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Original Research Article

Screening of Rice Germplasm for the Distribution of Rice Blast Resistance Genes and Identification of Resistant Sources

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Abstract - Rice blast, caused by a fungus *Magnaporthe oryzae*, is one of the most devastating diseases of rice worldwide. Analyzing the valuable genetic resources is important in making progress towards blast resistance. Molecular screening of major rice blast resistance (R) genes was determined in 2,509 accessions of rice germplasm from different geographic regions of Asia and Europe using PCR based markers which showed linkage to twelve major blast R genes, *Pik-p, Pi39, Pit, Pik-m, Pi-d(t)2, Pii, Pib, Pik, Pita, Pita/Pita-2, Pi5*, and *Piz-t.* Out of 2,509 accessions, only two accessions had maximum nine blast resistance genes followed by eighteen accessions each with eight R genes. The polygenic combination of three genes was possessed by maximum number of accessions (824), while among others 48 accessions possessed seven genes, 119 accessions had six genes, 267 accessions had five genes, 487 accessions had four genes, 646 accessions had two genes, and 98 accessions had single R gene. The *Pik-p* gene appeared to be omnipresent and was detected in all germplasm. Furthermore, principal component analysis (PCA) indicated that *Pita, Pita/Pita-2, Pi-d(t)2*, *Pib* and *Pit* were the major genes responsible for resistance in the germplasm. The present investigation revealed that a set of 68 elite germplasm accessions would have a competitive edge over the current resistance donors being utilized in the breeding programs. Overall, these results might be useful to identify and incorporate the resistance genes from germplasm into elite cultivars through marker assisted selection in rice breeding.

Key words - Rice blast, Disease resistance, Rice germplasm, Resistance gene

Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops that feed half of the global population (Khush, 2004). Due to different types of biotic and abiotic stresses, rice production is less (5 tons/ha) than its average yield potential (10 tons/ha) (Khush and Jena, 2009). Among the biotic stresses, blast caused by *Magnaporthe oryzae* has been reported as a major contributor to the yield gap (Sharma *et al*., 2012) in the rice growing ecosystems. Manipulation of disease resistance in rice has been a key objective in all rice breeding programs to maintain the production because many plant pathogens evolve quickly and may breakdown the resistance developed by resistance (R) genes (Pink, 2002) that will result in the form of disease spread. In this respect, different factors such as growth stage of the plant at the time of infection, its resistance level and prevailing environmental conditions contribute towards the crop losses. Previously, different yield losses have been reported ranging from 5 to 10%, 8%, 14%, and 50 to 85% in India, Korea, China, and the Philippines, respectively (Padmanabhan, 1965). Therefore, the most effective way to control blast is to use resistant cultivars.

In the past two decades, many advances have been attained by exploring the genetics of resistance to the blast disease. Both conventional and molecular genetic techniques have played their role to identify more than 100 genes for resistance to blast from japonica (45%), indica (51%), and other (4%) genotypes (Sharma *et al*., 2012). Generally, differential physiological races of *M. oryzae* are being used to identify R genes in landraces, cultivars, and wild rice collections (Tanksley *et al*., 1997). Resistance to blast is considered as a monogenic trait as some varieties are resistant

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to one race and susceptible to another. For example, *Piricularia* (*pi*) genes confer complete resistance to those strains which bear related avirulence (*Avr*) genes. This strategy of using single genes is successful with known and targeted strains but variable nature of *M. oryzae* pathogen breaks this resistance (Han *et al*., 2001). Such resistance is controlled by gene-for-gene hypothesis that leads towards incompatibility due to interaction between *R*:*Avr* genes. Thus, in the development of durable resistant varieties, both major and minor genes can contribute collectively towards persistent resistance (Wang *et al*., 1994).

The R genes are being widely used in crop breeding for protection against various diseases. According to a previous report, these R genes, except chromosome 3 showed presence on all 12 chromosomes (Yang *et al*., 2008) and most of them clustered on regions of chromosome 6, 11, and 12 (Ballini *et al*., 2008). Twenty-two R genes have been cloned and many have been mapped in different rice varieties of japonica, indica, and wild species. Some of the R genes such as *Pi39(t)*, *Pi5, Pik, Pik-p, Pita, Pita-2, and Piz-t* have been reported for broad-spectrum resistance (BSR) (Liu *et al*., 2007; Yang *et al*., 2008; Cho *et al*., 2007) and other genes such as *Pib*, *Pii*, *Pik-m*, *Pit*, and *Pi-d(t)2* confer race specific resistance (RSR) (Chen *et al*., 2006; Ashikawa *et al*., 2008; Hayashi *et al*., 2009; Yang *et al*., 2008). Molecular markers linked to these genes are used in marker assisted selection (MAS) to provide broad resistance in the field. Many R linked markers such as RM3, RM247, T311, JJ113-T3, and YL155/87 have been discovered in previous reports (Jia *et al*., 2002; Liu *et al*., 2007; Chen *et al*., 2004; Hayashi *et al*. 2006) that could be used in MAS programs. MAS has the advantage for the blast control, functioning in accordance with the gene-for-gene hypothesis, it contributes in selecting the traits of interest along with linked molecular markers. With the progress in knowledge about blast resistance, different strategies have been used for breeding blast resistance that includes multilines, mixtures, and pyramiding (Abe, 2004; Zhu *et al*., 2000). MAS has been used in pyramiding to confirm the presence of multiple genes and screening of populations to track the introgression of genes (Kelly, 1995). However, the changing nature of pathogen demands positive screening and identification for more blast R genes in germplasm.

