Indian Journal of Agricultural Sciences **88** (3): 361–71, March 2018/Article

Ferreting out blast disease resistance in advanced breeding lines of rice (*Oryza sativa*) by phenotypic evaluation and microsatellite markers

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Received: 05 September 2017; Accepted: 13 December 2017

ABSTRACT

Rice (*Oryza sativa* L.) is the second most important cereal crop of developing countries and the staple food of about 65% of the world's population. Rice blast is a serious disease affecting rice, caused by a fungal pathogen *Magnaporthe grisea* (anamorph - *Pyricularia grisea*). In this endeavor, it is important to identify durable resistance sources. Forty two elite advanced breeding lines containing local and IRRI cultivars in their parentage were screened for the blast disease in uniform blast nursery during wet season of 2012 and 2013 using local land race Pankhali-203 as an inoculum spreader. Four elite lines, viz. GR-7/CRMAS-2231-36(8-4-1-1-1), GR-7/DDR-8(1-5-1-1), IR-28/IET-16804(4-1-1-1-1,2) and Jaya/IR-64(52-2-3-2) and GR-7, a released cultivar, were found highly resistant. Screening of these lines was also done using gene specific eighteen SSR markers. All markers amplified a total of 757 bands with an average of 42.10 bands per marker. The SSR marker RM535 for *Pib* gene and RM28050 for *Pi20* gene produced maximum number (19) of alleles. Most of the breeding lines were found to have all the resistance genes with a diversity of allele range. The diversity analysis revealed the clustering pattern of these genotypes based on their parentage and not the genes present. The highest similarity index value of 0.53 was found between GR-7/NWGR-2002(3-1-1-1-1) and GR-7/NWGR-2002(2-3-1-2-1). Thus, present study revealed the resistance source for rice blast disease from advanced breeding lines simultaneously with the resistance genes present in them.

Key words: Blast disease, Disease resistance, Microsatellite markers, Resistance genes, Rice

Rice (*Oryza sativa* L.), the world's most important staple food crop needs continuous improvement to feed the fast growing population and in this context, enhancement of host plant resistance against biotic and abiotic stresses is a critical component. Rice blast caused by the filamentous ascomycete *Magnaporthe grisea*, is one of the most devastating diseases of cultivated rice. In India, it was first recorded in Thanjavur (Tanjore) delta of South India in 1918 by Mc Rae (1922). However, it attracted the attention only when epidemic occurred in 1919 (Padmanabhan 1965).

The use of resistant varieties is the cheapest and most effective method of controlling the disease, though development of such varieties is encountered with many difficulties.Broad-spectrum durable resistance against rice blast pathogen populations is one of the major objectives of rice breeding programs to overcome the damage caused by rice blast fungus (Wang *et al*. 1994). Resistance controlled by single gene may remain effective for many years, but this is typically not durable. The cellular and morphological development of *M. grisea* has been very adapted to the infected rice plants. The fungal pathogen also has a very high genetic variability (Dean *et al*. 1994). Therefore, development of rice cultivars having durable and horizontal resistances to blast is necessary. One of the strategies for developing the blast resistant rice cultivars may be done through the development of rice genotypes by gene pyramiding or multiple resistance gene to cover the continuously developing races of the blast pathogen in the field. Yu *et al.* (1987) used traditional cultivar Pankhali 203 (Selection made from local land race, released in 1955 from Nawagam, Dist. Ahmedabad, Gujarat, India) as a resistance source in his study at IRRI, Manila, Philippines for inheritance of blast disease resistance. However, the same traditional cultivar ranks highly susceptible and is used now days as an infector row which spreads inoculum for creating epiphytotic condition in blast screening nursery. This may be due to emergence of virulent races within pathogen population and it indicates the narrow genetic diversity for blast resistance within this variety that has been cultivated over the years. Hence, the best way to examine its mechanism is probably to examine a cultivar or genotype that has remained resistant for a long period of time, because durability of resistance is essentially unpredictable. In addition, phenotypic screening is influenced by environmental conditions and is cumbersome

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to detect and expensive to practice, because it requires the use of large field spaces. By identifying polymorphic markers associated with blast resistance genes, we established the basis for tracing the useful resistance alleles into advanced breeding lines.

The use of the wealth of rice genetic resources that has evolved over the millennia through various selection processes has an enormous impact on development of disease resistance in many countries including India. The aim of the present study is detection for presence or absence of six blast resistance genes based on simple sequence repeats (SSR) DNA markers in advanced breeding lines which were also evaluated phenotypically for two seasons.

MATERIALS AND METHODS

The investigation was carried out on 42 advanced breeding lines with the combination of local and foreign genotypes in their parentage. The material also includes six varieties with highly resistant (GR-7, IR-64) to highly susceptible (Pankhali-203) reaction to the blast disease. IR-64 is known as the most popular cultivated rice in Asia and as source of resistance to blast disease under irrigated lowland (Bonman *et al.* 1989, Roumen 1992). GR-7 is reported resistant against blast disease in almost all the centres in All India Coordinated Research Trials of India. The list of plant materials used is given in Table 1 and 2.

