oryza alata*. The highest number of InDels were found in *Pi37* allele of *O. punctata* (BB) followed by *Pi9* allele of *O. australiensis* and *Pi2* allele of *O. alata*. To dissect these nucleotide variations furthermore, Ka/Ks ratios were determined. Among all the alleles, *Pi37* allele of *O. australiensis*, *Pi9* allele of *O. rufipogon*, and *Pi37* allele of *O. alata* have shown the highest Ka/Ks ratios. The details of the nucleotide diversity of alleles of each gene are given in Table 2 and Supplementary Table 3. Among the alleles, the evolutionary distances ranged from 0.01 to 1.20. *Pi2* allele of *O. australiensis*, *Pi9* of *Oryza longistaminata*, *Pib* allele of *Oryza officinalis*, *Pita* allele of *Oryza minuta*, and *Pi37* allele of *O. alata* showed high evolution when compared to other alleles of each gene. To know the evolution of alleles, Tajima's D values were computed for all the genes. In the allele

Table 2. A summary of nucleotide diversity analysis of Blast R genes.

Oryza SPECIES	*Pi2*				*Pi9*				*Pib*				*Pita*				*Pi37*			
	SNPs	INDELS	π	$\theta\pi$	SNPs	INDELS	Π	$\theta\pi$	SNPs	INDELS	Π	$\theta\pi$	SNPs	INDELS	π	$\theta\pi$	SNPs	INDELS	π	$\theta\pi$
O. sativa (AA)	0	0	0	0	0	0	0.00	0.00	650	39	0.57	1.107	0	2	0.00	0	0	2	0.00	0
O. glaberrima (AA)	90	4	0.05	0.06	11	9	0.01	0.01	657	38	0.57	1.164	571	37	0.55	1.089	0	6	0.00	0
O. rufipogon (AA)	908	53	0.56	1.12	32	6	0.00	0.02	411	29	0.54	0.988	576	34	0.56	1.102	945	55	0.55	1.101
O. longistaminata (AA)	865	57	0.55	1.05	936	50	0.56	1.15	587	39	0.54	1.002	525	40	0.51	0.898	945	56	0.55	1.099
O. punctata (BB)	855	48	0.55	1.08	773	51	0.54	1.01	640	38	0.56	1.092	528	46	0.51	0.923	1414	91	0.53	0.99
O. officinalis (CC)	856	54	0.56	1.09	215	6	0.14	0.16	670	29	0.58	1.189	550	40	0.54	0.998	483	33	0.54	1.022
O. australiensis (EE)	899	55	0.57	1.2	926	62	0.55	1.03	649	50	0.54	1.037	8	7	0.01	0.008	621	29	0.55	1.066
O. brachyantha (FF)	891	59	0.54	1.04	55	17	0.04	0.04	651	37	0.57	1.166	567	37	0.55	1.06	1201	83	0.53	0.946
O. granulata (GG)	483	32	0.41	0.62	939	50	0.55	1.12	650	39	0.56	1.164	579	33	0.56	1.1	1066	85	0.51	0.915
O. minuta (BBCC)	903	48	0.56	1.12	115	9	0.08	0.09	617	44	0.54	1.018	578	34	0.56	1.12	957	64	0.54	1.018
O. redleyi (HHJJ)	859	50	0.56	1.09	127	77	0.15	0.17	637	36	0.56	1.084	560	35	0.55	1.054	859	63	0.52	0.963
O. alata (CCDD)	60	2	0.04	0.05	859	56	0.53	0.97	619	37	0.56	1.114	566	35	0.55	1.069	984	58	0.56	1.149
O. coarctata (KKLL)	656	23	0.53	0.97	92	9	0.08	0.09	602	35	0.52	0.949	525	40	0.51	0.898	959	59	0.55	1.091
Haplotypes (H)	7				11				7				5				8			
Tajima's D test	0.59				−0.38				−0.156				−0.5				−0.22			

Notes: Π, nucleotide diversity; $\theta\pi$, evolutionary distance.

