

Molecular Marker Validation for Bacterial Leaf Blight Resistant Genes Xa21 and Xa4 in Ld 99-12-38 / IRBB 60 Genetic Background

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ABSTRACT. Bacterial leaf blight (BLB) disease is an emerging issue in rice cultivation of Sri Lanka. *Xanthomonas oryzae* pv. *oryzae* is causal organism. There is no chemical control method practice in the world to control the disease. Use of resistance varieties is most appropriate mode of control. Bacterial blight resistance Xa21 and Xa4 are dominant genes responsible for durable resistance of rice varieties for the disease. The advanced rice line, Ld 99-12-38 is farmer demanding, non lodging type, high yielding rice line and is susceptible to BLB. Therefore, an attempt was taken to improve it resistant to BLB through marker-assisted backcross breeding (MAB) by crossing rice line with resistant variety IRBB60, and advanced plants up to BC₃F₂ level. At the beginning selected two molecular markers namely pTA248 (Xa21) and MP1+MP2 (Xa4) were tested for their polymorphism with parental materials. To identify BLB resistance Xa21 and Xa4 alleles, markers were validated in thirty five BC₃F₂ progenies derived from a cross between Ld 99-12-38/IRBB60. As the results selected two molecular markers were polymorphic with parental materials with the expected fragment sizes. Out of the tested 35 progenies, eight were confirmed with possessing both Xa21 and Xa4 with resistant to the disease, twenty one lines carried only the resistant allele Xa4. A paired t- test revealed that there is a significant difference in the resistance level exerted by progeny carrying resistance alleles of two genes than with one ($p = 0.0064$). In addition, six progeny lines were heterozygote for Xa4 locus; one progeny line was heterozygous at both loci. No lines were identified carrying only the Xa21. Therefore it can be concluded that resistant genes Xa4 and Xa21 has been pyramided to rice lines derived from cross between Ld 99-12-38/IRBB60 in BC₃F₂ progenies with similar morphological characters of Ld 99-12-38 to withstand BLB. The rice line Ld 99-12-38 does not carry any resistance genes regarding BLB and IRBB 60 consist of Xa21 and Xa4 alleles. The selected markers pTA248 and MP1+MP2 can be recommended to in marker- assisted selection of Xa21 and Xa4 genes in crosses involving the tested genetic background.

Key words: bacterial leaf blight, marker-assisted selection, rice, Xa21, Xa4

INTRODUCTION

Rice is the most important and most widely consumed staple food crop in the world. Demand for rice is steadily increasing, as the number of rice consumers is increasing, specially in developing countries (Khush and Jena, 2009). Though rice is world's most important food crop, there are many limiting factors for rice cultivation, such as socio economic, soil and land factors, water supply, availability of good clean and healthy seeds, weeds, pests and diseases, and other biological agents such as rats, birds etc. Rice blast and BLB are the major devastating diseases that limit rice yield significantly (Ou, 1985; Mew *et al.*, 1993). Bacterial Leaf Blight (BLB) is caused by bacterial pathogen called *Xanthomonas oryzae* pv. *oryzae* and is one of the devastating diseases of rice, causing yield losses ranging from 74 to 81% (Srinivasan and Gnanamanickam, 2005). There is no chemical control method practiced in the world to control BLB. Therefore development of resistant varieties is the most appropriate solution to the problem. Development of broad spectrum durable resistance through gene pyramiding for biotic stress resistance can be accelerated through the process of marker assisted selection (MAS) (Joshi and Nayak, 2010). So far, 40 BLB resistance genes have been reported (Khan, Naeem & Iqbal, 2014).

Ld 99-12-38 is a farmer demanding, 3½ month age group high yielding rice line. It is resistant to lodging, brown plant hopper and rice blast. This line was included in the national coordinated rice variety testing