The genotypes were evaluated for their spectrum of reaction against the leaf blast at the Uniform Blast Nursery (UBN) at the research farm of Main Rice Research Station, Anand Agricultural University, Nawagam, Gujarat, India (22^0 48' N latitude and 71^0 38' E longitude at an altitude of 32.4 meters above mean sea level). Weather condition during investigating years is presented in Table 3. Evaluation was conducted in three replications during wet seasons of 2012-13 and 2013-14 in uniform blast nursery. A 50-cm-long row of each entry was planted in nursery beds with a row spacing of 10 cm. One row of susceptible check (Pankhali-203) was planted after each entry and also along the borders to facilitate the uniform spread of the disease. For screening of genotypes under controlled conditions, additional inoculum was also spread. For this, diseased leaves were collected, chopped off into pieces of 3-6 cm long and was scattered over the plot at 10 days after sowing. Conidial spore suspension from infected rice kernels was also used for the same. This operation was carried out during prolonged wet weather to facilitate infections and polycyclic development of the disease. Relative humidity was also maintained through water sprinkling at early morning. The test entries were scored based on leaf blast severity using 0-9 scale of Standard Evaluation System (SES) for rice, IRRI (1996). It was done after 10-15 days of post infection depending on the severity of the infection on the susceptible infector rows. At least two readings on blast severity in entries were taken at 10 days intervals. Whenever there was different score values between the replications, only the higher score was considered for evaluation. The disease severity was calculated as given by Shrestha and Mishra (1994). The

Table 1 Pedigree record of the genotypes under study

Genotypic code	Pedigree	Progeny number
G1	GR-7/IR-22	1-2-1-2-2
G2	GR-7/IR 59656-5K-2	2-2-1-1-3
G3	GR-7/IR 71730-51-2	3-2-1-1-1
G4	GR-7/9-YOU-138	2-1-1-1
G5	GR-7/CRMAS-2231-29	6-1-2-2
G6	GR-7/IR-64	5-1-1
G7	GR-7/CRMAS-2231-36	8-4-1-1-1
G8	GR-7/CRMAS-2231-36	1-1-2-1-1
G9	GR-7/NWGR-2002 (NWGR-2002: IET-10750/IET-14714)	2-3-1-1-1
G10	GR-7/NWGR-2002	3-1-1-1-1
G11	GR-7/NWGR-2002	2-3-1-2-1
G12	GR-7/NWGR-2002	4-2-1-2-1
G13	GR-7/Ratna	1-1-1-1-1
G14	GR-7/Ratna	3-1-2-1
G15	GR-7/NWGR-3003 (NWGR-3003: IR-28/IET-13548)	3-1-2-1-1
G16	GR-7/NWGR-3003	4-1-1-1-1
G17	GR-7/IRBB-7	5-3-1-1-2
G18	GR-7/DDR-8	1-5-1-1
G19	GR-7/Mahi Sugandha	3-8-1-1-1
G20	GR-7/IET-17429	1-1-1-1-1-1
G21	GR-7/IET-17429	3-3-1-1-1
G22	GR-7/IET-17429	4-3-1-1-2
G23	GR-7/IET-17429	6-4-1-1-1
G24	IR-28/IET-16804	1-4-1-1-1-1,2
G25	IR-28/IET-16804	4-1-1-1-1,2
G26	IR-28/IET-16804	5-3-2-1
G27	IR-28/IET-17905	1-2-1-3-1
G28	IR-28/IET-16810	1-1-1-1-1,2
G29	IR-28/Gurjari	1-1-1
G30	IR-64/Gurjari	2-2-1
G31	IR-72/IR-38	1-1-1-2-1
G32	IR-72/ Pusa Sugandha-2	1-1-3-2-1
G33	Gurjari/IR-38	4-1-2-1-2,3
G34	Gurjari/IR-38	5-3-1-2-1
G35	Gurjari/IR-38	6-5-1-1-1
G36	Gurjari/IR-72	2-2-1-1-1
G37	Gurjari/IR-72	2-1-1-1-1
G38	Gurjari/IR-72	4-1-2-3-1
G39	GR-11/IR-64	3-1-1-1, 2
G40	GR-11/IR-64	4-1-3-1-2
G41	GAUR-100/IR-64	3-1-1-1
G42	Jaya/IR-64	52-2-3-2

Table 2 Name of varieties and their history

Genotypic code	Variety	Pedigree	Year of release	Leaf blast reaction
G43	GR-7	GR-3/ Basmati-370	2000	Resistant
G44	IR-64	IR5657-33-2-1/ IR2061-465-1-5-5	1985	Resistant
G45	Gurjari	Asha/Kranti	1997	Moderately Resistant
G46	GR-11	Z-31/IR-8-246	1977	Moderately Susceptible
G47	GAR-13	GR-11/IET-14726-22-1-8-1-1-1	2009	Resistant
G48	Pankhali-203	Selection from local land race Pankhali	1955	Susceptible

replicated data for all the 48 genotypes consisting of six checks for leaf blast disease severity for both the seasons individually and pooled were subjected to RBD analysis.

Total DNA was extracted from the leaves by cetyl trimethyl ammonium bromide (CTAB) method (Zidani *et al*. 2005) with minor modifications at Plant Biotechnology Laboratory, Department of Genetics and Plant Breeding, B. A. College of Agriculture, Anand Agricultural University, Anand, Gujarat, India. To estimate the quantity and quality (in terms of protein and RNA contamination) of isolated genomic DNA, spectrophotometry was performed and the data were analyzed using Nanodrop N. D. 1000 software (Ver. 3.3.0). The quantified DNA samples were used as stocks to prepare working concentration of 50 ng/µl by diluting with nuclease free water.

The genomic DNA was amplified using SSR primers listed in Table 4. The PCR profile was as follows: 94°C for 5 minutes (initial denaturation) followed by 34 cycles at 94°C for 1 minute (denaturation after every cycle), ΔT°C (50 – 65°C) for 1 min for primer annealing (depending on the melting temperature of the primer pair), 72°C for 1 min (extension of annealed primer) and 72°C for 5 min (final extension). The amplified products of SSR were analyzed electrophoretically using 2.5-2.8% agarose gel.

