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Genomic characterization of blast resistant lines in the background of Improved Samba Mahsuri using genome wide SSR markers

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Abstract
Rice (*Oryza sativa* L.) is the most extensively cultivated and consumed grain and it is considered as lifeline for more than half of world's population. Blast and Bacterial leaf blight (BLB) are major diseases among biotic stresses which seriously limiting crop productivity. Hence, developing disease resistance lines is important in sustainability of rice production. In the present study, five blast resistance lines developed in background of elite rice variety improved samba mahsuri were genotyped with genome wide SSR markers and genomic differences were established. Genomic differences were studied in four combinations of ISM-ML^{Pi54}/ ISM-ML^{Pi9}, ISM-ML^{Pi54}/ ISM-ML^{Pi20}, ISM-ML^{Pi54}/ ISM-ML^{P40} and ISM-ML^{Pi54}/ ISM-ML^{Pita}. The results have shown that, among 1800 screened SSR markers, 63 were found to be polymorphic markers between ISM-ML^{Pi54}/ISM-ML^{Pi9}, 57 polymorphic markers between ISM-ML^{Pi54}/ISM-ML^{Pi20}, 55 polymorphic markers between ISM-ML^{Pi54}/ISM-ML^{Pi40} and 60 polymorphic markers between ISM-ML^{Pi54}/ISM-ML^{Pita}. Number of polymorphic markers on each chromosome was found varying from 3 to 10 among four combinations with average of five markers per chromosome. Similarly, % polymorphism was varying from 1.67-5.98% with average of 3.16-3.52% per chromosome. Results of this study greatly enhance molecular insights in these blast resistance lines and their efficient use in further blast disease resistance breeding programmes.

Keywords: Bacterial leaf blight, Blast, Genomic differences, Improved samba mashuri, Polymorphic markers

1. Introduction
Rice is one of the principal food crops for more than half of the world population. India is one of the leading producers of rice and ranks first in area (43.39 million hectares), second in world's production (104.32 million tons) with an average productivity of 2.40 tons ha^{-1} (Agricultural Statistics at a Glance, 2016 [1]. Though rice plays significant role in achieving global food security, productivity of rice seriously limited by number of biotic and abiotic stresses.

Blast and Bacterial Leaf Blight (BLB) are the most destructive rice diseases in Asia and has become principal production constraint. Chemical control for blast and BLB is cost intensive and associated with environmental hazards (Jamaloddin *et al*., 2020) [8]. Alternatively, the most effective strategy is exploitation of the host-plant resistance. Accelerated efforts led to identification of resistant sources and their incorporation into modern cultivars through strong resistant breeding programmes. As a result many resistance varieties were developed in last three decades for blast and BLB (Singh *et al*., 2012) [22]. Further, advanced tools such as marker assisted selection aided by the molecular markers lead to the precise incorporation of desirable genomic regions from wild/relative species. The introgression lines developed through these tools will serve as valuable sources for resistance breeding programme. The simple sequence repeats (SSRs) are proven useful for marker assisted introgression of the resistance gene(s) into elite cultivars as well as to study parental polymorphism in rice for blast and BLB (Kumar *et al*. 2013) [10].

In the present study five blast resistance lines (ISM-ML^{Pi54}, ISM-ML^{Pi9}, ISM-ML^{Pi20}, ISM-ML^{Pi40} and ISM-ML^{Pita}) developed in background of ISM through marker assisted backcross breeding (Patroti *et al*., 2019 [18] and Madhav *et al*., Unpublished) were genotyped with genome wide SSR markers. This study helps to understand the genomic difference among blast resistance lines and their effective utilization in further blast resistance breeding programme.

2. Materials and Methods

2.1 Plant material

The present study was carried out at ICAR-Indian Institute of Rice Research (ICAR-IIRR), Hyderabad. Experimental material constituted of five blast resistance lines *i.e*., ISM-ML^{Pi54}, ISM-ML^{Pi9}, ISM-ML^{Pi20}, ISM-ML^{Pi40} and ISM-ML^{Pita} which were developed in the background of Improved Samba Mahsuri (ISM). These materials were obtained from the ICAR- IIRR, Rajendranagar, Hyderabad.