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(NCRVT), variety adaptability testing (VAT) and large scale variety adaptability testing (LSVAT) under different agro ecological zones of Sri Lanka and performed well. However, was not released as a variety due to its susceptibility to BLB. Recently, it was found that this line is tolerant to drought (Wasala *et al.*, 2015). Hence, its importance as a potential germplasm for the development of an elite variety has greatly increased. Therefore, backcross breeding procedure with marker-assisted selection (MAS) is suitable for improving line further with the available BLB resistant variety IRBB 60, containing *Xa4*, *xa5*, *xa13* and *Xa21* resistance genes (Huang *et al.*, 1997). Therefore the objectives of this research is phenotypically assess the disease response of IRBB60, Ld 99-12-38, BC₁F₁, BC₂F₁ and BC₃F₂ progenies of a cross between Ld 99-12-38/IRBB 60, to validate the use of markers *pTA248* and *MP1+MP2* for the presence of *Xa21* and *Xa4* in IRBB 60, Ld 99-12-38 and BC₃F₂ progenies of a cross between Ld99-12-38/IRBB 60, and to develop an advanced breeding lines pyramided with resistant alleles of *Xa21* and *Xa4* confirming resistant to BLB through MAB.

METHODOLOGY

A BLB susceptible Ld 99-12-38 rice line was used as the recipient parent and BLB resistant IRBB 60 variety was used as donor parent. Twenty one days old seedlings of parents were transplanted in the field, with one plant per hill maintaining a spacing of 15x20 cm. All the cultural practices were done as the Department of Agriculture recommendations. At the flowering stage hybridization was carried out by adopting a hot water emasculation method as described by (Garceia, Zaguirre, and Carreres, 2008). The resulted F₁ seeds were collected. Those seeds were established in a nursery and transplanted in field after 21 days along with parental lines. At late booting stage plants were inoculated with BLB inoculum collected from the BLB affected leaves in the field. Inoculum was prepared by cutting disease affected leaves into small pieces and suspended into distilled water about 1/2 hour. The inoculum density was adjusted to 10⁸ CFU/ml (OU, 1985) and the plants were inoculated by clipping tips of 5-10 fully opened leaves as described by Kauffman *et al.*, 1973. Disease reaction was scored two weeks after inoculation, the lesion length of upper most five leaves were measured (cm) using a ruler. At the same time, length of five non destructive corresponding leaves were also measured and diseased leaf area percentage was calculated based on the lesion length and the length of the whole leaf. Plants having lesion length of less than 5cm were considered as resistant (SES for rice IRRI, 2014). The selected resistance plants were back crossed with recipient parent Ld 99-12-38 and BC₁F₁ was obtained. Following the same procedure BC₂F₁, BC₃F₁ were developed. Finally BC₃F₁ population was allowed to selfing and BC₃F₂ was obtained. The selected resistant plants in BC₃F₂ similar characters with recipient parent were subjected for MAS to confirm the presence of BLB resistance *Xa4* and *Xa21* alleles.

DNA Extraction and PCR Amplification

DNA was extracted using two weeks old fresh leaf samples collected from nursery with C-TAB extraction method with few modifications. PCR was carried out and polymorphism was tested for parental lines against selected markers. The PCR was carried out for both markers with 15 µl reaction volumes, with 5 pico moles of each forward and reverse primers, 1x PCR master mix (GoTaq Green Master Mix) and 150ng of each genomic DNA sample. PCR conditions for *pTA248* as, initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation 94°C for 1 minute, 59.1°C for 1 minute annealing and extension 72°C for 2 minutes. Final extension was 72°C for 5 minutes, and PCR conditions for *MP1+MP2* was initial denaturation at 95°C for 4 minutes, and 35 cycles of denaturation 95°C for 30 seconds, annealing 56°C for 30 seconds and extension 72°C for 1 minute. Final extension was 72°C for 10 minutes. Marker polymorphism was visualized in a 3% agarose gel electrophoresis assayed for two hours at gel electrophoresis and post stained with ethidium bromide.

RESULTS AND DISCUSSION

Bacterial leaf blight is caused by the plant pathogenic bacteria *Xanthomonas oryzae* pv. *oryzae*, and is one of the devastating diseases of rice causing yield losses ranging from 74 - 81% (Srinivasan and Gnanamanickam, 2005). When plants are infected at booting stage it results poor quality grains with a high proportion of broken kernels. Breeding and development of resistant varieties carrying major resistance alleles have been the most effective and economical strategy to control BLB with a minimal adverse effect on the environment (Huang *et al.* 1997; Jena and Mackill, 2008; Singh *et al.* 2001).