Table 3 Weekly meteorological data during period of investigation at research location

Std. Week	Nawagam 2012					Nawagam 2013				
	Maximum temp. (°C)	Minimum temp. (°C)	Rainfall (mm)	Rainy days	RH (%)	Maximum temp. (°C)	Minimum temp.(°C)	Rainfall (mm)	Rainy days	RH (%)
26	34.7	26.9	40.0	4	80	36.6	22.9	52.8	2	67
27	29.4	26.8	101.0	3	82	32.4	25.4	15.2	2	76
28	31.1	25.7	72.2	4	87	30.3	25.4	48.3	3	86
29	32.8	26.4	0.0	0	76	33.0	25.4	0.0	0	74
30	33.1	26.0	33.3	3	76	32.7	25.3	139.2	3	79
31	31.1	25.8	23.7	4	83	30.7	25.2	49.8	3	86
32	30.1	25.3	70.2	6	79	29.8	25.2	52.8	2	89
33	31.2	25.5	5.1	1	76	28.6	24.6	52.6	3	88
34	31.8	25.4	11.0	2	78	31.6	24.7	6.2	1	79
35	31.9	25.0	70.2	4	75	31.4	25.0	6.9	1	78
36	31.9	25.7	56.8	2	80	34.2	23.6	0.0	0	75
37	31.8	25.3	11.0	1	78	33.6	24.5	59.6	4	83
38	32.4	25.3	66.4	4	85	29.8	24.6	164.9	4	85
39	30.5	25.2	113.5	4	82	31.3	23.2	0.0	0	79
40	32.2	23.5	0.0	0	79	33.3	24.6	0.0	0	79
41	34.1	23.8	0.0	0	75	35.2	22.8	0.0	0	68
42	34.6	19.7	0.0	0	69	35.4	20.0	0.0	0	59
43	33.9	19.7	0.0	0	60	35.4	17.8	0.0	0	53
44	34.4	20.5	0.0	0	56	34.5	17.2	0.0	0	56
45	35.1	21.1	0.0	0	55	34.5	16.4	0.0	0	50
46	34.3	18.3	0.0	0	50	32.7	13.5	0.0	0	54
47	32.1	14.5	0.0	0	40	31.0	15.8	0.0	0	46
48	32.2	15.6	0.0	0	39	29.9	16.9	0.4	0	47
49	30.9	15.6	0.0	0	42	33.2	19.1	0.0	0	53
50	29.2	14.5	0.0	0	48	30.5	16.8	0.0	0	63
51	30.3	15.9	0.0	0	43	25.4	18.4	0.0	0	57
52	31.2	13.0	0.0	0	46	29.0	11.2	0.0	0	61
Total			674.4	42				648.7	28	
Average	32.2	22.1			67	32.1	21.3			69

Table 4 List of microsatellite markers used in the study

Markers	F/R	Chromosome No.	Target gene	Primer sequence	Reference
RM 7654	F	11	*Pi1*	5' CTC ATG GTT GTG TCG TGG TC 3'	Liu *et al.* (2008)
	R			5' GTG CAG TGC CAG TGG TAC G 3'	
RM 224	F			5' ATC GAT CGA TCT TCA CGA GG 3'	
	R			5' TGC TAT AAA AGG CAT TCG GG 3'	
RM 208	F	2	*Pib*	5' AGT ACC ACC ACC ATT CTC TGC AAG C 3'	Eizenga e*t al.* (2006)
	R			5' TCG ATT GGC CAT GAG TTC TCG 3'	
RM 535	F			5' ACT ACA TAC ACG GCC CTT GC 3'	Kim *et al.* (2005)
	R			5' CTA CGT GGA CAC CGT CAC AC 3'	
RM 2265	F			5'AAC TGA CCG TAT ATT AGC CA 3'	
	R			5' TGA CCG CCT CTA TTA TAT TG 3'	
RM 144	F	11	*Pik*	5' TGC CCT GGC GCA AAT TTG ATC C 3'	Eizenga e*t al.* (2006)
	R			5' GCT AGA GGA GAT CAG ATG GTA GTG CAT G 3'	
RM7433	F			5' TGC TGC GTG TTA CTT TGG TG 3'	
	R			5' 5' AAC CCT TCA TCA GGC TAC GC 3'	
RM 7102	F	12	Pita	5' GGG CGT TCG GTT TAC TTG GTT ACT CG 3'	Jia *et al.* (2004)
	R			5' GGC GGC ATA GGA GTG TTT AGA GTG C 3'	
YL 155				5' AGC AGG TTA TAA GCT AGG CC3'	Jia *et al.* (2002)
YL 87				5' CTA CCA ACA AGT TCA TCA AA 3'	
YL 183		12	*Pita*	5'AGC AGG TTA TAA GCT AGC TAT3'	Jia *et al.* (2002)
YL 87				5' CTA CCA ACA AGT TCA TCA AA 3'	
RM 7488	F	6	*Piz*	5' GTG TTC GGA GAT GAG AGG ATA CG 3'	Rathour *et al.* (2008)
	R			5' GCG CTT TCT ATT GGA GAT GAA CC 3'	
MRG 2431	F			5'ATC CAA ATC CAA TGG TGC AG 3'	
	R			5' GTG GCG AAA GGG AAC ATT CT 3'	
MRG 4963	F			5' CGA AAA GTG GGA AGC AAA TG 3'	
	R			5'GCG TAC CCC TAG TGG CTG TA 3'	
RM 28050	F			5' GAT AAG ACT TGG GTG GAC ATC ACG 3'	
	R			5' CTT CTA TGG TCG CAA TTC AGA TGC 3'	
RM 5364	F			5' GTA TTA CGC TCC GAT AGC GGC 3'	
	R			5' GTA TCC TTT CTC GCA ATC GC 3'	
RM 21	F	11		5' ACA GTA TTC CGT AGG CAC GG 3'	Chen *et al.* (1997)
	R			5' GCT CCA TGA GGG TGG TAG AG 3'	
RM 168	F	3		5' TGC TGC TTG CCT GCT TCC TTT 3'	Chen *et al.* (1997)
	R			5' GAA ACG AAT CAA TCC ACG GC 3'	
RM 215	F	9		5' CAA AAT GGA GCA GCA AGA GC 3'	
	R			5' TGA GCA ACC TCC TTC TCT CTG TAG 3'	