Crop germplasm collections including landraces and wild relatives have been maintained in gene banks in different countries. These collections provide a favorable gene pool for specific traits such as resistance to blast. Previously, *Pi9* from *Oryza minuta* and *Pup1* locus from traditional cultivar Kasalath have been reported for their contribution towards resistance breeding (Qu *et al*., 2006; Gamuyao *et al*., 2012). Thus, it is vital to explore the germplasm for its function and potential use in future breeding and food security. Therefore, the present study was carried out to assess the distribution of blast R genes in recent collections of rice germplasm and selection of resistant sources to develop high yielding rice varieties.

Materials and Methods

Plant materials and DNA extraction

The experimental material was comprised of 2,509 accessions of rice (*Oryza sativa* L.) collected from seven geographical regions of the world (24 countries) (Table 1). All the germplasm accessions used in this study were acquired from National Agrobiodiversity Center (NAS, RDA, Republic of Korea). The seeds were germinated and grown in the green house. Young leaves from two week old plantlets were used for DNA extraction. Genomic DNA was extracted according to the Qiagen DNeasy Plant Mini Kit protocol (QIAGEN, Germany). The concentrations of DNA were estimated using Take3™ Micro-Volume Plate (BioTek Instruments, Inc., USA) and final adjustment was made at 100 ng/μL.

Gene specific markers

The whole germplasm collection of rice accessions was screened for the presence of twelve major blast resistance genes such as *Pik-p, Pi39, Pit, pik-m, Pi-d(t)2, Pii, Pib, Pik, Pita, Pita/Pita-2, Pi5*, and *Piz-t* using a set of SNP, SSR, and InDel markers (Table 2). All the markers were selected to identify the blast resistance genes in the germplasm used in this study.

Marker analysis

Gene specific markers were employed for genotyping by examining the presence or absence of an amplified product.

Table 1. Details of 2,509 accessions of rice (*Oryza sativa* L.) acquired from National Agrobiodiversity Center (NAS, RDA, Republic of Korea)

Region	Country	Abbreviation	Number of accessions	Region	Country	Abbreviation	Number of accessions
Eastern Asia	Japan	JPN	756	Central Asia	Uzbekistan	UZB	12
	South Korea	KOR	748		Azerbaijan	AZE	05
	China	CHN	401		Kazakhstan	KAZ	02
	Taiwan	TWN	100		Kyrgyzstan	KGZ	02
	Hong Kong	HKG	01	Northern Asia	Russia	RUS	12
Southeastern Asia	Philippines	PHL	323	Western Asia	Turkey	TUR	04
	Viet Nam	VNM	17		Iran	IRN	03
	Myanmar	MMR	02	Europe	Italy	ITA	20
	Cambodia	KHM	06		Hungary	HUN	05
Southern Asia	Afghanistan	AFG	02		Spain	ESP	04
	Bangladesh	BGD	09				
	India	IND	35				
	Pakistan	PAK	29				
	Nepal	NPL	11				

Table 2. List of blast resistance genes and specific primers used for the amplification of genes in 2509 accessions of rice

Gene	Linked marker	Type of marker	Primer Sequences	Specificity[z]	Expected size (bp)	Chromos-ome locus	Donor varieties	Type of rice	References
Pik-p	k39575	SNP	F-GGTGTTTGGGAACCTGAACCTGAACCCTA F-GGTGTTTGGGAACCTGAACCCTG R-TTTCTGTTCGTCGGATGCTC	+ -	158	11	K60	Japonica	Hayashi *et al.* 2006
Pi39	RM247	SSR	F-TAGTGCCGATCGATGTAACG R-CATATGGTTTTGACAAAGCG		155	12	Q15	Japonica	Liu *et al.* 2007
Pit	t311(k59)	SNP	F-CGTGAACCCAATGCACCAGTATTA F-CGTGAACCCAAGGCACCAGTATTC R-CATGTAGTTCTGGATGTTGTAGCTACTC	+ -	287	1	K59	Indica	Hayashi *et al.* 2006
Pik-m	k4731	SNP	F-GCAGATGCATCAGCCAGTGAGTT F-GCAGATGCATCAGCCAGTGAGTG R-GTGCAGGACCGGCACGCAG	+ -	171	11	Tsuyuake	Japonica	Hayashi *et al.* 2006
Pi-d(t)2	RM3	SSR	F-ACACTGTAGCGGCCACTG R-CCTCCACTGCTCCACATCTT		120	6	Digu	Indica	Chen *et al.* 2004
Pii	JJ113-T3	SNP	F-GGATGATGTGATCTGCAGAG R-CTCTTGGTGATCTTTGTTAC	+	484	9	Ishikari shiroke	Japonica	Yi *et al.* 2004, Ise 1991
Pib	Pibdom	SSR	F-GAACAATGCCCAAACTTGAGA R-GGGTCCACATGTCAGTGAGC	+	365	2	Te-Qing	Indica	Fjellstrom *et al.* 2004
Pik	k6438	SNP	F-GCGACCCTGTCTTTGGACTGG F-GCGACCCTGTCTTTGGACTGC R-GAATGATGAGGAGAGAAGGCTGTCG	+ -	226	11	Kanto 51	Japonica	Hayashi *et al.* 2006
Pita[y]	YL155/87 YL183/87		F-AGCAGGTTATAAGCTAGGCC F-AGCAGGTTATAAGCTAGCTAT R-CTACCAACAAGTTCATCAAA	+ -	1042	12	Katy	Japonica	Jia *et al.* 2002, 2004
Pita/Pita-2	ta5	InDel	F-CAGCGAACTCCTTCGCATACGCA F-CAGCGAACTCCTTCGCATACGCG R-CGAAAGGTGTATGCACTATAGTATCC	+ -	515	12	Yashiromochi (Pita), Pi No. 4 (Pita-2)	Japonica	Hayashi *et al.* 2006
Pi5	JJ817	SNP	F-GATATGGTTGAAAAGCTAATCTCA R-ATCATTGTCCTTCATATTCAGAGT	+	1450	9	RIL260	Japonica	Cho *et al.* 2007
Piz-t	zt56591	SNP	F-TTGCTGAGCCATTGTTAAACA F-TTGCTGAGCCATTGTTAAACG R-ATCTCTTCATATATATGAAGGCCAC	+ -	257	6	Toride 1	Japonica	Hayashi *et al.* 2006

