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Molecular characterization of bacterial leaf blight resistant near isogenic lines of rice (*Oryza sativa L.*) using RAPD and SSR markers

Ankita A. Patel[1*], G.C. Jadeja[1], N. Subhash[2] and Y. M. Shukla[3]

[1]*Department of Genetics and Plant breeding, Anand Agricultural University, Anand-388110, Gujarat, India*
[2]*Department of Plant Tissue Culture, Anand Agricultural University, Anand-388110, Gujarat, India*
[3]*Department of Biochemistry, Anand Agricultural University, Anand-388110, Gujarat, India*

Corresponding author: Ankita A. Patel; anki.4404@gmail.com

Abstract

Rice (*Oryza sativa* L.) is nutritionally, one of the most important cereal crops. Rice production is mostly reduced due to different biotic stresses. Bacterial leaf blight, caused by *Xanthomonas oryzae* pv. *oryzae* bacteria, is one of the major biotic destructive diseases throughout the world. This disease affects the rice production because it reduces the photosynthesis by causing leaf wilting. The preferred strategy for disease management is through varietal resistance because it is not fully controlled by any chemical treatments. Gene pyramiding is the most effective strategy; in which, pyramided lines with three to four different bacterial leaf blight resistance genes have been developed in the genetic background of popular rice cultivars by using marker-assisted selection. The present study was carried out with an aim to study the molecular characterization of 30 rice genotypes for BLB resistance using RAPD and SSR markers. Overall results on the basis of clustering pattern of SSR and RAPD pooled data analysis revealed genetic similarity between some of the pyramided lines with their respective recurrent parent. Some of the SSR markers are very informative and can be useful for marker-assisted selection.

Highlights

- DNA extraction was done using modified CTAB method from 30 different rice samples
- Study themolecular characterization of 30 rice genotypes for BLB resistance using RAPD and SSR markers
- Overall results on the basis of clusteringpattern of SSR and RAPD pooled data analysis revealed genetic similarity between some of the pyramided lines with their respective recurrent parent
- Some of the SSR markers are very informative and can be useful for marker-assisted selection

Keywords: Rice, Bacterial Leaf Blight (BLB), Near Isogenic Lines (NILs), Disease resistance genes, Molecular markers, RAPD (Randomly Amplified Polymorphic DNA), SSR (Simple Sequence Repeat) Marker assisted selection.

Rice (*Oryza sativa* L.) is the staple food for nearly 50 percent of the world population and serves as a major carbohydrate source. After China, India is the second largest rice producing country in the world. In India, mostly rice growing states include: West Bengal, Andhra Pradesh, Uttar Pradesh, Punjab, Orissa, Tamil Nadu, Chhattisgarh, Bihar, Karnataka and Haryana. In India, rice production was recorded 96 million tonnes in 2010–11, 103.4 million tonnes in 2011–12 and 100 million tonnes in 2012-13 (FAO, Agricultural Outlook and Situation Analysis Reports, United Nations, 2012-13). Rice is a self-pollinated crop with a monocot seed. The basic chromosome number of the genus is n=12. The Oryza species are either diploid (2n=24) or tetraploids (2n=48). It is an ideal model plant for the study of grass genetics and genome organization due to its diploid

genetics, relatively small genome size of 430 Mb (Causse *et al*., 1994; Kurata *et al*., 1994), significant level of genetic polymorphism, large amount of well conserved genetically diverse material and compatible wild species (Tanksley *et al*., 1989; Wang *et al*., 1992).