Clear and distinct bands amplified by SSR primers were scored for the presence (designated as '1') and/or absence (designated as '0') of the corresponding band among the genotypes for computer analysis. The molecular weight of the PCR products was estimated by Alpha Ease FC4.0.0 software (Alpha Innotech Corporation, USA) for each primer to analyze alleles range. The data generated by SSR assay were entered into binary matrix and subsequently analyzed using NTSYSpc version 2.02 (Rohlf 1994). Coefficients of similarity were calculated by using Jaccard's (1908) coefficient (GD_J) by SIMQUAL function. Cluster analysis was performed by agglomerative technique using the UPGMA method by SAHN clustering function of NTSYS-pc. Relationships between the rice cultivars were portrayed graphically as dendrograms.

RESULTS AND DISCUSSION

Reaction to blast disease in field

The evaluation of 42 elite breeding lines with 6 varieties of rice was carried out during *kharif* 2012 and *kharif* 2013 to study field response to blast disease. The screening in

2012 revealed total eight lines to be highly resistant and they are G4, G7, G18, G22, G25, G21 G33 and G42 with disease incidence of 2.96, 2.96, 0.00, 2.22, 1.48, 3.70, 2.96 and 0.74 %, respectively (Table 5 and 6). While, four lines like G7 (2.96%), G18 (2.22%), G25 (3.70%) and G42 (4.07%) found to be highly resistant in 2013. Variety GR-7 was reacted as highly resistant in both the seasons with 0.00% (2012) and 4.44% (2013) disease incidence. IR-64, Gurjari and GAR-13 reacted as highly resistant (2.96, 2.22 and 0.00%, respectively) in 2012 and resistant (6.30, 8.89 and 6.67%, respectively) in 2013. Sixteen entries like G9 (16.30%), G10 (14.81%), G11 (16.30%), G12 (14.07%), G13 (14.07%), G16 (14.07%), G17 (11.85%), G19 (14.07%), G26 (15.56%), G27 (11.85%), G28 (14.07%), G29 (8.89%), G34 (11.11%), G35 (8.89%), G38 (14.81%), G39 (16.30%) and seventeen entries like G1 (15.56%), G4 (7.41%), G9 (14.07%), G10 (12.59%), G12 (14.07%), G13 (14.07%), G16 (16.30%), G17 (7.41%), G22 (6.67%), G26 (11.11%), G27 (11.85%), G28 (14.07%), G29 (8.89%), G31 (10.37%), G33 (7.41%), G34 (7.78%), G35 (13.33%) were found resistant for blast correspondingly in 2012 and 2013. Eight (G1, G2, G3, G5, G14, G20, G24 and G30) and eleven (G2, G3, G5, G11, G14, G19, G20, G24, G30, G38, G39) lines were found moderately resistant in 2012 and 2013, respectively. Four lines reacted as moderately susceptible in both the seasons (G15, G23, G37, G40 in 2012 and G15, G23, G37, G41 in 2013). Four (G6, G8, G32, G41) and three (G8, G21, G40) lines were susceptible in 2012 and 2013, respectively. While, only two (G21, G36) and three (G6, G32, G36) lines reacted as highly susceptible in 2012 and 2013, correspondingly. Local landrace Pankhali-203 was found highly susceptible with highest blast incidence (89.63% in 2012 and 94.07 in 2013).

Microsatellite markers study

The purity of the DNA samples extracted from all 48 genotypes was giving values which are in coherence with the prescribed purity standards ranging from 1.8 to 2.0 of 260/280, 260/230 absorbance ratio using Nanodrop spectrophotometer. The six different resistance imparting genes were targeted for the SSR study for which eighteen SSR markers were used. The molecular weight range produced by different markers is given in Table 7. They amplified a total of 757 bands with an average of 42.10 bands per marker. The SSR marker RM2265 for *Pib* gene produced maximum number of 49 bands, while RM7654 for *Pi1* gene produced minimum number of 32 bands. The average numbers of allele amplified by 18 markers were 12.17. The SSR marker RM535 for *Pib* gene and RM28050 for *Pi20* gene produced maximum number (19) of alleles. YL155/YL87 which is positive control for *pita* gene produced single allele of 1050 bp and YL183/YL87 which is used for negative control for *pita* gene produced single allele of 1046 bp. Thus, the highest PIC (Polymorphic Information Content) value obtained was 1.0 in YL155/YL87 and YL183/YL87 for *Pita* gene and lowest PIC value was 0.62 for RM208 for *Pib* gene.