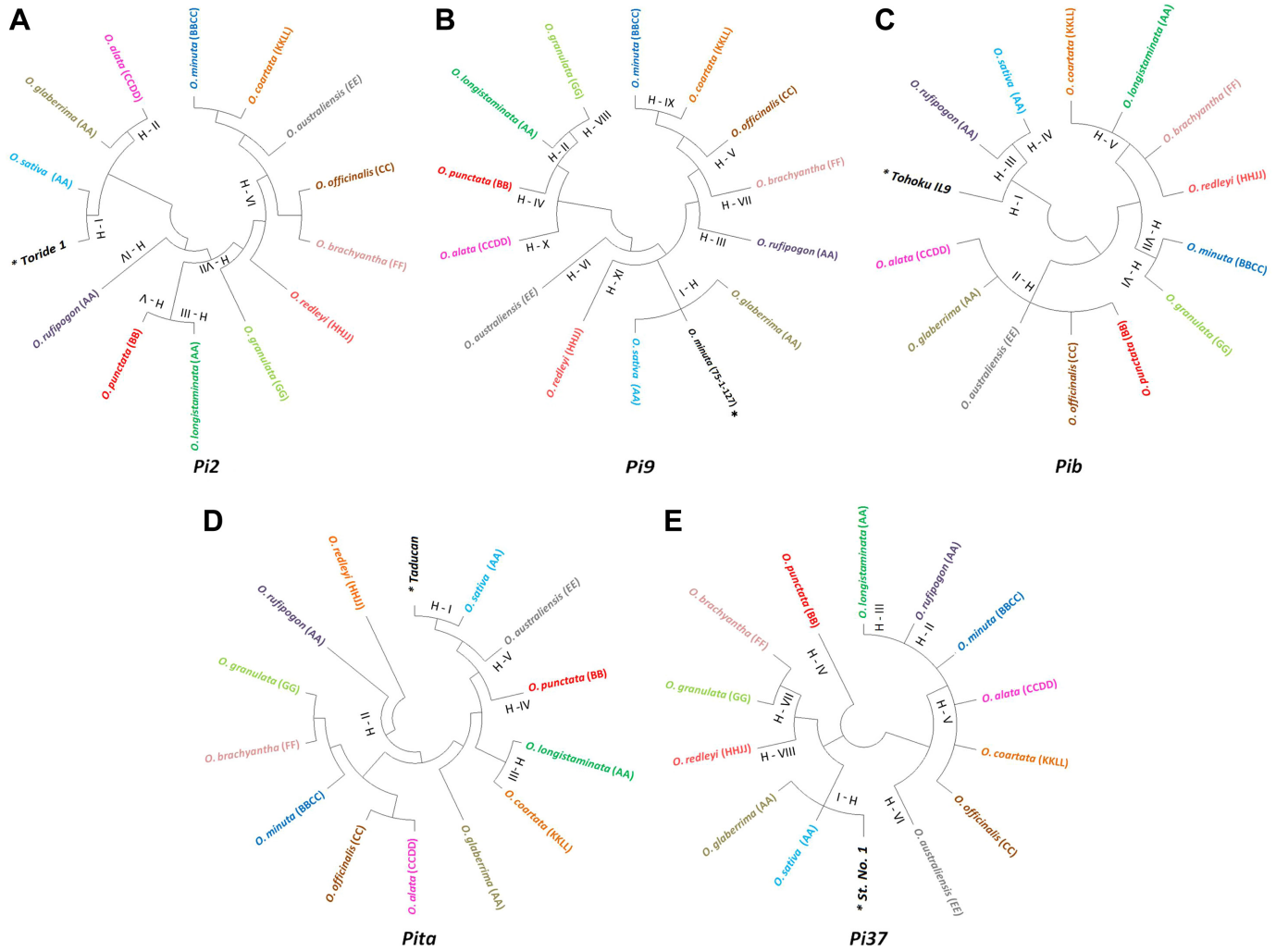

Figure 1. Phylogenetic analysis of five major blast resistance genes explains about the relationship among 13 *Oryza* species at each loci. **A** represents the *Pi2* gene, **B** represents the *Pi9* gene, **C** represents the *Pib* gene, **D** represents the *Pita* gene, and **E** represents the *Pi37* gene. The reference gene sequence used for analysis is indicated by *.

of *Pi2*, the Tajima's D value was positive (0.59), and for other genes, it was negative, ranging from −0.15 to −0.50.