[z]+: Resistant specific and -: susceptible specific primers, [y]dominant marker.

The amplifications were carried out in 20 μL of reaction mixture containing 40 ng of genomic DNA, 0.5 μL of each primer (10 picomoles), 10x buffer 2 μL, 1 U of Taq DNA polymerase (Promega Co, USA), 1 μL of dNTP (2.5 mM), and 14.85 μL nuclease-free water. DNA amplifications were performed in PTC-100 thermal controller (MJ Research Watertown, MA, USA). Thermal cycling program involved an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 50-55°C for 30 sec, primer extension at 72°C for 30 Sec, followed by a final extension at 72°C for 10 min. The amplifications from SSR markers were sized using high-resolution capillary electrophoresis on a QIAxcel system (Qiagen, Germany), while all other amplifications were performed using 2% agarose gel to visualize the banding pattern at >20 bp product size between the alleles. The amplified fragments using markers were scored as presence (1) or absence (0) of amplicon linked to each gene DNA fragment.

Data analysis

The data was analyzed by principal component analysis (PCA) in Microsoft excel by add-in "Multibase" package (http://www.numericaldynamics.com). Rice blast resistance genes and rice accessions were examined for identifying the presence of principal components that may segregate germplasm into different regional classes. There were 12 variables (values of rice blast genes) in our data.

Results

Screening of rice blast resistance genes

The whole collection of rice germplasm was subjected to PCR-based markers to get data for the amplification patterns of resistance genes against rice blast using BSR (*Pik-p, Pi39, Pik, Pita, Pita/Pita-2, Pi5, Piz-t*) and RSR (*Pit,Pik-m, Pi-d(t)2, Pii, Pib*) genes. All the 2,509 accessions of different geographic origins possessed one or more blast resistance genes on the basis of positive bands for different markers (Fig. 1a). Among these R genes, *Pik-p* was widely distributed in 2,509 (100 %) accessions. The second most disseminated R gene was *Pi39* in 1,680 accessions followed by *Pit* in 1,007, *Pik-m* in 836, *Pib* in 470, *Pi-d(t)2* in 462, *Pii* in 456, *Pita* in 275, *Pik* in 254, and *Pita/Pita-2* in 259 accessions, whereas *Pi5* and *Piz-t* were comparatively less amplified in 90 and 59 accessions, respectively.

Most of the accessions in the entire collection contained one to nine different R genes. Only two accessions had maximum nine blast resistance genes, while 18 accessions possessed eight blast resistance genes (Fig. 1b). Among other R genes, forty-eight accessions had seven genes, 119 accessions had six genes, 267 accessions had five genes, 487 accessions had four genes, 824 accessions had three genes, 646 accessions had two genes, and 98 accessions had only one gene. There was not a single accession found without any R gene in the present study. All the 98 monogenic accessions held *Pik-p* gene to resist the rice blast. Two accessions which possessed the highest number of R genes belong to Chinese germplasm, while eighteen accessions, each of them with eight R genes belong to different countries including the Philippines, South Korea, China, Cambodia, and Viet Nam bearing six, five, three (both for CHN and KHM) and one accession, respectively (Fig. 2).

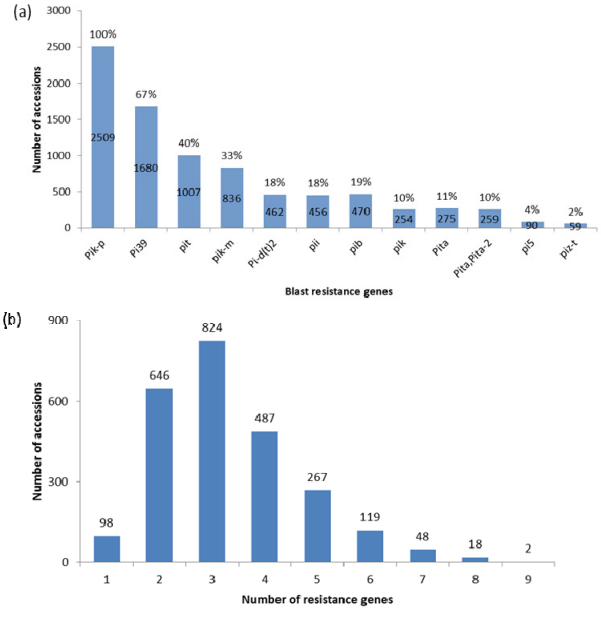

Fig. 1. Frequency distribution of blast resistance genes. (a) Frequency of 12 R genes among 2,509 accessions of rice (*Oryza sativa* L.), (b) frequency of rice accessions bearing variable number of blast resistance genes.