Genetic diversity and principal component analysis

Based on the SSR data, cluster analysis was performed using genetic similarity values and a dendrogram was generated showing genetic relationships among these genotypes. The highest similarity index value of 0.53 was found between G10 and G11, while the least similarity index value of 0.00 was found between many numbers of genotypes. Clustering pattern of dendrogram generated by using the pooled molecular data of 16 markers of six genes produced two main clusters namely A and B (Fig 1). Cluster A was divided into two sub clusters A1 and A2. Sub cluster A1was again divided in to two clusters, viz. A1a and A1b. The cluster A1a consisted of G1, G2, G3, G4, G5, G16, G17, G18 and G19 and A1b comprised of G6, G7, G8, G9, G15, G13, G14, G10, G11 and G12. Sub cluster A2 consisted of G20, G21, G22, G24 and G23. Cluster B was divided into two sub clusters B1 and B2. Sub cluster B1 was again divided into two clusters, viz. B1a and B1b. The cluster B1a consisted of G25, G26, G27 and G28 and B1b comprised G29, G30, G31, G32, G33, G34, G35, G36, G37 and G38. Sub cluster B2 consisted of G39, G40, G41, G42, G43, G44, G45, G46, G47 and G48. The principle component analysis (PCA) carried out with 48 genotypes almost coincided with the results of cluster analysis. The clustering pattern of 2D of PCA analysis was in accordance with the dendrogram clustering pattern. The 2D plot (Fig 2) showed that the genotypes scattered in two different groups. Genotype G43 and G44 overlapped to each other while, G48 was far away from them which was found highly susceptible to the blast.

The screening of the rice genotypes against blast disease showed that breeding lines GR-7/CRMAS-2231-36(8-4-1-1-1), GR-7/DDR-8(1-5-1-1), IR-28/IET-16804(4-1-1-1-1,2) and Jaya/IR-64(52-2-3-2) including variety GR-7 were highly resistance in both the years and thus may have resistance durability. Total 16 breeding lines GR-7/9-YOU-138(2-1-1-1), GR-7/NWGR-2002(2-3-1-1-1), GR-7/NWGR-2002(3-1-1-1-1), GR-7/NWGR-2002(4-2-1-2-1), GR-7/Ratna(1-1-1-1-1), GR-7/NWGR-3003(4-1-1-1-1), GR-7/IRBB-7(5-3-1-1-2), GR-7/IET-17429(4-3-1-1-2), IR-28/IET-16804(5-3-2-1), IR-28/IET-17905(1-2-1-3-1), IR-28/IET-16810(1-1-1-1-1,2), IR-28/Gurjari(1-1-1), IR-72/IR-38(1-1-1-2-1), Gurjari/IR-38(4-1-2-1-2,3), Gurjari/IR-38(5-3-1-2-1) and Gurjari/IR-38(6-5-1-1-1) were found either highly resistant or resistant in both the years. This may be due to the presence of a resistant genotype as one of the parent in their pedigree. All these sources of resistance identified, can be exploited in breeding programs for the development of disease resistant commercial cultivars after determining their genetics, if these are found to possess other desirable agronomic characters. Ravikumar *et al*. (2014) also screened 91 advanced breeding lines along with one resistance check NLR 145 and susceptible check MTU 7029 to classify the material into resistance and susceptible lines for leaf blast resistance. The advanced breeding lines AE258, AE279, AE280, E408 and the resistant check NLR 145 showed complete resistant

Table 5 Blast disease scale, severity and reaction of 48 genotypes of rice under epiphytotic condition

Codes	Resistance scale			Disease incidence (%)			Reaction		
	2012	2013	Mean	2012	2013	Mean	2012	2013	Mean
G1	1.60	1.40	1.50	17.78	15.56	16.67	MR	R	MR
G2	2.33	1.93	2.13	25.93	21.48	23.70	MR	MR	MR
G3	2.20	2.40	2.30	24.44	26.67	25.56	MR	MR	MR
G4	0.27	0.67	0.47	2.96	7.41	5.19	HR	R	HR
G5	3.33	3.13	3.23	37.04	34.81	35.93	MR	MR	MR
G6	7.47	7.67	7.57	82.96	85.19	84.07	S	HS	HS
G7	0.27	0.27	0.27	2.96	2.96	2.96	HR	HR	HR
G8	5.73	6.13	5.93	63.70	68.15	65.93	S	S	S
G9	1.47	1.27	1.37	16.30	14.07	15.19	R	R	R
G10	1.33	1.13	1.23	14.81	12.59	13.70	R	R	R
G11	1.47	1.87	1.67	16.30	20.74	18.52	R	MR	MR
G12	1.27	1.27	1.27	14.07	14.07	14.07	R	R	R
G13	1.27	1.27	1.27	14.07	14.07	14.07	R	R	R
G14	3.07	3.47	3.27	34.07	38.52	36.30	MR	MR	MR
G15	4.20	4.40	4.30	46.67	48.89	47.78	MS	MS	MS
G16	1.27	1.47	1.37	14.07	16.30	15.19	R	R	R
G17	1.07	0.67	0.87	11.85	7.41	9.63	R	R	R
G18	0.00	0.20	0.10	0.00	2.22	1.11	HR	HR	HR
G19	1.27	1.67	1.47	14.07	18.52	16.30	R	MR	R
G20	1.53	1.93	1.73	17.04	21.48	19.26	MR	MR	MR
G21	7.80	7.20	7.50	86.67	80.00	83.33	HS	S	HS
G22	0.20	0.60	0.40	2.22	6.67	4.44	HR	R	HR
G23	4.60	4.60	4.60	51.11	51.11	51.11	MS	MS	MS
G24	1.53	1.53	1.53	17.04	17.04	17.04	MR	MR	MR
G25	0.13	0.33	0.23	1.48	3.70	2.59	HR	HR	HR
G26	1.40	1.00	1.20	15.56	11.11	13.33	R	R	R
G27	1.07	1.07	1.07	11.85	11.85	11.85	R	R	R
G28	1.27	1.27	1.27	14.07	14.07	14.07	R	R	R
G29	0.80	0.80	0.80	8.89	8.89	8.89	R	R	R
G30	1.73	2.03	1.88	19.26	22.59	20.93	MR	MR	MR
G31	0.33	0.93	0.63	3.70	10.37	7.04	HR	R	R
G32	7.33	7.63	7.48	81.48	84.81	83.15	S	HS	HS
G33	0.27	0.67	0.47	2.96	7.41	5.19	HR	R	HR
G34	1.00	0.70	0.85	11.11	7.78	9.44	R	R	R
G35	0.80	1.20	1.00	8.89	13.33	11.11	R	R	R
G36	7.73	8.03	7.88	85.93	89.26	87.59	HS	HS	HS
G37	5.33	5.03	5.18	59.26	55.93	57.59	MS	MS	MS
G38	1.33	1.93	1.63	14.81	21.48	18.15	R	MR	MR
G39	1.47	2.07	1.77	16.30	22.96	19.63	R	MR	MR
G40	5.20	5.80	5.50	57.78	64.44	61.11	MS	S	S
G41	5.60	5.20	5.40	62.22	57.78	60.00	S	MS	MS
G42	0.07	0.37	0.22	0.74	4.07	2.41	HR	HR	HR
G43	0.00	0.40	0.20	0.00	4.44	2.22	HR	HR	HR
G44	0.27	0.57	0.42	2.96	6.30	4.63	HR	R	HR
G45	0.20	0.80	0.50	2.22	8.89	5.56	HR	R	R
G46	1.73	2.03	1.88	19.26	22.59	20.93	MR	MR	MR
G47	0.00	0.60	0.30	0.00	6.67	3.33	HR	R	HR
G48	8.07	8.47	8.27	89.63	94.07	91.85	HS	HS	HS
SEm				1.55	1.55	1.79			
CD				4.34	4.34	5.10			
CV (%)				10.54	9.88	9.67			