Severe loss in rice production occurs due to damage by pathogens such as fungus, bacteria and pests. Major diseases of rice are rice blast *(Magnaporthe grisea)*, Sheath blight (*Rhizoctonia solani)* and bacterial leaf blight (BLB) (*Xanthomonas oryzae* pv. *oryzae) (Xoo*-family Pseudomonadaceae, a rod-shaped gram-negative bacterium). Bacterial leaf blight (BLB) is one of the major biotic destructive diseases throughout the world (Khan, 1996). It causes leaf wilting, affects photosynthesis and reduces 1000-grain weight and generally results in yield loss upto 20-50%. The chemical control of bacterial leaf blight is not effective. Therefore, the preferred strategy for disease management is through varietal resistance. Plant disease resistance is often controlled by Mendelian genetics and follows a gene-for-gene relationship in many plant species and their pathogens (Flor, 1971). In rice, single-gene resistance has been the primary means of control of bacterial leaf blight, but unfortunately, due to continuous and large-scale use of single-gene resistance, there has been a shift in the virulence pattern of the strains, leading to breakdown of resistance (Mew *et al.,* 1992). One method, to delay breakdown of resistance is to provide a broad-spectrum of resistance by combining multiple genes (gene pyramiding) having complementary resistance spectra, relative to the pathogen sub-populations, into a single plant genotype (Babujee and Gnanamanickam, 2000). Gene pyramiding would result in genotypes with more durable resistance through both ordinary gene action and quantitative complementation (Ogawa *et al.,* 1987; Yoshimura *et al.,* 1995). A total of thirty five bacterial leaf blight resistance genes have been identified in cultivated rice and the wild relatives (Nino-Lui *et al*., 2006; Wang *et al*., 2009). Eleven of them are recessive resistance genes including *xa5*, *xa8*, *xa13*, *xa15*, *xa19*, *xa20*, *xa24*, *xa28*, *xa31* and *xa32* (Nino *et al*., 2006; Singh *et al*., 2007; Rao, 2003) and some are dominant genes such as *Xa1, Xa2, Xa3, Xa4, Xa7, Xa10, Xa11, Xa12, Xa14, Xa16, Xa17, Xa18, Xa21, Xa22* etc (Kinoshita, 1991; Lin *et al.,* 1996; Chun *et al.,* 2007). *Xa21* is a dominant resistant locus that confers resistance to all Indian and Philippine races of *Xanthomonas oryzae* pv. *oryzae* tested (Ikeda *et al.,* 1990) and encodes leucine-rich repeat (LRR) receptor kinase-like proteins. It is mapped on chromosome 11. The *xa5* is the recessive resistant locus which encodes the gamma subunit of transcription factor IIA (TFIIAγ) and mapped on the short arm of chromosome 5. The *xa13* is the recessive resistant locus which specifically confers resistance to the Philippine *Xanthomonas oryzae* pv. *oryzae* race 6. The *xa13* gene was first discovered in the rice variety BJ1 and mapped on the long arm of rice chromosome 8 (Ogawa *et al.,* 1987; Zhang *et al.,* 1996; Sanchez *et al.,* 1998). The *xa13* has been shown to be a mutation in the promoter region of a gene that is a homolog of the nodulin MtN3 (Chu *et al*., 2006). *Xa4* is a dominant resistant locus and mapped on the terminal region of long arm of chromosome 11. The pyramided rice lines used in this study, possess R genes *viz*, *Xa4*, *xa5*, *xa13*, *Xa21,* in the genetic background of popular rice cultivars, IR 64, Swarna, Lalat, Tapaswini, Pusa Basmati-1 and Samba Masuri.

Detection and analysis of genetic variation can help us to understand the molecular basis of various biological phenomena in plants. Since the entire plant Kingdom cannot be covered under sequencing projects, molecular markers and their correlation to phenotypes provide us with requisite landmarks for elucidation of genetic variation. Genetic or DNA based marker techniques such as, RAPD (random amplified polymorphic DNA), SSR (simple sequence repeats) and AFLP (amplified fragment length polymorphism) are routinely being used in ecological, evolutionary, taxonomical, phylogenic and genetic studies of plant sciences. The basis of RAPD technique is differential PCR amplification of genomic DNA. It deduces DNA polymorphisms produced by ‘‘rearrangements or deletions at or between oligonucleotide primer binding sites in the genome’’ using short random oligonucleotide sequences (mostly ten bases long) (Williams *et al*., 1991). RAPD analysis is simple, quick to perform, requires only small amount of DNA, relatively less expensive, no need of genome sequence information, can be employed across species using universal primers and provides variation data at multiple loci (Williams *et al*., 1991). Microsatellite or SSR are monotonous repetitions of very short (one to five) nucleotide motifs,