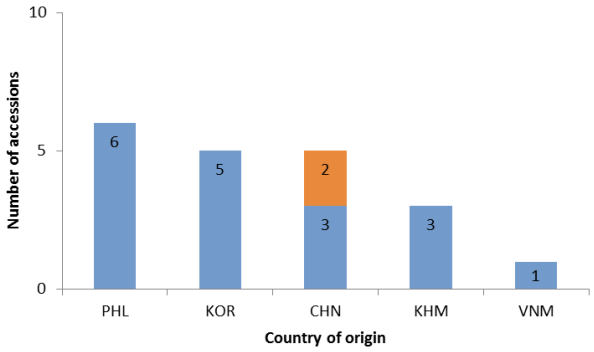

Fig. 2. Twenty rice accessions with highest number of resistance genes along with country of origin. Blue, and brown bars show 8, and 9 genes per accession, respectively.

Genetic diversity of BSR genes

The *Pik* multi-gene family comprised of three genes including *Pik-p, Pik-m*, and *Pik* and located on chromosome 11. Among them *Pik-P* was the most dominant gene, having been detected in all germplasm accessions from Asia and Europe (Table 3). Similarly, *Pik* gene was detected in 234, 13, and 4 accessions from Eastern, Southeastern, and Southern Asia, respectively, whereas a single accession from each of the Northern, and Western Asia and Europe expressed *Pik* gene. As a whole, 836 (33.3%) accessions had a combination of *Pik-p,* and *Pik-m* (RSR) genes. Furthermore, seventy-nine accessions showed positive results for all the three *Pik* multi genes. The *Pi39* was the second most dominant gene with its presence from minimum 5 accessions in Western Asia to maximum 1,499 accessions in Eastern Asia. The *Pita* gene was expressed in 155, 111, and 9 accessions, while *Pita/Pita-2* was detected in 166, 85, and 8 accessions in Eastern, Southeastern, and Southern Asian germplasm, respectively. Moreover, a combination of *Pita* and *Pita/Pita-2* genes was observed in 199 accessions (7.9%). However, both *Pita* and *Pita/Pita-2* genes did not amplify in the rest of the germplasm studied here (Table 3). The *Pi5* gene was present in 51, 32, and 5 accessions from Southeastern, Eastern, and Southern Asia, respectively, including two accessions from Western Asia. Similarly, *Piz-t* was detected in 30, and 28 accessions from Eastern and Southeastern Asian germplasm, respectively. There was also a single accession from Bangladesh in the Southern Asia, which held *Piz-t* gene.

Genetic diversity of RSR genes

The *Pit* gene showed its presence in the whole set of germplasm in various frequencies except Northern Asia. Eastern Asia had highest number of accessions (747) with *Pit* gene followed by Southeastern, and Southern Asia, and Europe with 202, 49, and 6 accessions, respectively. Whereas, two accessions from Western Asia and a single accession from Central Asia expressed *Pit* gene. The *Pik-m* gene was also expressed in the germplasm collected from all seven regions showing lowest presence (4 accessions) in Northern Asia and highest (618 accessions) in eastern Asia. The *Pi-d(t)2* gene was possessed in 283, 157, and 19 accessions from Eastern, Southeastern, and Southern Asia, respectively. Among other regions, each of the Central, and Western Asia, and Europe demonstrated a single accession with *Pi-d(t)2* gene. Similarly, the *Pii* gene was observed in Eastern, Southeastern, and Southern Asian and European germplasm with 349, 80, 12, and 8 accessions, respectively. Central Asian germplasm depicted only one accession, while Northern and Western Asia each possessed three accessions bearing the *Pii* gene. The RSR genes *Pit* and *Pi-d(t)2* were present in the same single accession from Uzbekistan in Central Asia,

Table 3. Region-wise distribution of resistance genes in present collection of rice germplasm

Region	*Pik-p*	*Pi39*	*Pit*	*Pik-m*	*Pi-d(t)2*	*Pii*	*Pib*	*Pik*	*Pita*	*Pita, Pita-2*	*Pi5*	*Piz-t*
Eastern Asia	2006	1499	747	618	283	349	321	234	155	166	32	30
Southeastern Asia	348	102	202	153	157	80	130	13	111	85	51	28
Southern Asia	86	28	49	33	19	12	13	4	9	8	5	1
Central Asia	21	14	1	12	1	1	0	0	0	0	0	0
Northern Asia	12	8	0	4	0	3	0	1	0	0	0	0
Western Asia	7	5	2	5	1	3	0	1	0	0	2	0
Europe	29	24	6	11	1	8	6	1	0	0	0	0

while the *Pii* gene was detected in an another accession from the same country. The *Pib* gene was amplified in 321, 130, and 13 accessions from Eastern, Southeastern, and Southern Asia, respectively, while it did not amplify in germplasm from Central, Northern, and Western Asia. There were also six accessions from Europe, which expressed *Pib* gene (Table 3).