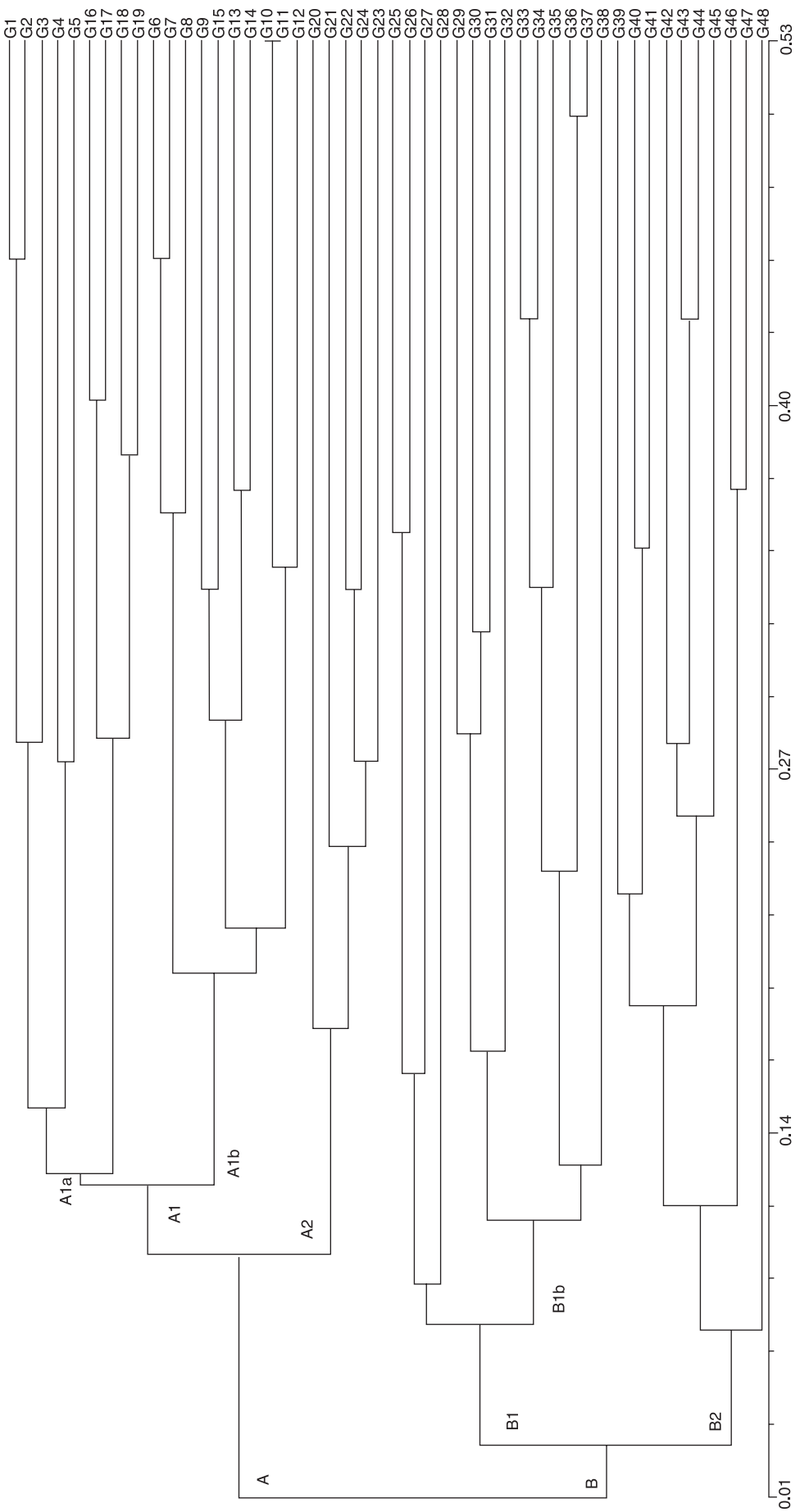

Fig 1 Dendrogram showing clustering of 48 rice genotypes constructed using UPGMA based on Jaccard's Coefficient obtained from pooled SSR analysis.