Table-1: Rice genotypes used for molecular analysis

Sr. No.	Genotypes	Sr. No.	Genotypes
Bacterial leaf blight resistant NILs and recurrent parents		16	IET 21067 (NIL of Tapaswini) (xa5, xa13, Xa21)
1	IR 64 (Recurrent parent)	17	IET 21069 (NIL of Tapaswini) (*xa5, xa13, Xa21*)
2	IET 20667 (NIL of IR 64) (*Xa4, xa5, xa13, Xa21*)	18	IET 21070 (NIL of Tapaswini) (*xa5, xa13, Xa21*)
3	IET 20668 (NIL of IR 64) (*Xa4, xa5, xa13, Xa21*)	19	IET 21071 (NIL of Tapaswini) (*xa5, xa13, Xa21*)
4	IET 20669 (NIL of IR 64) (*Xa4, xa5, xa13, Xa21*)	20	IET 21072 (NIL of Tapaswini) (*xa5, xa13, Xa21*)
5	SWARNA (Recurrent parent)	21	IRBB 60 (NIL of IR24) (*Xa4, xa5, xa13, Xa21*)
6	IET 20670 (NIL of Swarna) (*xa5, xa13, Xa21*)	22	SAMBA MASURI (Recurrent parent)
7	IET 20671(NIL of Swarna) (*xa5, xa13, Xa21*)	23	IET 19046 (NIL of Samba Masuri) (*xa5, xa13, Xa21*)
8	IET 20672 (NIL of Swarna) (*xa5, xa13, Xa21*)	24	PUSA BASMATI -1 (Recurrent parent)
9	LALAT (Recurrent parent)	25	IET 18990 (NIL of Pusa Basmati) (*xa13, Xa21*)
10	IET 21063 (NIL of Lalat) (*xa5, xa13, Xa21*)	**Bacterial leaf blight susceptible rice genotypes**	
11	IET 21064 (NIL of Lalat) (*xa5, xa13, Xa21*)	26	GR 11
12	IET21065 (NIL of Lalat) (*xa5, xa13, Xa21*)	27	GR 4
13	IET 21066 (NIL of Lalat) (*xa5, xa13, Xa21*)	28	Jaya
14	IET 21068 (NIL of Lalat) (*xa5, xa13, Xa21*)	29	GR 3
15	TAPASWINI (Recurrent parent)	30	TN 1

which occur as interspersed repetitive elements in all eukaryotic genomes (Tautz and Renz, 1984). Variation in the number of tandemly repeated units is mainly due to strand slippage during DNA replication. Microsatellites are highly popular genetic markers because of their co-dominant inheritance, high abundance, locus-specificity, high reproducibility, multi-allelic nature and the ease of assessing SSR size variation by PCR with pairs of flanking primers.

Materials and Methods

The seeds of 30 rice genotypes (Table 1) which included, 19 NILs, six recurrent parents and five susceptible check cultivars used in the study were obtained from the Main Rice Research Station, Anand Agricultural University, Nawagam, Gujarat, India.

Plant material and DNA extraction

The seeds of 30 rice genotypes (Table-1) were grown in pots, 15 days old seedlings were collected and used for isolating genomic DNA. DNA was extracted using a modified CTAB method (Zidani *et al*., 2005). All of the required reagents were prepared as per Sambrook *et al*., (1989). Fresh leaf tissues (0.3 g) was ground in liquid nitrogen and taken into a 2 ml microcentrifuge tube. The ground sample was extracted with 0.8 ml of CTAB extraction buffer. [To prepare 10 ml of CTAB extraction buffer, 1 M Tris HCL (1ml), 0.5 M EDTA (1ml), 5 M NaCl (2.8ml) were mixed with 4 ml of distilled water. Then, 4% CTAB (0.4g) and 1% PVP (0.1g) were added to this mixture. Dissolved PVP and CTAB properly and heated this mixture at 60°C (about 20-30 minute). Two percentage of β-mercaptoethanol (200µl) was added, just before use.] Sample was mixed well with extraction buffer by inversion. The sample was incubated for one hour at 65°C in water bath (allowed it to cool down). Equal amount of 800 µl of chloroform: isoamylalcohol (24:1) was added to centrifuge tube and mixed by inversion. Centrifugation was carried out it for 20 minutes at 10,000 rpm at 4°C. Supernatant was transferred into a new tube and further extracted with chloroform: isoamyl alcohol (24:1), and the DNA was precipitated with 80% ethanol. The pellet was air dried and resuspended in 100µl of Tris–EDTA

Table-2: List of RAPD primers, range of molecular weight amplified by primers, total number of bands and loci, number of polymorphic loci and% polymorphism, Polymorphism Information Content (PIC) values obtained by analyzing 30 genotypes of rice