2.2 Genotyping with SSR markers

Twenty five days old seedlings of ISM-ML^{Pi54}, ISM-ML^{Pi9}, ISM-ML^{Pi20}, ISM-ML^{Pi40} and ISM-ML^{Pita} were transplanted into the main field (Irrigated). Leaf samples were collected 40 days after transplanting for genotyping. The step-by-step genomic DNA isolation procedure is presented below for the CTAB mini-prep method (Zheng *et al.*, 1995) [29]. 2-4 cm length of tender healthy rice leaf samples were collected and approximately 0.5 g leaf tissue was ground by using a spot test plate, after which 400 µl of CTAB buffer [100 mM Tris HCl (pH 8.0), 20 mM EDTA, 1.4 mM NaCl] was added to the each well and collected into 2 ml eppendorf tube and were incubated in water bath at 65ºC for 55 minutes then equal volume of chloroform: isoamyl alcohol (24:1) was added to the sample which was then centrifuged at 12,000 rpm for 12 minutes. After centrifugation, supernatant was transferred into new eppendorf tube and added equal volumes of chilled isopropanol. Then samples were incubated at -20ºC refrigerator for 1 hour. After 1 hour, the tubes were taken out from refrigerator, and centrifuged for 10 minutes at 10,000 rpm to pellet out DNA. Then pellets were washed using 150 µl of 70% ethanol by centrifugation at 8,000 rpm for 5 minutes. Then DNA pellets were allowed to dry at room temperature. Dried DNA pellets were dissolved in 200 µl 1XTE buffer [10 mM Tris HCl (pH 8.0), 1 mM EDTA]. DNA quantification was done by analyzing the purified DNA measured by nanodrop, the concentration and quality of DNA in individual samples was determined. After that the pure DNA samples were then genotyped by using SSR or microsatellite markers.

A set of 1800 SSR markers covering all the 12 chromosomes of rice were used for parental polymorphism survey. About, 3 µl of diluted template DNA (40 ηg / µl) of parental lines was dispensed in the bottom of 96 well PCR plates. Then 1µl of forward and 1µl of reverse primers (10 µM) were added to each well in PCR plate. PCR master mix, 1µl of dNTPs (2.5 mM), 1.5 µl of taq buffer (10X), 0.5 µl of taq DNA polymerase enzyme (3U) and 7 µl of sterile water was prepared in an Eppendorf tube and added. About 10 µl of the PCR cocktail mix was added to each well to make final volume to 15 µl. The polymerase chain reaction (PCR) comprised of one cycle of denaturation at 94ºC for 5 minutes, followed by 35 cycles at 94ºC for 30 seconds, 55ºC for 30 seconds and 72ºC for 1 minute, with a final extension of 72ºC for 5 minutes. The amplified products were run on 3% Agarose (Lonza USA) gel containing 0.1 mg/ml of ethidium bromide along with DNA ladder (50 bp) at 120 V for 1-2 hours until the bands are clearly separated and DNA fragments visualized with the help documentation unit (Alpha Imager 1200, Alpha Innotech Corp., USA) which was stored for further scoring and permanent records.

2.3 Statistical analysis

The number of polymorphic markers between each lines combination ISM-ML^{Pi54}/ ISM-ML^{Pi9}, ISM-ML^{Pi54}/ ISM-ML^{Pi20}, ISM-ML^{Pi54}/ ISM-ML^{P40} and ISM-ML^{Pi54}/ ISM-ML^{Pita} were estimated chromosome wise and % polymorphism was obtained (No. of polymorphic markers/ Total No. of markers used) X 100 (Mohanty *et al*., 2017) [15]. The polymorphic marker alleles were scored in following fashion. Allele "A" was assigned to ISM-ML^{Pi54} in all four combinations and allele "B" was assigned to ISM-ML^{Pi9}, ISM-ML^{Pi20}, ISM-ML^{P40} and ISM-ML^{Pita} in respective combinations. The genotyping was subjected to graphical genotypes (GGT 2.0) programme (Lachyan *et al*., 2019) [12].