Table 6 Number of genotypes reacted against blast disease incidence in both the seasons

Disease reaction	Number of genotypes					
	Advanced breeding lines			Varieties		
	2012	2013	Based on mean scale	2012	2013	Based on mean scale
Highly resistant	8	4	7	4	1	3
Resistant	16	17	14		3	1
Moderately resistant	8	11	11	1	1	1
Moderately susceptible	4	4	4			
Susceptible	4	3	2			
Highly susceptible	2	3	4	1	1	1
Total	42	42	42	6	6	6

reaction to leaf blast in seedling stage with 1 score and the advanced breeding lines AE257, AE259, AE275, AE278, AE281, BE325 and E 409 showed resistant reaction for leaf blast with 2 score. Varieties IR-64, Gurjari and GAR-13 were found highly resistant in 2012 and resistant in 2013. This type of non-consistency in disease resistance was also recorded in many other breeding lines also. Bonman (1992) observed that a diverse set of environmental factors can

Table 7 Results of candidate genes and respective molecular markers in SSR analysis

Gene name	Marker	No. of alleles amplified	Molecular Weight Range	Total No. of bands	PIC value
Pi1	RM7654	9	160-225	32	0.84
	RM224	16	107-196	47	0.91
Pib	RM208	4	282-339	35	0.62
	RM535	19	432-768	35	0.93
	RM2265	10	203-349	49	0.86
Pik	RM144	15	210-386	47	0.88
	RM7433	15	134-265	47	0.90
Pita	RM7102	12	321-449	42	0.83
Piz	MRG7488	14	204-357	47	0.87
	MRG2431	17	247-692	47	0.90
	MRG4963	12	165-285	48	0.85
Pi20	RM28050	19	119-254	47	0.92
	RM5364	11	118-219	34	0.79
	RM21	18	115-235	47	0.93
	RM168	10	103-170	48	0.86
	RM215	16	140-233	44	0.89
Pita	YL155/YL87	1	1050	35	1.00
	YL183/YL87	1	1046	26	1.00
Total		219		757	
Average		12.17		42.10	0.8766

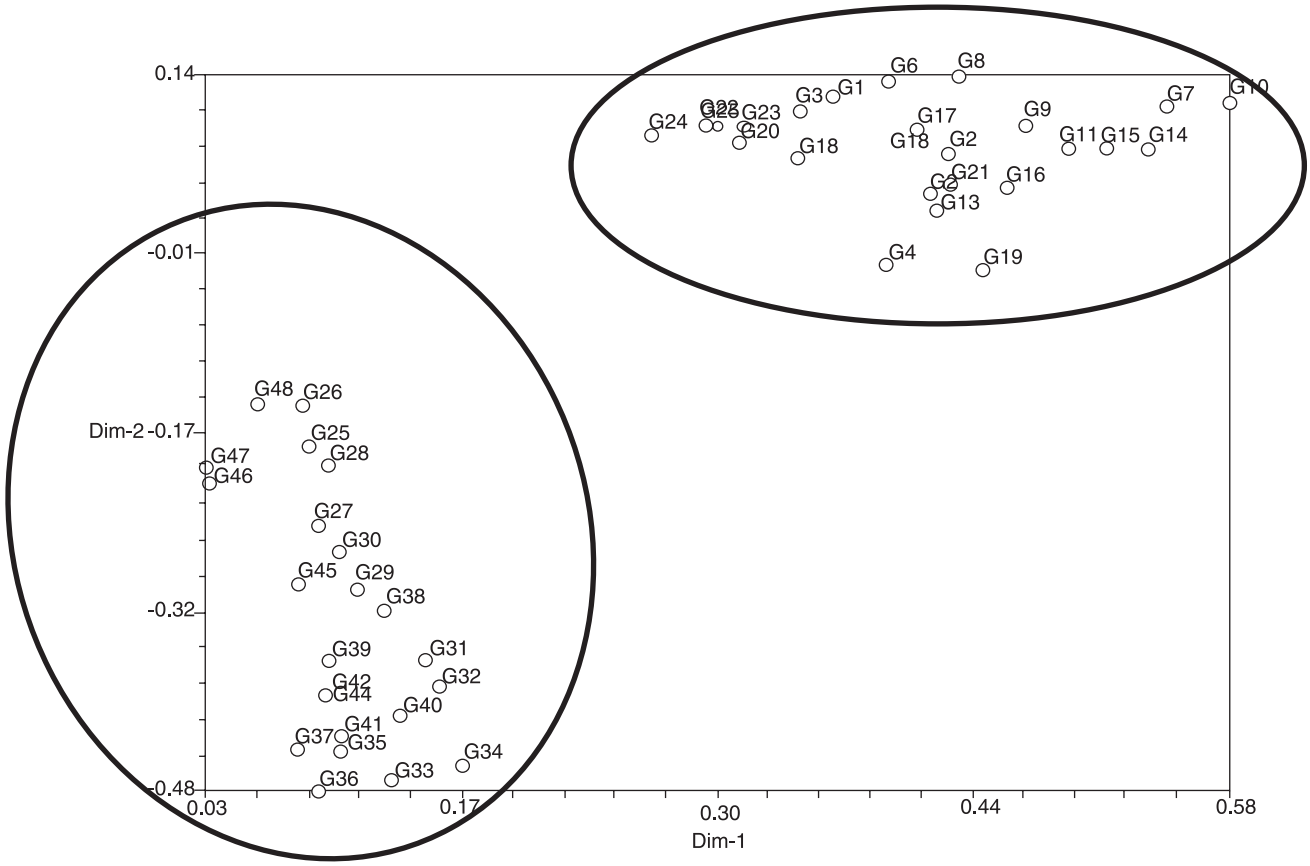

Fig 2 Two dimensional plot by PCA using SSR primers.