Principal component analysis (PCA)

The PCA has been reported useful to determine the important variables and genetic variation among plant accessions (Shankar *et al*., 2009). Therefore, the general pattern of variation of rice blast resistance genes and rice germplasm was observed by PCA, which revealed the contribution and relation among 12 R genes (Fig. 3). The first two principal components (PCs) accounted for 19.5 and 12.4% of total variability, respectively. The PC1 was correlated with all R genes except *Pi39* and *Pik*, however these R genes showed correlation in PC2. Among R genes *Pi-d(t)2, Pib, Pita*, and *Pita/Pita-2* were more important than other variables in constructing PC1 (Fig. 3b). The overall analysis indicated that North Asian germplasm clustered on the left part of the PCA graph with a higher proportion of accessions holding *Pi39, Pik-p, Pik-m*, and *Pii* genes, while Central and West Asian, and European germplasm was located on the lower right part with more accessions showing *Pi39, Pik-m, Pii*, and *Pit* genes, including *Pi-d(t)2* which was present in one accession from each region (Fig. 3, Table 3). The majority of accessions from Southern, Southeastern, and Eastern Asia were located on the upper right side of the graph. These three regions were influenced by *Pi-d(t)2, Pita, Pita/Pita-2, Pib*, and *Pit* genes in wider extent and *Pi5, Pik-m, piz-t, Pii, Pi39,* and *Pik* genes from moderate to lesser extent. The *Pik-p* gene remained with no influence on resistance holding its position at zero scale in PCA analysis (Fig. 3).

Selection of elite sources for resistance breeding

Based on the findings from this study a set of 68 accessions of elite germplasm expressing higher number of R genes was identified from Eastern, Southeastern, and Southern Asia. Among fourteen selected accessions from Chinese germplasm, two accessions (K115566, IT114581) held highest number (9) of R genes followed by three and nine accessions each with eight and seven genes, respectively. The *Piz-t* gene was absent in Chinese germplasm, whereas *Pik* gene was present in a single accession (Table 4). Korean germplasm also contributed with five and eleven accessions bearing eight and seven genes (*Pik-p, Pi39, Pii, Pik-m, Pi-d(t)2, Pib, Pita*, and *Pita/Pita-2*), respectively, whereas only single accession (IT17707) from Japanese collection exhibited seven genes. Among the selected germplasm, 23% are the landraces collected from various parts of South Korea. The germplasm from Southeastern Asia contributed 32 accessions that include twenty-five from Philippines, five from Cambodia, and two from Viet Nam bearing seven to eight genes. All the germplasm from Philippines were obtained from International Rice Research Institute (IRRI). Southern Asia was represented

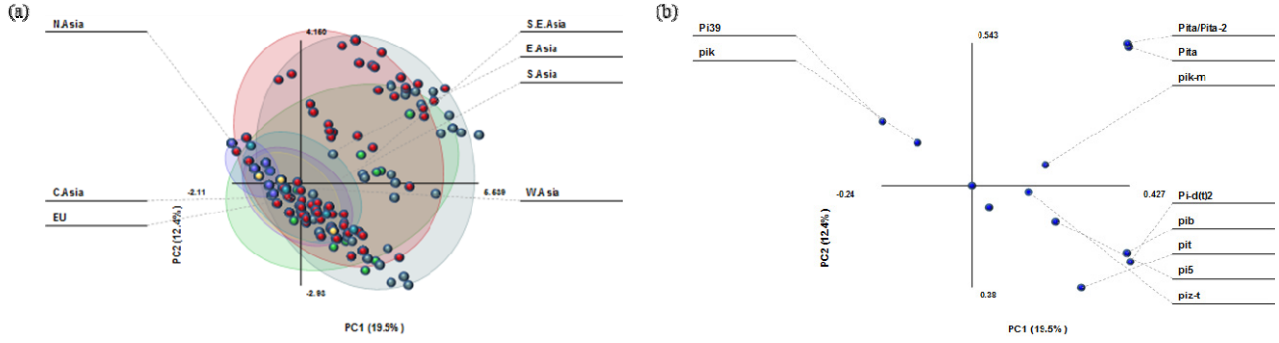

Fig. 3. Principal component analysis of randomly selected rice germplasm (653 accessions) based on twelve resistance genes. PC1 and PC2 are the first and second principal components, respectively. (a) PCA was done among germplasm collected from seven different geographical regions of Asia and Europe. The colors were coded according to regions: Eastern Asia, red; Southern Asia, green; Southeastern Asia, light blue; Europe, purple; Central Asia, yellow; Western Asia, blue-green; Northern Asia, slate-blue. (b) The overall variance and 12 mostly contributing resistance genes in these two components.