3. Results and Discussion

A total of 1800 SSR markers which are distributed on 12 chromosomes were employed for the genomic characterization of five blast resistant lines (ISM-ML^{Pi54}, ISM-ML^{Pi9}, ISM-ML^{Pi20}, ISM-ML^{Pi40} and ISM-ML^{Pita}) in ISM background. The genomic differences were established in comparison to ISM-ML^{Pi54} i.e., ISM-ML^{Pi54}/ ISM-ML^{Pi9}, ISM-ML^{Pi54}/ ISM-ML^{Pi20}, ISM-ML^{Pi54}/ ISM-ML^{P40} and ISM-ML^{Pi54}/ ISM-ML^{Pita}. The ISM-ML^{Pi54} is the major blast resistant lines which has wide spectrum resistance against the major isolates of *M. oryzae* in India (Madhav *et al*., 2005 and Ramkumar *et al*., 2011) [13, 20].

The utilization of molecular markers has substantially increased the efficiency and made it much easier to introgression of gene(s) from wild sources. Polygenic markers that were previously difficult to assess using traditional breeding procedures, could now be easily identified utilizing molecular markers. The tagging of gene of interest and fine mapping of gene are based on the screening of markers for parental polymorphism among rice cultivars. The markers which are found to be polymorphic can be used in molecular breeding study for biotic and abiotic stresses of rice (Vishalakshi *et al*., 2020) [26].

3.1 Genomic differences among the blast resistance lines

The details of markers used, polymorphic markers obtained and % polymorphism among blast resistant lines (chromosome wise) are provided in Table 1 and Figure 1. The study showed the clear cut differences among the ISM-ML^{Pi54}, ISM-ML^{Pi9}, ISM-ML^{Pi20}, ISM-ML^{Pi40} and ISM-ML^{Pita} lines as significant differences observed in polymorphic markers obtained on each chromosome for blast resistance lines. Identified polymorphic markers on each chromosome are depicted in Figure 2.

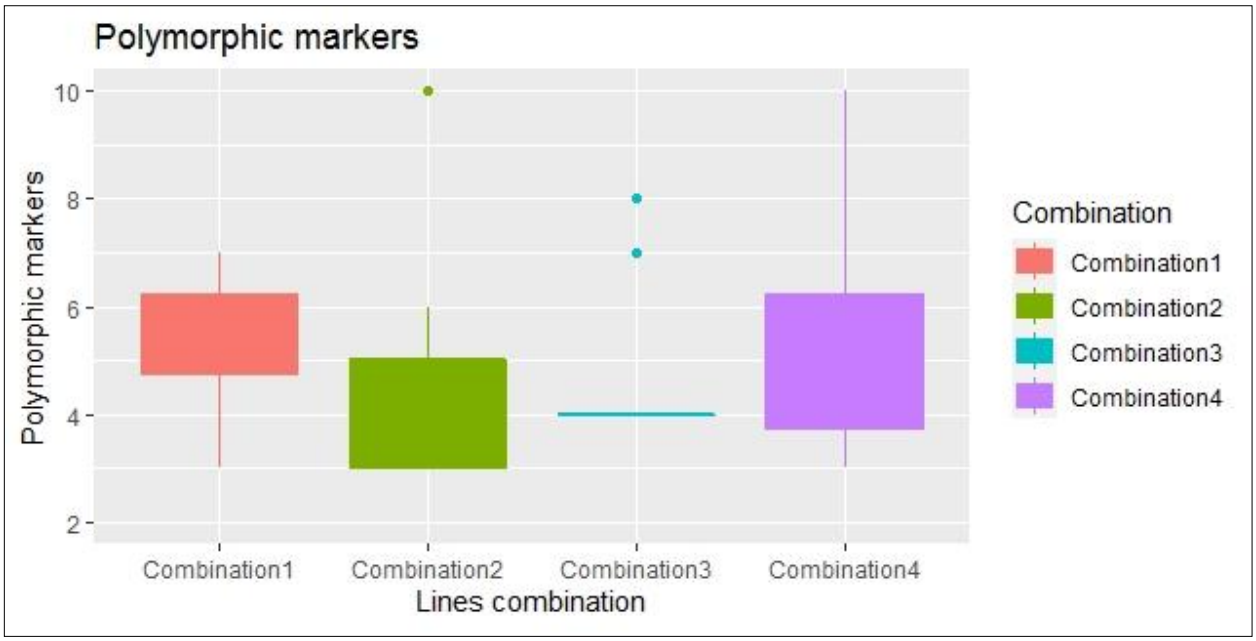
The total number of SSR markers used for genomic characterization on each chromosome were ranging from 102 (Chromosome 2) to 240 (Chromosome 1) with average of 150 SSR markers. The number of polymorphic markers obtained for the ISM-ML^{Pi54}/ ISM-ML^{Pi9} combination were found to be 63 (3.52%) which is ranging from 3 (chromosome 4) to 7 (chromosome 1, 6 and 11) with average of 5.25 markers per chromosome. Similarly, % polymorphism on each chromosome was ranging from 2.52% (chromosome 4) to 4.79% (chromosome 6), which indicate that, genetic variability for ISM-ML^{Pi54} and ISM-ML^{Pi9} was more on the 6th chromosome and very less on 4th chromosome. The number of polymorphic markers obtained for the combination ISM-ML^{Pi54}/ ISM-ML^{Pi20} were found to be 57 (3.16%) which is ranged from 3 (chromosomes 2, 3, 4 and 9) to 10 (chromosome 12) with an average of 4.75 markers per chromosome. Similarly, % of polymorphism on each chromosome was ranged from 2.08% (chromosome 1) to