Host plant resistance has been considered as the most economical and eco-friendly strategy for management of biotic stresses (Hulbert *et al.*, 2001). For BLB there is no effective chemical control method practiced, hence, the only durable strategy is to grow resistant varieties. There are many BLB resistant germplasm available in the world, carrying multiple resistance alleles to the disease. So far 40 genes have been identified with alleles contributing to resistance against BLB (Khan, Naeem, & Iqbal, 2014). However, in Sri Lankan rice varieties an assessment has not been carried out so far. In this investigation, the resistant germplasm IRBB 60 carrying the resistance dominant alleles of *Xa4* and *Xa21* (Huang *et al.*, 1997) were successfully pyramided to the backcross progeny of IRBB 60/Ld 99-12-38.

The gene *Xa21* conveys broad spectrum resistance against BLB, and through wide hybridization *Xa21* was transferred from the wild species *Oryza logistaminata* to the susceptible rice cultivar IR 24, generating the near isogenic line IRBB 21 (Ikeda *et al.*, 1990; Kush *et al.*, 1990). When tested for disease resistance spectrum, IRBB 21 conferred a high level of resistance to many diverse *Xoo* strains from India and the Philippines (Kush *et al.*, 1990). The resistant parent IRBB 60 is a descendent of IRBB 21. The BLB resistance allele *Xa4* identified by Petpisit *et al.*, 1977, is one of the widely exploited resistance genes in many rice breeding programs, and it confers durable resistance at all stages of plant growth in many commercial rice varieties (Mew *et al.*, 1992; Sun *et al.*, 2003).

The advanced breeding line Ld 99-12-38 is a high yielding line with good plant architecture. It is resistant to blast disease, and it is drought tolerant. Though it is important as a valuable germplasm with a potential of has been developed as a variety, its release is hindered as a result of its susceptibility to the BLB. Given its prospectus as a high yielding variety with multiple resistances against major biotic and abiotic stresses. Based on the data collected over four consecutive seasons, it was confirmed that the Ld 99-12-38 advance line was susceptible to BLB, and the IRBB 60 variety was resistant, at the late booting stage to *X.oryzae* pv. *oryzae*. The disease development was observed as early as fourteen days after inoculation in both Ld 99-12-38 and IRBB 60 rice varieties (Fig. 1).

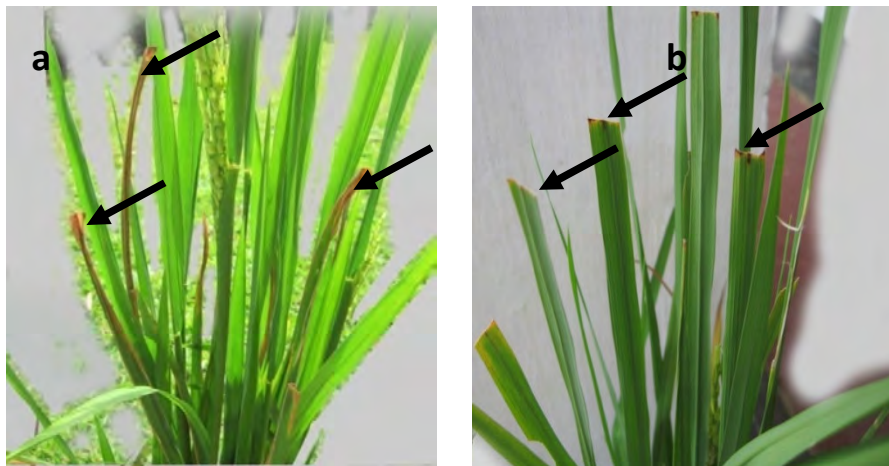


Fig.1. Bacterial leaf blight leaf lesion of (a) Ld 99-12-38 (susceptible parent) and (b) IRBB60 (resistant parent) at 14 days after inoculation with *Xanthomonas oryzae* pv. *oryzae*

A paired *t*- test revealed that the two parents were significantly different ($p = 0.001$) in their response to BLB in all four seasons (Table 01). In all four seasons, IRBB 60 (quantified in terms of BLB disease %) showed a significantly higher level of resistance (0.1%) compared to the Ld 99-12-38 (44%) against *X. oryzae* pv. *oryzae*. Hence, the advanced line Ld 99-12-38 was identified as susceptible in all four field trials, and agreeing with the previous reports IRBB 60 was identified as resistant to BLB. The IRBB 60 rice variety is known to carry resistant alleles *Xa21*, *Xa4*, *xa5*, and *xa13* (Huang *et al.*, 1997) against BLB, however, the Ld 99-12-38 advance line was not characterized to identify the allelic haplotype it carried with respect to the above mentioned genes conferring BLB resistance.