Table 4. Details of sixty-eight elite germplasm accessions bearing seven to nine rice blast resistance genes

Accession number	Country	Pik-p	Pi39	Pit	Pik-m	Pi-d(t)2	Pii	Pib	Pik	Pita	Pita/ Pita-2	Pi5	Piz-t	Total
K115566	CHN	+	-	+	+	+	+	+	-	+	+	+	-	9
IT114581	CHN	+	+	+	+	+	+	-	-	+	+	+	-	9
K115565	CHN	+	+	-	+	-	+	+	-	+	+	+	-	8
IT110068	CHN	+	-	+	+	+	+	-	-	+	+	+	-	8
IT219967	CHN	+	-	+	-	+	+	+	-	+	+	+	-	8
K174878	CHN	+	+	+	+	-	-	+	-	+	+	-	-	7
IT275266	CHN	+	+	-	+	-	+	+	-	+	+	-	-	7
IT275268	CHN	+	+	-	+	-	+	+	-	+	+	-	-	7
IT275307	CHN	+	+	-	+	-	+	+	-	+	+	-	-	7
K179078	CHN	+	+	+	+	+	-	+	+	-	-	-	-	7
K115661	CHN	+	-	+	+	+	-	+	-	+	+	-	-	7
IT219970	CHN	+	-	+	-	+	+	-	-	+	+	+	-	7
IT251604	CHN	+	+	+	-	+	-	+	-	+	+	-	-	7
IT264912	CHN	+	+	+	-	+	+	-	-	+	+	-	-	7
IT212003	KOR	+	+	-	+	+	-	+	-	+	+	-	+	8
IT259452	KOR	+	+	+	+	-	-	+	-	+	+	-	+	8
K115016	KOR	+	-	+	+	+	-	+	-	+	+	+	-	8
IT283524	KOR	+	+	+	+	+	-	+	-	+	+	-	-	8
K115407	KOR	+	+	+	+	+	-	+	-	+	+	-	-	8
IT192004	KOR	+	+	-	+	+	-	+	-	+	+	-	-	7
IT212028	KOR	+	+	+	-	+	-	+	-	+	+	-	-	7
K023073	KOR	+	+	+	+	+	-	-	-	+	+	-	-	7
IT251452	KOR	+	-	+	+	+	-	+	-	+	+	-	-	7
IT268126	KOR	+	+	+	+	+	+	-	-	+	-	-	-	7
K179257	KOR	+	+	-	+	-	-	+	+	+	+	-	-	7
K115021	KOR	+	-	+	+	+	-	+	-	+	+	-	-	7
K115413	KOR	+	-	+	+	+	-	+	-	+	+	-	-	7
K115417	KOR	+	-	+	+	+	-	+	-	+	+	-	-	7
K115418	KOR	+	-	+	+	+	-	+	-	+	+	-	-	7
K115419	KOR	+	+	+	+	+	-	+	-	+	-	-	-	7
IT17707	JPN	+	+	+	+	-	+	+	+	-	-	-	-	7
IT102255	PHL	+	+	+	+	-	-	+	-	+	+	-	+	8
IT122817	PHL	+	+	+	+	-	-	+	-	+	+	-	+	8
IT122884	PHL	+	+	+	+	-	+	+	-	-	-	+	+	8
IT265437	PHL	+	+	+	+	-	-	+	-	+	+	-	+	8
IT284228	PHL	+	+	+	-	-	+	-	-	+	+	+	+	8
IT102017	PHL	+	-	-	+	+	+	+	-	+	+	+	-	8
IT9592	PHL	+	-	+	+	+	+	+	-	-	-	+	-	7
IT122675	PHL	+	-	+	+	+	-	+	-	+	+	-	-	7
IT122759	PHL	+	-	+	+	+	-	+	-	+	+	-	-	7
IT122772	PHL	+	+	+	+	-	-	+	-	+	+	-	-	7
IT122848	PHL	+	+	+	+	+	+	+	-	-	-	-	-	7
IT228658	PHL	+	+	+	+	+	-	+	-	+	-	-	-	7
IT268017	PHL	+	-	-	+	+	+	+	-	-	+	+	-	7
K115081	PHL	+	+	+	+	+	-	-	-	+	+	-	-	7
IT267996	PHL	+	+	+	+	+	-	+	-	+	-	-	-	7
IT267999	PHL	+	+	+	+	-	-	+	-	-	-	+	+	7
K115166	PHL	+	-	+	+	-	-	+	-	+	-	+	+	7

Table 4. Details of sixty-eight elite germplasm accessions bearing seven to nine rice blast resistance genes (Continued)

Accession number	Country	Pik-p	Pi39	Pit	Pik-m	Pi-d(t)2	Pii	Pib	Pik	Pita	Pita/ Pita-2	Pi5	Piz-t	Total
IT265427	PHL	+[y]	-[x]	-	-	+	+	+	-	+	+	-	+	7
IT101911	PHL	+	-	-	-	+	+	+	+	+	+	-	-	7
IT101970	PHL	+	-	-	+	+	+	+	-	+	+	-	-	7
IT102078	PHL	+	-	+	+	+	-	-	-	-	+	+	+	7
IT102183	PHL	+	-	-	-	+	+	+	-	+	+	+	-	7
IT102193	PHL	+	-	-	-	+	+	+	-	+	+	+	-	7
IT102256	PHL	+	-	-	+	+	+	+	-	-	+	+	-	7
IT102265	PHL	+	+	-	+	+	-	-	-	+	+	+	-	7
IT265931	VNM	+	-	-	+	+	+	+	-	+	+	+	-	8
IT265530	VNM	+	-	+	+	+	-	+	-	+	+	-	-	7
IT268279	KHM	+	-	-	+	+	+	+	-	+	+	+	-	8
IT268280	KHM	+	+	+	+	+	+	+	-	+	-	-	-	8
IT268278	KHM	+	+	+	+	+	+	+	-	+	-	-	-	8
IT227062	KHM	+	+	+	-	+	-	+	-	+	+	-	-	7
IT268289	KHM	+	-	-	+	+	+	-	-	+	+	+	-	7
IT219152	IND	+	+	+	+	+	-	-	-	+	+	-	-	7
IT219153	IND	+	+	+	+	+	-	-	-	+	+	-	-	7
K177612	IND	+	-	+	+	+	-	+	-	+	+	-	-	7
IT275312	IND	+	-	+	+	+	-	+	-	+	+	-	-	7
IT265398	IND	+	+	+	+	+	-	+	+	-	-	-	-	7

[z]Total number of genes per accession, [y]+: Presence of amplicon linked to R gene, [x]-: Absence of amplicon linked to R gene.

by only five Indian accessions bearing seven R genes such as *Pik-p, Pit, Pik-m, Pi-d(t)2, Pib, Pita*, and *Pita/Pita-2* (Table 4).