Phylogenetic analysis was also carried out among the alleles of each gene, and the observed phylogenetic groups were compared with the identified haplotypes (Fig. 1). It revealed the presence of a minimum of five groups in case of *Pita* to the maximum of 11 groups in case of *Pi9*.

LRR domains were observed in all the alleles. The xxLxLxx motif is the main motif embedded in the LRR region. These motifs function as an interaction site for the pathogen with the R gene. Hence, the distributions of this motif among the alleles of tested genes were observed, and it was found that xxLxLxx motif was highly abundant between 100 and 600 amino acids in the alleles of *Pi2* (400–500), *Pi9* (400), *Pib* (200–300), *Pita* (400; 600–800), and *Pi37* (600–800; 1000–1200; Supplementary Fig. 1). In addition to this, various functional sites such as N-glycosylation site, casein kinase II (CK2) phosphorylation site and N-myristoylation site were also observed in all the deduced proteins of various alleles, which are known to involve in the post-translation modifications (Supplementary Table 4).

Discussion

The majority of the plant disease resistance genes were encoded by NBS and LRR domain proteins.[19] In NBS–LRR class of R genes, LRR domain is the most important domain since it determines the host–pathogen interactions. Although many reports explained about the diversity of R genes and NBS–LRR genes,[20] but there are few studies existing on the diversity of LRR domains of R genes.

Although many blast R genes and quantitative trait loci (QTLs) were reported till date, the five blast resistance genes that were known to be effective in India and also in many countries were selected which are being used routinely in breeding programs.[13,21–32] *Pita* gene is studied by many other researchers for the DNA polymorphism among landraces and wild species.[28]

The *Oryza* species are valuable reservoirs for many biotic and abiotic stress resistance genes.[33] Many studies have shown

the importance of wild *Oryza* species in the improvement of rice crop.[34] However, few studies have exploited these *Oryza* species for isolation of blast resistance genes.[33] Till now, only two genes, namely, *Pi9* and *Pi40*, were identified from wild *Oryza* species *O. minuta* and *O. australiensis*.[11,35] Novel alleles of blast genes and evolutionary studies for two major blast R genes, *Pita* and *Pi54*, from wild species were determined.[28,36] Systematic attempts have not been made to utilize wild species for mining of LRR alleles among important blast resistance genes. Here, we made an attempt to isolate and analyze the nucleotide diversity of various LRR alleles of five major blast resistance genes using 13 wild *Oryza* species comprising of different genomes. Thirteen *Oryza* species were used to obtain novel and divergent alleles of these genes. The present study focused on the sequence polymorphisms along with protein functional sites for five major blast resistance genes to know their evolution and phylogenetic relationships among the deduced alleles. It is a known fact that SNPs and InDels form the basis of genetic variation in nature, which is measured by nucleotide diversity. High polymorphisms (SNPs and InDels) were observed in the LRR of *Pi37*, whereas least was observed in *Pita*. Most of the SNPs are non-synonymous changes, which revealed by showing higher Ka/Ks values. Tajima D test values of the four genes were negative, which indicates that these genes are under purifying selection across the species of *Oryza*, whereas the *Pi2* showed positive Tajima value, which may be under balancing selection, suggesting that *Pi2* alleles are maintained in a given population. Purifying selection was also evidenced in *Pi54* alleles, while *Pita* and *Pib* alleles were deduced from landraces, diverse set of rice accessions, and wild species.[36–39] Similar observations were made in the case of *Xa21*, *Xa26*, and *xa5* alleles obtained from wild species.[40,41] Most of the alleles originating from five blast resistance genes showed non-synonymous (*Ka*) to synonymous (*Ks*) ratios <1, indicating that they were under purifying selection, which was also evident from Tajima's D values.

Phylogenetic analysis of the alleles of each gene revealed that the reference (reported) alleles of the five blast resistance genes were grouped with the *Oryza sativa*. We observed alleles derived from four genes distributed into five to eight haplotypes except *Pi9* alleles where in more haplotypes were found.