Sr. No.	Primer Name	Range of molecular weight (bp)	Total No. of bands	Total No. of Loci	No of polymorphic loci	Percent Polymorphism (%)	PIC value
1	OPA-03	245-4702	277	13	10	76.92	0.91
2	OPA-04	210-4939	263	11	7	63.64	0.90
3	OPA-05	165-1283	129	9	9	100	0.86
4	OPA-09	247-3771	100	7	7	100	0.82
5	OPA-12	227-1892	138	12	12	100	0.88
6	OPA-15	168-2055	157	11	10	90.91	0.88
7	OPAB-09	194-1837	215	12	12	100	0.90
8	OPAC-05	212-1164	138	9	7	77.78	0.84
9	OPAE-03	201-5226	150	9	9	100	0.86
10	OPB-17	319-1130	193	9	9	100	0.88
11	OPC-11	442-1602	88	4	2	50	0.72
12	OPH-03	182-1411	160	8	6	75	0.84
13	OPH-04	217-768	100	7	7	100	0.84
14	OPK-03	278-1439	109	5	4	80	0.78
15	OPK-07	107-1047	138	9	7	77.78	0.84
16	OPL-11	310-1313	115	9	8	88.89	0.83
17	OPM-02	617-5193	216	15	14	93.33	0.91
18	OPP-01	95-930	136	8	7	87.5	0.84
19	OPP-03	155-1732	183	18	18	100	0.93
20	OPP-06	138-5713	335	18	16	88.89	0.94
21	OPP-17	135-1685	200	15	15	100	0.92
22	S-34	140-2932	155	11	9	81.82	0.87
23	S-70	177-2287	133	10	9	90	0.87
24	ES-13	134-1838	201	13	11	84.62	0.89
25	ES-14	172-1359	254	16	14	87.5	0.92
26	ES-15	132-2072	251	12	11	91.67	0.90
27	ES-17	179-3210	378	16	14	87.5	0.93
28	ES-19	281-1614	78	7	7	100	0.78
29	ES-22	410-1001	128	5	2	40	0.79
TOTAL			**5118**	**308**	273	2513.75	25.07
AVERAGE			176	10.62	9.41	86.68	0.86

Table 3: Jaccard's similarity coefficient of 30 rice genotypes based on RAPD data analysis