5.98% (chromosome 12) which is indicated that genetic variability for ISM-ML^{Pi54} and ISM-ML^{Pi20} was more on the 12th chromosome and less on 1st chromosome. The gene combination ISM-ML^{Pi54}/ ISM-ML^{Pi40}, had a total of 55 (3.16%) polymorphic markers which ranged from 4 (chromosomes, 1,2,3,4,5,7,8,9,10 and 12) to 8 (chromosome 6) with an average of 4.58 markers per chromosome. The % of polymorphism on each chromosome was ranging from 1.67% (cromosome1) to 5.48% (chromosome 6) which indicated that genetic variability for ISM-ML^{Pi54} and ISM-ML^{Pi40} was high on the 6th chromosome and very less on 1st chromosome. Similarly, the total number of polymorphic markers obtained for the combination ISM-ML^{Pi54}/ ISM-ML^{Pita} were found to be 60 (3.29%) which is ranged from 3 (chromosomes 2, 4 and 9) to 10 (chromosome 12) with an average of 5 markers per chromosome. Similarly, % of polymorphism on each chromosome was ranged from 2.34% (chromosome 9) to 5.98% (chromosome 12) which indicated that genetic variability for ISM-ML^{Pi54} and ISM-ML^{Pita} was high on the 12th chromosome and very low on 9th chromosome.

Table 1: Number of polymorphic SSR markers and % polymorphism among the improved samba mashuri blast resistant lines (Chromosome wise)

Chromosome	Number of SSRs	ISM-ML^{Pi54}/ ISM-ML^{Pi9}		ISM-ML^{Pi54}/ ISM-ML^{pi20}		ISM-ML^{Pi54}/ ISM-ML^{pi40}		ISM-ML^{Pi54}/ ISM-ML^{pita}	
		Polymorphic	% Polymorphism	Polymorphic	% Polymorphism	Polymorphic	% Polymorphism	Polymorphic	% Polymorphism
Chr1	240	7	2.92%	5	2.08%	4	1.67%	6	2.50%
Chr2	102	4	3.92%	3	2.94%	4	3.92%	3	2.94%
Chr3	142	5	3.52%	3	2.11%	4	2.82%	4	2.82%
Chr4	119	3	2.52%	3	2.52%	4	3.36%	3	2.52%
Chr5	141	5	3.55%	5	3.54%	4	2.84%	4	2.84%
Chr6	146	7	4.79%	5	3.42%	8	5.48%	7	4.80%
Chr7	143	5	3.50%	4	2.80%	4	2.79%	5	3.50%
Chr8	160	5	3.13%	5	3.13%	4	2.50%	4	2.50%
Chr9	128	4	3.12%	3	2.34%	4	3.13%	3	2.34%
Chr10	150	6	4.00%	5	3.33%	4	2.67%	4	2.67%
Chr11	162	7	4.32%	6	3.70%	7	4.32%	7	4.32%
Chr12	167	5	2.99%	10	5.98%	4	2.40%	10	5.98%
Total	1800	63	42.28%	57	37.8%	55	37.9%	60	39.43%
Average	150	5.25	3.52%	4.75	3.16%	4.58	3.16%	5	3.29%