Table 1. Percentage of affected leaf area in Ld 99-12-38 and IRBB 60 rice variety 14 days after inoculation with *Xanthomonas oryzae* pv. *oryzae*

Parental Line	Bacterial leaf blight affected leaf area %					disease response
	14 <i>yala</i>	14/15 <i>maha</i>	15 <i>yala</i>	15/16 <i>maha</i>	average	
Ld 99-12-38	39.5	43.3	44.9	48.4	44.0	susceptible
IRBB 60	0.4	0.3	1.1	00.4	0.5	resistant

Backcrossing is a well-known and long established breeding scheme, where a desired character is introgressed from a donor parent into the genomic background of a recurrent parent. Hence, the main purpose of backcrossing is to introduce the desired character while restoring the elite donor's genetic background in the resultant progeny. With the assistance of MAS backcrossing process will accelerate the selection of desired plants in early generations. This is beneficial in rice breeding programs because it will reduce the number of unnecessary phenotypes from the population, and target only the desired in consecutive crosses. The reduced number of plants in the trial saves land, labor and input cost. In this investigation backcrossing was performed to introduce the desired resistance alleles of *Xa21* and *Xa4* present in IRBB 60 to IRBB 60/Ld 99-12-38 derived progeny, and restore the genetic background of elite Ld 99-12-38. In each backcrossing the selection was made to pick lines with desirable traits along with low BLB affected leaf area percentage, indicating resistance to BLB.

Here, out of 105 plants in BC₁F₁ inoculated with *X. oryzae* pv. *oryzae*, 31 plants were categorized as resistant and 21 plants as moderately resistant (Fig.2). Out of these 35 plants carrying the desired attributes of the recipient parent Ld 99-12-38 were identified, and was advanced up to BC₃F₂ through backcrossing.