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	1																													
2	0.74	1																												
3	0.66	0.77	1																											
4	0.67	0.82	0.72	1																										
5	0.62	0.74	0.73	0.74	1																									
6	0.62	0.7	0.64	0.69	0.63	1																								
7	0.66	0.71	0.73	0.68	0.67	0.63	1																							
8	0.54	0.63	0.64	0.56	0.64	0.6	0.6	1																						
9	0.64	0.75	0.72	0.72	0.71	0.68	0.73	0.7	1																					
10	0.64	0.78	0.68	0.78	0.72	0.69	0.68	0.63	0.75	1																				
11	0.58	0.7	0.69	0.68	0.73	0.64	0.67	0.64	0.7	0.7	1																			
12	0.53	0.64	0.62	0.64	0.66	0.59	0.59	0.59	0.65	0.64	0.69	1																		
13	0.61	0.71	0.71	0.69	0.73	0.71	0.67	0.7	0.72	0.71	0.75	0.66	1																	
14	0.59	0.69	0.65	0.7	0.77	0.61	0.67	0.61	0.67	0.68	0.71	0.66	0.73	1																
15	0.59	0.66	0.67	0.65	0.7	0.58	0.64	0.58	0.68	0.7	0.72	0.76	0.67	0.69	1															
16	0.38	0.35	0.37	0.36	0.37	0.38	0.4	0.37	0.39	0.4	0.39	0.42	0.42	0.4	0.4	1														
17	0.44	0.45	0.44	0.45	0.42	0.43	0.49	0.42	0.48	0.47	0.43	0.42	0.43	0.46	0.44	0.65	1													
18	0.38	0.42	0.44	0.44	0.46	0.39	0.43	0.41	0.45	0.46	0.44	0.43	0.45	0.46	0.45	0.6	0.63	1												
19	0.43	0.46	0.47	0.48	0.47	0.46	0.53	0.42	0.51	0.5	0.45	0.43	0.48	0.5	0.46	0.64	0.74	0.64	1											
20	0.41	0.39	0.4	0.39	0.4	0.44	0.44	0.38	0.43	0.44	0.39	0.4	0.44	0.45	0.38	0.61	0.58	0.62	0.67	1										
21	0.44	0.47	0.48	0.48	0.5	0.45	0.53	0.43	0.51	0.52	0.5	0.46	0.49	0.52	0.5	0.51	0.62	0.64	0.69	0.65	1									
22	0.41	0.46	0.48	0.47	0.48	0.41	0.48	0.44	0.48	0.49	0.48	0.45	0.48	0.49	0.48	0.52	0.59	0.65	0.63	0.62	0.81	1								
23	0.46	0.45	0.44	0.46	0.47	0.45	0.47	0.42	0.49	0.48	0.47	0.42	0.49	0.5	0.46	0.59	0.61	0.57	0.71	0.63	0.68	0.73	1							
24	0.41	0.48	0.48	0.49	0.5	0.42	0.48	0.41	0.49	0.51	0.49	0.44	0.5	0.48	0.48	0.5	0.6	0.64	0.67	0.6	0.75	0.78	0.71	1						
25	0.41	0.47	0.5	0.46	0.48	0.44	0.49	0.45	0.5	0.48	0.49	0.46	0.48	0.47	0.47	0.54	0.64	0.61	0.66	0.6	0.71	0.76	0.67	0.78	1					
26	0.38	0.41	0.41	0.42	0.44	0.37	0.42	0.39	0.43	0.45	0.4	0.41	0.42	0.43	0.43	0.49	0.52	0.6	0.59	0.54	0.64	0.6	0.6	0.67	0.63	1				
27	0.44	0.47	0.48	0.49	0.47	0.41	0.5	0.41	0.48	0.49	0.46	0.45	0.45	0.5	0.48	0.52	0.58	0.57	0.67	0.54	0.67	0.67	0.65	0.67	0.71	0.68	1			
28	0.44	0.48	0.51	0.48	0.48	0.42	0.49	0.44	0.49	0.49	0.48	0.45	0.47	0.49	0.47	0.58	0.65	0.57	0.67	0.57	0.67	0.69	0.67	0.73	0.77	0.67	0.74	1		
29	0.44	0.47	0.51	0.48	0.48	0.42	0.47	0.42	0.47	0.48	0.45	0.44	0.44	0.48	0.44	0.53	0.62	0.58	0.68	0.57	0.66	0.69	0.67	0.68	0.71	0.65	0.73	0.75	1	
30	0.38	0.4	0.41	0.4	0.41	0.39	0.4	0.43	0.43	0.42	0.42	0.43	0.43	0.43	0.41	0.54	0.54	0.54	0.57	0.51	0.53	0.59	0.59	0.56	0.58	0.56	0.59	0.59	0.6	1

1) IR-64	2) IET-20667	3) IET-20668	4) IET-20669	5) SWARNA	6) IET-20670
7) IET-20671	8) IET-20672	9) LALAT	10) IET-21063	11) IET-21064	12) IET-21065
13) IET-21066	14) IET-21068	15) TAPASWINI	16) IET-21067	17) IET-21069	18) IET-21070
19) IET-21071	20) IET-21072	21) IRBB-60	22) GR-11	23) SAMBAMASURI	24) IET-1904
25) PUSA BASMATI	26) IET-18990	27) GR-4	28) JAYA	29) GR-3	30) TN-1

(TE) buffer. To estimate the quantity and quality (in terms of protein and RNA contamination) of isolated genomic DNA, spectrophotometry was performed. One microliter of DNA sample was loaded on to the well of Nanodrop instrument. The concentration of DNA and absorbance at 260 nm and 280 nm were measured. To check the DNA quality of isolated genomic DNA, electrophoresis was done using 0.8% agarose gel prepared in 1X TBE buffer.

RAPD amplification

Amplification of RAPD fragments was performed according to Williams *et al.* (1990) using decamer arbitrary primers (Operon technologies Inc, USA; SIGMA-D, USA) (Table 2). Amplification were performed in a 25 µl reaction volume containing 1.5 µl DNA (50 ng/µl), 2.5 µl of PCR buffer (10 x) with 15 mM $MgCl_2$, Bangalore Genei, India, 1 µl of Primer (10 p moles/µl) (MWG), 0.5 µl of dNTPs (2.5 mM) Bangalore Genei, India, 0.5 µl of *Taq* DNA polymerase (3U/µl) and 19 µl of sterile distilled water. Amplification was performed in a programmed thermocycler with initial denaturation at 94°C for 5min, 35 cycles of denaturation at 94°C for 1min, primer annealing at 38°C for 1min, extension at 72°C for 1min, and final extension at 72°C for 5 min. Amplified products were electrophoresed on 1.5% agarose in 1*x*TBE buffer. The gels were stained with ethidium bromide and documented using gel documentation system (Bio-Rad, Hercules, California).