The principle domain (xxLxLxx) in the LRR region was observed among all the alleles, as this motif is crucial and known to be involved in protein–protein interactions. Polymorphisms in this domain leading to different blast genes were evidenced in the case of *Pi2* and *Piz-t* where a single amino acid difference in this xxLxLxx motif has a crucial role in resistance specificity toward blast pathogen.[10] In addition, the posterior portion of the LRR domain is reported to be important, where a lot of variations among *Pi2* and *Pi9* cultivars have been found.[42] Similar results have been observed in two genes, *i.e.*, *Pita* and *Pi37* alleles (xxLxLxx motifs were enriched in 600–800 and 1000–1200 regions), whereas in other three genes, middle portion of LRR seems to be more important.

The various functional sites in the LRR region revealed the presence of CK2 phosphorylation sites, N-glycosylation sites, N-myristoylation sites, and protein kinase C phosphorylation sites. The frequency of these functional sites varies across the species based on their polymorphisms caused due to SNPs and InDels.[43] Interestingly, of the four functional sites, two were phosphorylation sites, which are known to play key role in host–pathogen interactions, abiotic stresses, and photosynthesis.[44] Furthermore, LRR regions are known to involve in photomorphogenesis, which may help in better adaptation.[36] The other two protein functional sites include N-glycosylation sites and N-myristoylation sites, which are known to play a pivotal role in disease resistance mechanisms. Earlier studies explained that the N-glycosylation sites involved in bacterial blight resistance governed by *Xa3* and *Xa26* genes[45] and tomato leaf mould disease resistance mediated through *Cf-9*,[46] whereas N-myristoylation site has been reported in the NBS–LRR protein of *Arabidopsis* and also in *Pi54*, which helps in recognition of its cognate bacterial Avr proteins *AvrPphB* and *Avr-Pi54*.[47,48] We have also observed the pattern of repeats of amino acids such as cysteine, lysine, proline, glycine, phenylalanine, and arginine. These patterns have multiple functions with distinct nature, which generally depends on the adjacent motifs or sequences that together form recognition sites for bacterial or fungal *Avr* genes. The variations at the post-translation sites may not lead to loss of functionality, but the variation in pathogen interaction sites within the gene results in modified proteins that may lead to susceptibility.[43] This kind of systematic study of LRR regions would be highly beneficial for further experimental validation.

Conclusion

We have successfully cloned and sequenced the LRR alleles of five major blast resistance genes in *Oryza* species. Based on nucleotide and protein variations, extensive polymorphisms were observed among various LRR alleles and many of polymorphisms were non-synonymous in nature and many alleles are undergoing purifying selection. It appears that xxLxLxx motif is necessary for host–pathogen interaction and hence found in all LRR alleles of five genes. The positional conservation of this motif also reveals that the middle and posterior parts of the LRR region are the most important. The appearance of post-translational sites in these LRR regions also indicates their importance among the alleles. The deduced LRR alleles of these five genes from divergent *Oryza* sources species can be explored for providing resistance; thereby these can be deployed in rice breeding programs. The present work offers clues in selecting the LRR alleles for broadening the blast resistance.

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Author Contributions

Conceived and designed the experiments: MSM. Analyzed the data: SJSRD, BU, SBA, and BV. Wrote the first draft of the manuscript: BV. Contributed to the refinement of the manuscript: SJSRD. Agreed with manuscript results and conclusions: MSM and VRB. Developed the structure and arguments for the article, made critical revisions, and approved the final version: MSM. Reviewed and approved the final manuscript: SJSRD, BU, BV, and VRB.

Supplementary Materials

Supplementary figure 1. The pictorial representation of LRR motif (xxLxLxx) distribution among all *Oryza* species in R genes. **A** represents the *Pi2* gene, **B** represents the *Pi9* gene, **C** represents the *Pib* gene, **D** represents the *Pita* gene, and **E** represents the *Pi37* gene.

Supplementary table 1. A list of primer sequences used to amplify five blast resistance genes.

Supplementary table 2. Gene sequences of five major blast resistance genes used in the present study.

Supplementary table 3. A list of synonymous sites and non-synonymous sites among the different blast R genes.

Supplementary table 4. Distribution of functional sites among the different blast R genes.

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