Previously, several studies employed molecular markers to study the genomic differences among the parental lines Neeraja *et al*., 2007 [16]; Channamallikarjuna *et al*., 2010 [5]; Kanagaraj *et al*., 2010 [9]; Marathi *et al*., 2011 [14]; Salunkhe *et al* 2011 [21]; Vikram *et al*., 2011 [25]; Kumar, 2011 [11]; Nguyen *et al*., 2012 [17]; Kumar *et al*., 2013 [10]; Ahmadikhan *et al*., 2015 [2]; Yadav *et al*., 2015 [28]; Dixit and Paul, 2018 [6]; Raghavendra and Hittalmani, 2018 [19]; Tripathi *et al*., 2018 [24]; Lachyan *et al*., 2019 [12]; Vishalakshi *et al*., 2020 [26]. Similar to our studies, Sruthi *et al*. (2016) [23] reported 454 polymorphic SSR primers between parents APMS-6B and BF-16B. Challa and Kole (2019) [4] conducted polymorphism survey between parents, Kasturi and Chaw Khao using 721 SSR markers and identified 95 polymorphic SSR markers with overall 13.17% of polymorphism.



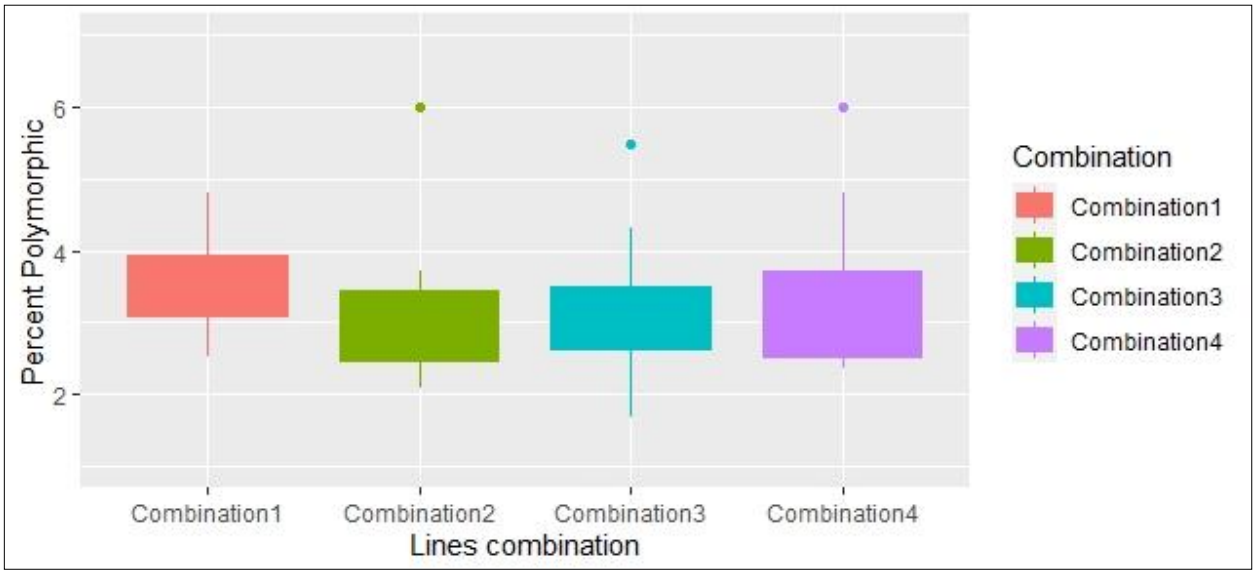


Fig 1: Box plots representing the number of polymorphic markers and percent polymorphism among the blast line combinations (Combination 1- ISM-ML^{Pi54}/ ISM-ML^{Pi9}, Combination 2- ISM-ML^{Pi54}/ ISM-ML^{Pi20}, Combination 3- ISM-ML^{Pi54}/ ISM-ML^{Pi40} and Combination 4- ISM-ML^{Pi54}/ ISM-ML^{Pita}).