Table 4: List of SSR primers predicted for specific bacterial leaf blight resistance genes, Position of genes on chromosomes, total number of amplified bands, range of molecular weight amplified by primers, number of amplified alleles, Polymorphism Information Content (PIC) values obtained by analyzing 30 genotypes of rice

Sr. No.	Primer name	Predicted gene	Chromosome No.	Total No. of bands	Range of molecular weight (bp)	No. of amplified alleles	PIC value
1	RM 167	*Xa4*	11	30	128-133	3	0.39
2	RM 144	*Xa4*	11	38	120-325	3	0.55
3	RM 13	*xa5*	5	36	124-400	4	0.67
4	RM 31	*xa5*	5	31	137-172	4	0.59
5	RM 122	*xa5*	5	30	230-250	3	0.46
6	RM 164	*xa5*	5	30	246-256	2	0.39
7	RM 390	*xa5*	5	37	166-185	3	0.39
8	RM 611	*xa5*	5	67	130-250	4	0.66
9	RM 39	*xa5*	5	30	113-122	2	0.39
10	SR 6	*xa13*	8	28	234-259	2	0.07
11	SR 11	*xa13*	8	40	202-785	8	0.46
12	RM 21	*Xa21*	11	43	110-400	6	0.73
13	RM 349	*Xa2*	4	75	108-435	7	0.81
14	RM 317	*Xa2*	4	33	78-170	3	0.73
15	M3	*Xa7*	6	16	513-560	3	0.54
16	RM 500	*xa8*	7	30	153-200	3	0.49
17	RM 533	*xa8*	7	33	270-279	2	0.50
18	RM 206	*Xa10*	11	34	127-175	3	0.64
19	RM 139	Not gene specific	11	107	65-1289	12	0.86
20	RM 331	Not gene specific	8	30	173-184	2	0.39
TOTAL				**798**		75	10.71
AVERAGE				39.9		4	0.53

SSR amplification

SSR amplification reactions were carried out in 25µl volume containing 1.5 µl of DNA (50 ng/µl), 2.5 µl of PCR buffer (10 x) Bangalore Genei, India, 0.5 µl of Forward Primer and 0.5 µl of Reverse Primer (10 p moles/µl) (MWG) (Table-3), 0.5 µl of dNTPs (2.5 mM) Bangalore Genei, India, 0.5 µl of *Taq* DNA polymerase (3U/µl) and 19 µl of sterile distilled water. The amplification reaction consisted of an initial denaturation step at 94°C for 5min, followed by 30 cycles of 1 min at 94°C (denaturation), 1min at a specific annealing temperature, and 1min at 72°C (extension) followed by a final extension step at 72°C for 5min. Amplification products were electrophoresed in 2.6% agarose in 1*x*TBE buffer. The gels were stained with ethidium bromide

Table-5: Jaccard's similarity coefficient of 30 rice genotypes based on SSR data analysis