Table 8 Blast disease reaction with presence/absence of R-gene

Genotypic code	Disease reaction in field						Resistance genes present (+)/absent (-)						
	HR	R	MR	MS	S	HS	*Pib*	*Piz*	*Pi1*	*Pik*	*Pita*	*Pi20*	Unknown
G1			*				+	+	+	+	+	+	+
G2			*				+	+	+	+	+	+	+
G3			*				+	+	+	+	+	+	+
G4	*						+	+	+	+	+	+	+
G5			*				+	+	+	+	+	+	+
G6						*	-	+	+	+	+	+	+
G7	*						+	+	+	+	+	+	+
G8					*		+	+	+	+	-	+	+
G9		*					+	+	+	+	+	+	+
G10		*					+	+	+	+	+	+	+
G11			*				+	+	+	+	+	+	+
G12		*					+	+	+	+	+	+	+
G13		*					+	+	+	+	+	+	+
G14			*				+	+	+	+	+	+	+
G15				*			+	+	+	+	+	+	+
G16		*					+	+	+	+	+	+	+
G17		*					+	+	+	+	+	+	+
G18	*						+	+	+	+	+	+	+
G19		*					+	+	+	+	+	+	+
G20			*				+	+	+	+	+	+	+
G21						*	+	+	+	+	+	+	+
G22	*						+	+	+	+	+	+	+
G23				*			+	+	+	+	+	+	+
G24			*				+	+	+	+	+	+	+
G25	*						+	+	+	+	+	+	+
G26		*					-	+	+	+	-	+	+
G27		*					+	+	+	+	+	+	+
G28		*					+	+	+	-	-	-	+
G29		*					+	+	+	+	+	+	+
G30			*				+	+	+	+	+	+	+
G31		*					+	+	+	+	+	+	+
G32						*	+	+	+	+	-	+	+
G33	*						+	+	+	+	+	+	+
G34		*					+	+	+	+	+	+	+
G35		*					+	+	+	+	+	+	+
G36						*	+	+	+	+	+	+	+
G37				*			+	+	+	+	+	+	+
G38			*				+	+	+	+	+	+	+
G39			*				+	+	+	+	+	+	+
G40					*		+	+	+	+	+	+	+
G41				*			+	+	+	+	+	+	+
G42	*						+	+	+	+	+	+	+
G43	*						+	+	+	+	+	+	+
G44	*						+	+	+	+	+	+	+
G45		*					+	+	+	+	+	+	+
G46			*				+	+	+	+	-	+	+
G47	*						+	+	+	+	+	+	+
G48						*	+	+	+	+	+	+	+

influence the expression of partial resistance for rice blast disease, including temperature, duration of leaf-wetness, nitrogen fertilization, soil type, and water deficit. Because of the great diversity of rice-growing environments, resistance that proves durable in one system may or may not prove useful in another. Idowu *et al.* (2013) also found significant genotype-by-environment interaction which had the highest contribution to the total sum of squares for disease development and severity scores.

Based on the microsatellite markers, screening of these advanced breeding lines were performed by observing presence or absence of a resistance gene. The result showed that whole the population under study contained at least four resistance genes. High levels of resistance under artificial epiphytotic conditions and the presence of marker bands with more number of gene specific SSR primer tested in the screening of rice genotypes indicate the presence of more number of *R*-genes (Table 8). This may be due to the presence of a resistance genotype as one of the parents in their pedigree. Only some of the breeding lines were found absence for resistance genes. *Pib* gene was found absent in GR-7/IR-64(5-1-1) and IR-28/IET-16804(5-3-2-1). *Pik* gene was absent in IR-28/IET-16810(1-1-1-1-1,2). *Pita* was absent in GR-7/CRMAS-2231-36(1-1-2-1-1), IR-28/IET-16804(5-3-2-1), IR-28/IET-16810(1-1-1-1-1,2) and IR-72/Pusa Sugandha-2(1-1-3-2-1). While, *Pi20* was found absent in IR-28/IET-16810(1-1-1-1-1,2). Some of the genotypes, which were susceptible or highly susceptible under artificial epiphytotic conditions, carried blast resistance genes as indicated by gene linked markers. This may be due to prevailing different races for the pathogen. Pathotype specificity for gene specific SSR markers of rice blast was reported by Ashkani *et al.* (2012).

Pooled SSR analysis revealed that, in general cluster A consisted of the lines selected from cross combinations in which one parent GR-7 was common except IR-28/IET-16804 (1-4-1-1-1-1,2). While, cluster B comprised the lines selected in which IRRI derived cultivars were present along with the check varieties. Using SSR markers close to blast resistance genes, the genotypes studied did not cluster based on the resistance genes present in them. From this it can be assumed that these markers were not tightly linked to blast resistance genes in the genotypes studied. But the dendrogram generated from cluster analysis depicted clear relationship between parents and varieties from which they were developed and/or their geographical origin. Ghaley *et al.* (2012), in cluster analysis of the landraces, revealed two distinct rice cultivar groups, which separated at dissimilarity of 0.84 according to origin of the cultivars from low, mid and high altitude zones in Bhutan.

Conclusion

The present investigation was performed to estimate the durable resistance in the advanced breeding lines. Breeding lines GR-7/CRMAS-2231-36(8-4-1-1-1), GR-7/DDR-8(1-5-1-1), IR-28/IET-16804(4-1-1-1-1,2) and Jaya/IR-64(52-2-3-2) and cultivar GR-7 were found to be highly resistant against blast disease in both the test years and microsatellite marker analysis confirmed the presence of six resistance genes in each one of them. Given confirmation of such a broad genetic base, these lines are likely to exhibit durable resistance and prove highly valuable for commercial breeding programs.

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