Discussion

Genetic analysis of blast resistance by several researchers have pointed out the possible nature of resistance controlled by single dominant or recessive gene, two dominant independent or complementary genes, and/or parental resistance controlled by minor genes (Rath and Padmanahan, 1972; Padmanabhan, 1965; Higashi *et al*., 1985). Moreover, genetic instability of *M. oryzae* as well as host-specificity has made it difficult to breed for blast resistance (Pink, 2002). Host resistance has resulted in the form of short-life even in the improved varieties due to changes in the pathogen's race composition. Therefore, employment of multiple genes should be the target of breeding for stable resistance along with continuous search of new gene resources for gene pyramiding. This notion inspired us to undertake this large scale screening for the selection of resistant sources for future breeding of rice.

In search of good genetic resources of rice, we employed gene specific markers to study blast resistance genes which showed a frequency range of 2 (*Piz-t*) to 100% (*Pik-p*) (Fig. 1). A similar outcome has been reported earlier by Kim *et al*. [(6 R genes) 2010], and Imam *et al*. [(9 R genes) 2014] in the different select set of germplasm with frequency ranges of 30 to 99%, and 6 to 97%, respectively. As the breakdown of blast resistance occurs due to the emergence of stronger strains of fungus (Han *et al*., 2001), a higher frequency of R genes in the plants might be helpful in combating with a range of virulent strains.

The *Pik* multi genes including *Pik-p, Pik-m*, and *Pik,* located on chromosome 11 were possessed by 100, 33, and 10% accessions, which were collected from different ecosystems across the globe (Fig. 1a). The same set of *Pik* multi genes showed higher presence in germplasm from Eastern Asia, while lowest in Northern Asia (Table 3). According to Song et al. (2014) in Korean landraces (*Pik-m* 36.2%) and Zhai *et al*. (2011) in Chinese germplasm, *Pik-m* was responsible for the stable resistance. Whereas *Pik-p* was more frequent in the present study with the possibility of minimum influence alone (Fig. 3). Though the *Pik-m* gene is composed of two or

more genetic factors and has an RSR spectrum compared to *Pik* (Ashikawa *et al*., 2008), probability of overlap between *Pik* and *Pik-m* becomes higher and leads to a similar level of response to blast isolates. Considering the race specificities of these alleles, a "stair-type" resistance was observed among *Pik* alleles in Japanese blast pathogen population and strength of these alleles was ranked in order *Pik-m* > *Pik* > *Pik-p* (Kiyosawa, 1987). In contrast, Wang *et al*. (2009) found a variation of this pattern in Chinese isolates. These findings indicated that the *Pik* alleles *Pik-p, Pik-m*, and *Pik* are independent R genes which produce differential reactions against various isolates. Hence, *Pik-p* in combination with *Pik-m*, and *Pik* in rice growing areas could be effectively used in selection of resistant germplasm.

The resistance pattern for *Pi39* was observed in large number of accessions, 1,499 (75%), and 102 (29%) from Eastern and Southeastern Asia, respectively. Among Eastern Asia, Japanese and Korean germplasm followed by Chinese showed higher frequencies of *Pi39* compared to Taiwan and Southeastern countries such as the Philippines, Cambodia, and Viet Nam (data not shown). Our results for *Pi39* are comparable with the findings of Liu *et al*. (2007), who tested 475 isolates collected from different regions of China and revealed that *Pi39* is responsible for broad-spectrum resistance in Chinese germplasm. However, Song *et al*. (2014) had reported <10% frequency distribution of *Pi39* in Korean landraces of rice that might be due to less number of accessions used in their study. The *Pi39* gene is part of a big cluster with other R genes on Chromosome 12 against same pathogen that indicates involvement of a common genetic mechanism (Liu *et al*., 2007). Thus germplasm bearing *Pi39*, a broad-spectrum resistance gene could be utilized for resistance breeding.

The *Pit*, a major resistance gene on chromosome 1 has been reported for resistance in *indica* rice varieties 'Tjahaja' in Indonesia (Hayashi and Yoshida, 2009) and 'K59' in Northern China (Song *et al*. 2009). The *P*it gene was present in the whole germplasm collection (40%) except Northern Asia in the present study and it was in higher percentage than the outcome of Song et al. [(13.6%) 2014], while less than that reported by Li *et al*. [(100% from 35 accessions) 2013]. All the Eastern Asian germplasm studied here, including Korean, Japanese, and Chinese germplasm possessed *Pit* gene in the range of 30 to 54% (data not shown). However, Cho *et al*. (2007) couldn't identify *Pit* gene in any major Korean rice variety. The *Pit* gene is also a single copy gene in rice genome and functions in a gene for gene manner against pathogen. Thus germplasm bearing the *Pit* gene alone or in combination with other R genes could be a better parental source for breeding.