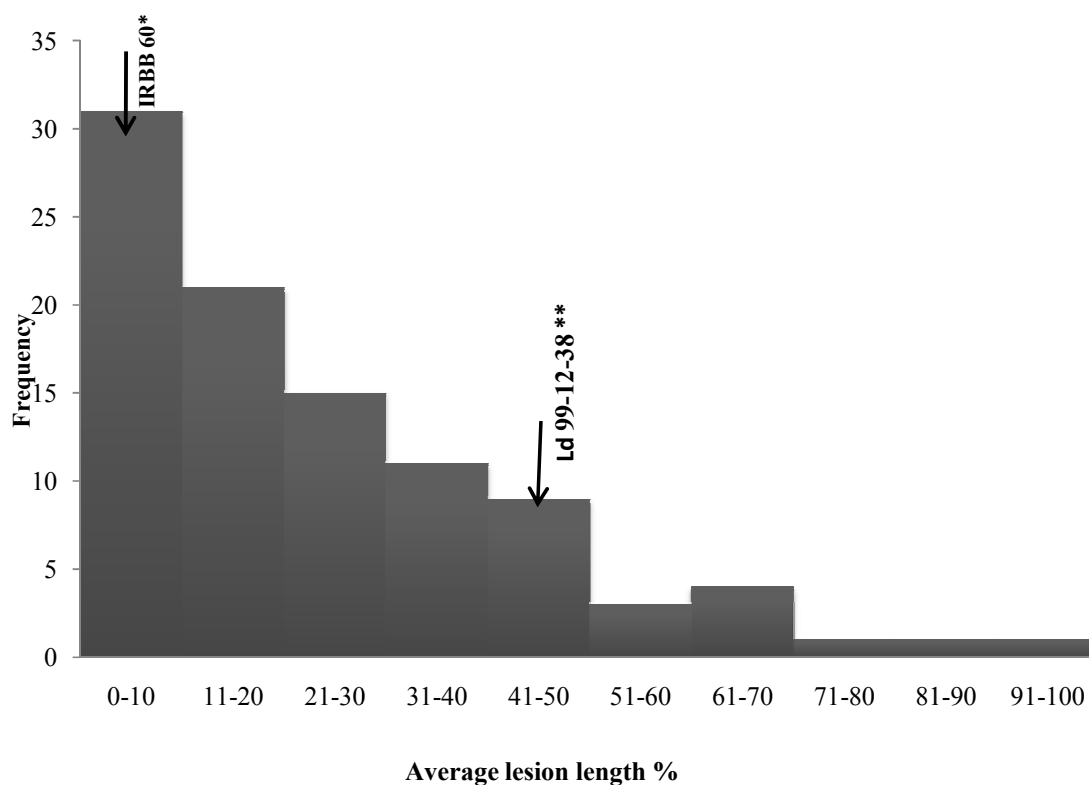


Fig. 2. Histogram of percentage disease severity in 105 BC₁F₁ plants and their parents (IRBB 60 and Ld 99-12-38) at 14 days after inoculation with *Xanthomonas oryzae* v. *oryzae*. The BLB scale is given as resistant (1-5%), moderately resistant 6-12%, moderately susceptible (13-25%), susceptible (25-50%), and susceptible (>50%).

Marker-assisted backcrossing (MAB) with accurate phenotypic selection is the most effective method for a selective transfer or pyramiding of resistance alleles into elite rice varieties, eventually restoring the desired recurrent parent genotype (Joseph *et al.* 2004; Shanti *et al.* 2010; Singh *et al.* 2001; Suh *et al.*, 2011). It is reported that MAB has been successfully used to introduce a wide spectrum of resistance alleles against *Xoo* isolates/races in many newly developed rice varieties.

According to the morphological data analysis of the 35 BC₃F₂ advanced lines and the resistant parent IRBB 60 (Fig.03), 29 lines were categorized as resistant, and six lines as moderately resistant to BLB.

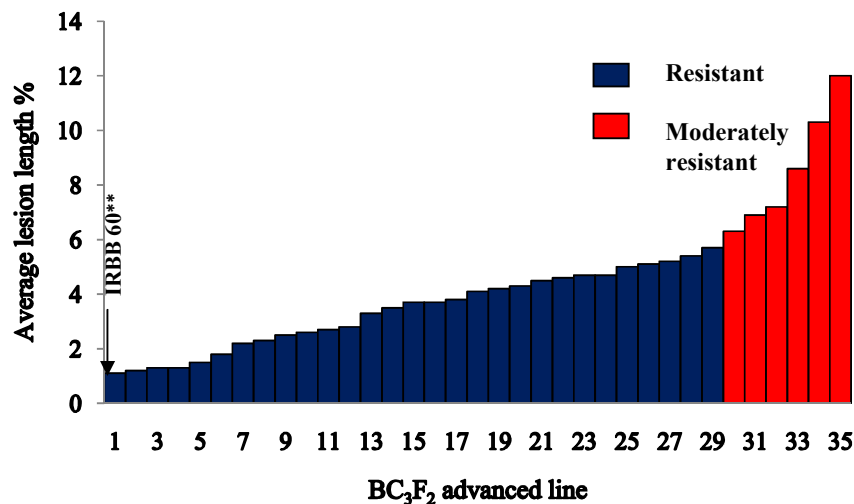


Fig. 3. Bacterial leaf blight affected average leaf lesion length % of 35 BC₃F₂ advanced lines

Molecular markers can be used to identify and pyramid favorable alleles in to a selected genetic background (Jena and Mackill, 2008; Lee *et al.* 2003; Singh *et al.* 2001; Suh *et al.*, 2009). The molecular level variations of the genes *Xa21* and *Xa4* exists as a result of the allelic differences, which gets expressed in terms of resistance/susceptibility to BLB. Out of the two genes *Xa21* contributes the largest to BLB resistance. These alleles can be tracked by using molecular markers to identify desired alleles and eliminate the undesired during selection.

According to the results the IRBB 60 variety amplified a fragment for 925 bp for the marker *pTA248*, confirming the presence of the resistance allele of *Xa21* (Table.2; Fig.4a). Further, the Ld 99-12-38 line amplified a fragment of 730 bp, which was previously identified as the fragment getting amplified by the susceptible allele (Table.2; Fig.4a). The marker *MP1+MP2* amplified a fragment of 150 bp from the variety IRBB 60 for the *Xa4* gene (Table.2; Fig.4b). Further, the Ld 99-12-38 line amplified a fragment of 120 bp, which was previously identified as the fragment getting amplified by the susceptible-allele carrier (Table.2; Fig.4b). Based on the fragments amplified by the linked - markers *pTA248* (*Xa21*) and *Mp1+Mp2* (*Xa4*), it can be confirmed that the variety IRBB 60 and advance breeding line Ld 99-12-38 carried the resistance and susceptibility allele, respectively.