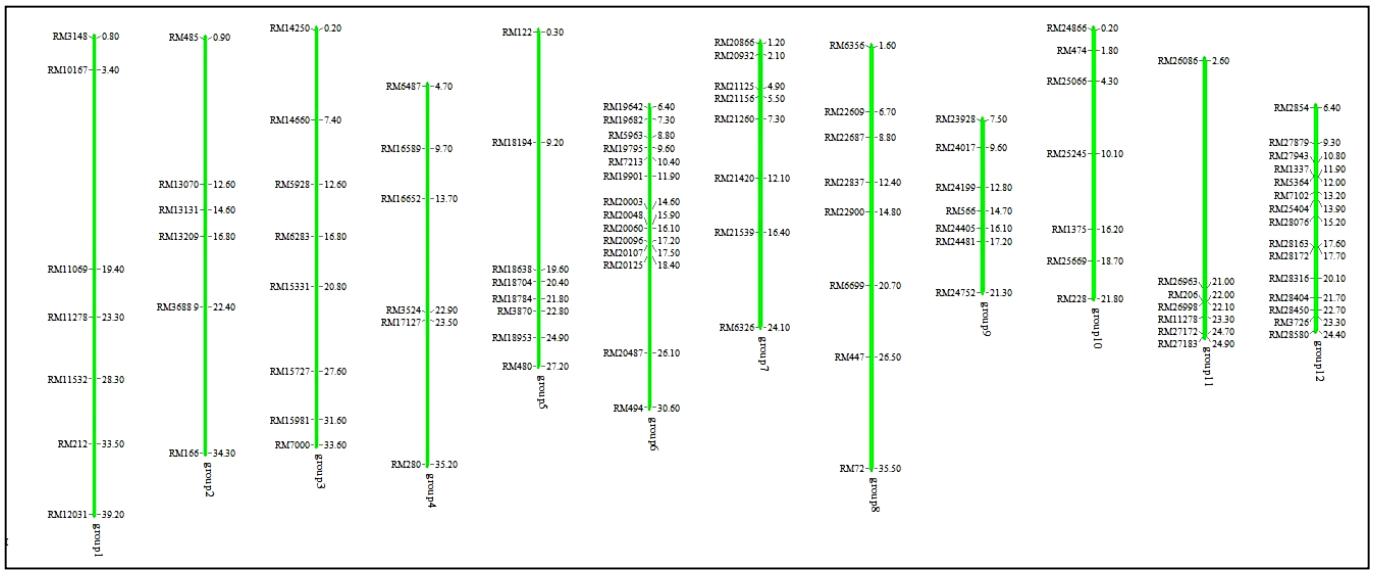


Fig 2: Graphical representation of the polymorphic markers distributed on the each chromosome between blast resistance lines.

The highest and lowest polymorphism was observed in chromosome 5 (17.02%) and chromosome 10 (5.36%), respectively. Recently, Ishwarya *et al*. (2021) [7] reported parental polymorphic survey among two BPH resistant donors (M229, 10-3) and a recipient (Telangana Sona) parent using 494 of SSR markers. Among these, 87 markers were showed polymorphic between M229 and Telangana Sona, while 93 were polymorphic between 10-3 and Telangana Sona. The highest polymorphism percentage was reported in chromosome 12 (30.59%) for Telangana Sona and M229, while chromosome 1 (30.61%) recorded the highest percentage of polymorphism for Telangana Sona and 10-3 indicating that these chromosomes are useful in studying the variation between the parents. Based on these reports, it was observed that number of polymorphic markers and % polymorphism on each chromosome varies due to different genetic background, number of markers employed and number of backcrosses during the line development.

In this study, it was found that low number of polymorphic markers due to the fact that all blast resistance lines had similar background *i.e.*, Improved Samba Mahsuri (ISM), which was also noticed in previous studies by Xu *et al*. (2002) [27] and Biradar *et al*. (2004) [3]. They found less number of polymorphic markers because both the parents were indica genotype. As results indicated less donor genome in (~3%) in blast resistance lines, compared to the recurrent genome, the linkage drag will be less from the donor segments. As blast resistance lines found to have much similar background genome they can be used for pyramiding of two or more blast resistance genes through marker assisted backcross breeding programme.

Conclusion

In the present study, blast resistance lines ISM-ML^{Pi54}, ISM-ML^{Pi9}, ISM-ML^{Pi20}, ISM-ML^{Pi40} and ISM-ML^{Pita} were genotyped with 1800 genome wide SSR markers. Based on results, an average five markers were found polymorphic with % polymorphism ~3%. These results indicates, these blast resistance lines carries less donor genome except targeted blast genes and amenable for developing the pyramided lines

with two or more blast resistance with much reduced effect of linkage drag.

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