The *Pi-d(t)2*, also known as *Pi-d2* has been reported in Chinese *indica* rice variety 'Digu' for conferring resistance against a blast strain ZB15. Previously, Chen *et al*. (2006) employed transgenic plants carrying *Pi-d(t)2* and found strong resistance against the pathogen. All the germplasm used in the present study showed presence of *Pi-d(t)2* in multi gene manner except Northern Asia, where this gene was absent (Table 3). Our results regarding *Pi-d(t)2* in combination with other R genes support the outcome of Chen *et al*. (2006). These authors identified three blast R genes *Pi-d1, Pi-d2*, and *Pi-d3* in 'Digu' and found that these genes confer race specific disease resistance, instead Digu showed broad spectrum resistance (Chen *et al*., 2006) that indicates the involvement of two or more genes to confer resistance against *M. oryzae* strains.

The *Pii* and *Pib* genes were moderately distributed in the present study with least representation of *Pii* in Centeral Asia and non in case of *Pib* in the Central, Northern, and Western Asia. Previously, *Pii* has been reported among the weaker effect R genes carried by native Japanese cultivar Fujisaka 5 (Kiyosawa, 1974) and *Pib* couldn't exhibit resistance in the bearer germplasm and near isogenic lines evaluated by Variar *et al*. (2009). Therefore, gene pyramiding with other weaker or stronger R genes might be a good strategy to assemble better combinations that possess durable resistance without damaging other agronomic traits.

The *pita*, and *Pita/Pita-2* genes were diverse in the germplasm from Eastern, Southeastern, and Southern Asia (Table 3). Furthermore, within the respective regions Korean, Philippines, and Indian rice germplasm showed diverse nature and potential source for resistance breeding against blast (data not shown). Similar findings were reported earlier that *Pita-2* was more effective in thwarting infection (Imam *et al*., (2014), while *Pita* was validated against rice blast in the Indian rice (Shikari

et al., 2013). Jia (2009) also tried to estimate the resistance spectra of *Pita* by using recombinant inbred lines. However, spectrum of *Pita* mediated resistance is not clear due to linkage drag. The *Pita* genes have been originated from several cultivars of *indica* such as Tetep and *japonica* such as Katy, and Yashiromochi (Cho *et al*., 2008; Jia *et al*., 2002; Hayashi *et al*., 2006). These genes can be assessed in the selected germplasm for further introgression by MAS at an early stage of selection with accuracy.

The *Pi5* and *Piz-t* were less frequently detected by marker amplifications in the geographical regions studied here. There was no monogenic accession found with either of the two genes in the present collection of germplasm. The *Pi5* has been reported to confer resistance to many isolates of *M. oryzae* collected from Philippines and Korea, however *Pi5* needs two gene products (*Pi5-1, Pi5-2*) to mediate resistance (Lee *et al*., 2009). Similar to many other R genes, both *Pi5* and *Piz-t* work in a combination of other R genes as emphasized by Hayashi *et al*. (2004) and evidenced from a previous study of Imam *et al*. (2014), where *Piz-t* and *Piz* have been used to show resistance in Japanese cultivars. Therefore, understanding the diversity of the specific gene is essential for integration of genes into rice breeding using MAS.

Furthermore, the PCA of cultivated rice accessions explained region wise variation of R genes. The resistance against blast was incurred by *Pi-d(t)2, Pib, Pita, Pita/Pita-2, Pit,* and *Pik-m* genes in Southern, Southeastern, and Eastern Asian regions including Western Asia. Similarly, in Northern and Central Asia including Europe the resistance was due to *Pi39, Pik, Pii*, and *Pi5* genes. Interestingly the accessions from Eastern and Southeastern Asia were relatively rich with major R genes such as *Pi-d(t)2*, *Pita, Pita/Pita-2* and *Pik-m*. It means that genetic diversity of rice accessions originated from Eastern and Southeastern Asia is much richer than Europe and rest of Asian regions that needs to be exploited.

Genotyping with gene specific markers helped us to identify 12 major blast resistance genes in germplasm collected from Asia and Europe. Monogenic lines are best to determine resistance spectra of individual R genes (Tsunematsu *et al*., 2000) but polygenic lines are necessary to study the epistatic interactions among different combinations of R genes such as *Pik-p, Pik-m, Pita*, and *Pi39, Pita/Pita-2* or *Pii* and *Pi5*. Several germplasm studied here had multiple resistance gene grouping that require virulence analyses using specific isolates to unravel the response of these genes. Previously, Cho *et al.* (2007) screened the major R genes such as *Pi5* and *Pik-m* in only two Korean rice varieties Taebaeg and Seogan, respectively. Similarly, *Piz-t* was present in three varieties such as Baegunchal, Hangangchal, Samgang. The elite material is necessary to recover a competitive and resistant progeny against pathogen. Hence, the presence of all the major R genes in the proposed list of germplasm (Table 4) could be used as donor parents to fulfil the gap of missing R genes, while developing new cultivars with multiple genes integrated and resistance enhanced to rice blast fungus in Korean rice. Furthermore, gene pyramiding of *Pik-m* [or *Pik*], *Pita* [or *Pita-2*], *Pii* [or *Pi5*], *Pi-d(t)2* and *Pi39* can possibly be effective to gain broad-spectrum blast resistance.

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