Table 2. PCR primers, primer sequence, linked genes, expected resistance and susceptible fragment sizes of *pTA248 (Xa21)* and *MPI+Mp2 (Xa4)* primers.

Marker/ Linked gene	Sequence	Resistant fragment size	Suscep fragment size	Reference
<i>pTA248</i> (<i>Xa 21</i>)	F- 3'AGACGCGGAAGGGTGGTTCCCGGA5'	925 bp	730 bp	Ronald <i>et al.</i> ,1992 Huang <i>et al.</i> , 1997 Sudarman <i>et al.</i> ,2008
	R- 3'AGACGCGTAATCGAAAGATGAAA 5'			
<i>MPI+MP2</i> (<i>Xa4</i>)	F- 3'ATCGATCGATCTTCACGAGG5' R- 3'TGCTATAAAAAGGCATTCGG5'	150 bp	120 bp	Sun <i>et al.</i> ,2003

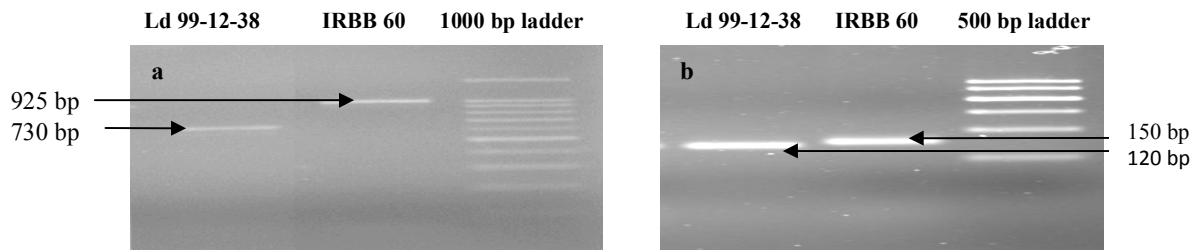


Fig.4. Amplified fragments of (a) Ld 99-12-38 and IRBB 60 for marker *pTA248 (Xa21)*, and (b) Ld 99-12-38 and IRBB 60 for marker *Mp1+Mp2 (Xa4)*.

When the thirty five BC₃F₂ lines derived from a cross between Ld 99-12-38 / IRBB 60 were tested with the BLB resistance gene (*Xa21* and *Xa4*) linked molecular markers *pTA248* and *MPI+ MP2* it was confirmed that each line at least carried one of the resistance alleles from either *Xa21* or *Xa4* (Fig. 5a;5b;Table. 3)

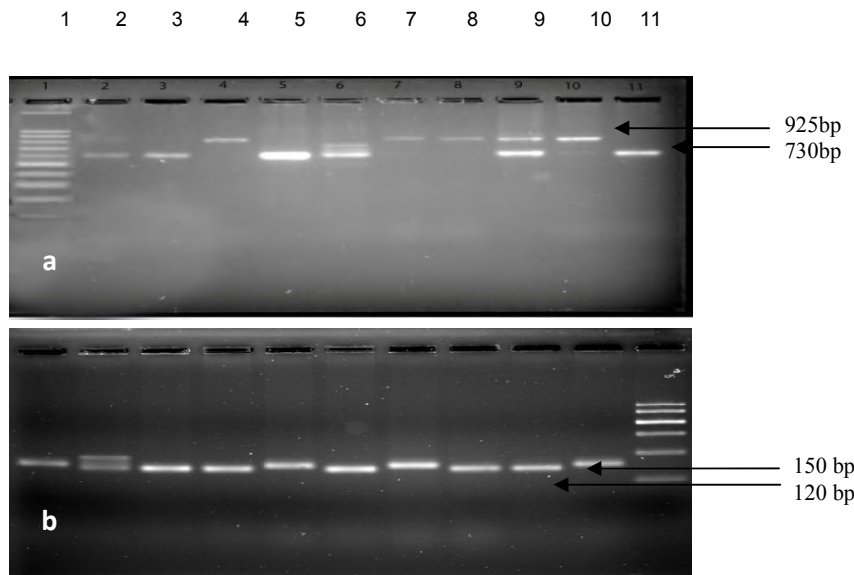


Fig. 05. (a). Amplified fragments of parental lines Ld 99-12-38 and IRBB 60 with BC₃F₂ advanced lines for marker *pTA248 (Xa21)* and (b) Amplified fragments of parental lines and BC₃F₂ progenies for marker *MPI+MP2 (Xa4)*

Out of the tested 35 lines eight lines identified as resistant in the phenotypic trial were confirmed to carry the resistance alleles of both *Xa21* and *Xa4* genes (Table 3). Twenty one progeny carried only the resistant allele for *Xa4*, among which was a combination of resistant and moderately resistant. A paired *t* test revealed that there is a significant difference in the resistance level exerted by progeny carrying resistance alleles of two genes than with one ($p = 0.0064$). Among the tested line none carried only *Xa21* resistance allele. In addition, six progeny were heterozygote for *Xa4* locus, and one progeny line was heterozygous at both loci, and these heterozygotic progeny were found to be resistant and hence, has an importance as a genetic resource for further studies.

Table 3 Molecular screening of BLB resistance in BC₃F₂ rice progenies with Parental lines for markers *pTA248 (Xa21)* and *MPI+MP2 (Xa4)*

BC ₃ F ₂ progenies	<i>Xa21(pTA248)</i>		<i>Xa4(MPI+MP2)</i>		Allele	
	925 bp	730 bp	150 bp	120 bp	<i>Xa21</i>	<i>Xa4</i>
Ld 99-12-38	-	+	-	+	S	S
IRBB60	+	-	+	-	R	R
10R	+	-	+	-	R	R
93MR2	+	-	+	-	R	R
100MR	+	-	+	-	R	R
59MR	+	-	+	-	R	R
63MR	+	-	+	-	R	R
93MR1	+	-	+	-	R	R
Fiii	+	-	+	-	R	R
101R	+	-	+	-	R	R
C	-	-	+	-	-	R
I	+	+	+	+	-	R
9iii	-	-	+	-	-	R
3	-	-	+	-	-	R
6	-	-	+	-	-	R
H	-	-	+	-	-	R
97R	-	-	+	-	-	R
8Rii	-	-	+	-	-	R
72R	-	-	+	-	-	R
8R	-	-	+	-	-	R
94MR	-	-	+	-	-	R
9ii	-	-	+	+	-	R
54MR	-	-	+	-	-	R
92MR	-	-	+	-	-	R
92MR2	-	-	+	-	-	R
55	-	-	+	-	-	R
99MR2	-	-	+	-	-	R
11	-	-	+	+	-	R
18	-	-	+	+	-	R
02	-	-	+	+	-	R
9i	-	-	+	+	-	R
Fi	-	-	-	+	-	MR
89R	-	-	+	+	-	MR
12	-	-	+	-	-	MR
Fii	-	-	+	-	-	MR
89Ri	-	-	+	-	-	MR
8	-	-	+	-	-	MR

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R-resistance, MR-moderately resistance, S-susceptible, + fragment present, - fragment absent

The eight lines carrying the resistance allele for both *Xa21* and *Xa4* were re-evaluated for their resistance responses. They were confirmed to carry the resistance alleles for *Xa21* and *Xa4* and was categorized as resistant based on the disease response and carried the elite characteristics of Ld 99-12-38. These eight advance breeding lines can be used in future rice breeding programs towards releasing an elite variety with BLB resistance and as a valuable germplasm resource for breeders.

CONCLUSION

The advance breeding line Ld 99-12-38 was susceptible to *X. oryzae* pv *oryzae*, while IRBB 60 was resistant. The molecular markers *pTA248* and *Mpl+MP2* can be successfully used in MAB to pyramid the resistance alleles of *Xa21* and *Xa4* to achieve sustainable resistance. Eight BC₃F₂ lines pyramided with resistance alleles of *Xa21* and *Xa4* were developed, and was made available for future research at the Regional Rice Research and Development Centre, Bombuwela, Sri Lanka.

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