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	1																													
2	0.84	1																												
3	0.64	0.77	1																											
4	0.78	0.85	0.71	1																										
5	0.53	0.59	0.7	0.5	1																									
6	0.88	0.88	0.73	0.88	0.55	1																								
7	0.48	0.58	0.75	0.55	0.69	0.55	1																							
8	0.65	0.54	0.54	0.55	0.43	0.62	0.48	1																						
9	0.45	0.55	0.55	0.52	0.55	0.47	0.7	0.45	1																					
10	0.81	0.81	0.68	0.81	0.62	0.92	0.56	0.57	0.48	1																				
11	0.6	0.6	0.78	0.67	0.66	0.68	0.65	0.55	0.47	0.75	1																			
12	0.55	0.55	0.66	0.47	0.66	0.47	0.59	0.41	0.61	0.53	0.61	1																		
13	0.64	0.64	0.64	0.66	0.64	0.73	0.53	0.48	0.37	0.81	0.85	0.55	1																	
14	0.45	0.45	0.53	0.5	0.53	0.5	0.53	0.44	0.43	0.56	0.58	0.46	0.57	1																
15	0.41	0.41	0.49	0.38	0.44	0.34	0.41	0.29	0.46	0.36	0.42	0.69	0.37	0.49	1															
16	0.42	0.42	0.57	0.44	0.47	0.44	0.52	0.47	0.4	0.41	0.48	0.4	0.42	0.47	0.39	1														
17	0.53	0.48	0.39	0.55	0.31	0.55	0.36	0.54	0.33	0.52	0.45	0.26	0.48	0.41	0.27	0.74	1													
18	0.38	0.34	0.38	0.36	0.42	0.35	0.35	0.42	0.29	0.37	0.44	0.36	0.42	0.47	0.36	0.66	0.62	1												
19	0.32	0.32	0.38	0.37	0.29	0.33	0.39	0.34	0.37	0.31	0.44	0.33	0.35	0.47	0.36	0.57	0.54	0.49	1											
20	0.35	0.35	0.39	0.37	0.39	0.36	0.43	0.42	0.37	0.34	0.41	0.27	0.35	0.37	0.27	0.65	0.61	0.5	0.57	1										
21	0.3	0.33	0.39	0.31	0.39	0.27	0.4	0.29	0.34	0.29	0.4	0.37	0.36	0.4	0.4	0.45	0.36	0.49	0.44	0.49	1									
22	0.2	0.2	0.23	0.2	0.29	0.18	0.24	0.22	0.25	0.2	0.28	0.28	0.26	0.29	0.28	0.35	0.29	0.42	0.42	0.46	0.64	1								
23	0.27	0.27	0.3	0.26	0.27	0.24	0.28	0.26	0.32	0.23	0.29	0.32	0.24	0.3	0.29	0.39	0.3	0.36	0.36	0.44	0.5	0.69	1							
24	0.32	0.32	0.35	0.33	0.35	0.29	0.36	0.28	0.33	0.31	0.4	0.37	0.35	0.43	0.36	0.38	0.32	0.38	0.44	0.41	0.55	0.56	0.62	1						
25	0.23	0.23	0.23	0.22	0.26	0.21	0.27	0.22	0.31	0.23	0.22	0.34	0.2	0.29	0.31	0.32	0.26	0.42	0.27	0.33	0.49	0.54	0.69	0.49	1					
26	0.22	0.24	0.27	0.29	0.22	0.22	0.28	0.2	0.29	0.24	0.26	0.32	0.22	0.35	0.35	0.27	0.22	0.33	0.33	0.28	0.49	0.5	0.59	0.56	0.7	1				
27	0.22	0.19	0.19	0.21	0.22	0.17	0.23	0.2	0.28	0.21	0.25	0.31	0.22	0.31	0.31	0.24	0.22	0.24	0.37	0.29	0.49	0.47	0.48	0.56	0.5	0.5	1			
28	0.22	0.22	0.22	0.25	0.22	0.2	0.23	0.2	0.23	0.24	0.28	0.25	0.24	0.31	0.31	0.26	0.24	0.26	0.27	0.29	0.4	0.41	0.42	0.56	0.41	0.5	0.66	1		
29	0.24	0.22	0.24	0.23	0.27	0.22	0.23	0.18	0.23	0.27	0.32	0.35	0.3	0.3	0.38	0.27	0.22	0.33	0.28	0.28	0.4	0.5	0.48	0.49	0.43	0.4	0.44	0.57	1	
30	0.3	0.3	0.24	0.32	0.24	0.28	0.2	0.23	0.26	0.33	0.32	0.29	0.3	0.25	0.24	0.24	0.27	0.27	0.28	0.22	0.26	0.37	0.41	0.36	0.34	0.35	0.41	0.44	0.53	1

1) IR-64 2) IET-20667 3) IET-20668 4) IET-20669 5) SWARNA 6) IET-20670
7) IET-20671 8) IET-20672 9) LALAT 10) IET-21063 11) IET-21064 12) IET-21065
13) IET-21066 14) IET-21068 15) TAPASWINI 16) IET-21067 17) IET-21069 18) IET-21070
19) IET-21071 20) IET-21072 21) IRBB-60 22) GR-11 23) SAMBA MASURI 24) IET-1904
25) PUSA BASMATI 26) IET-18990 27) GR-4 28) JAYA 29) GR-3 30) TN-1

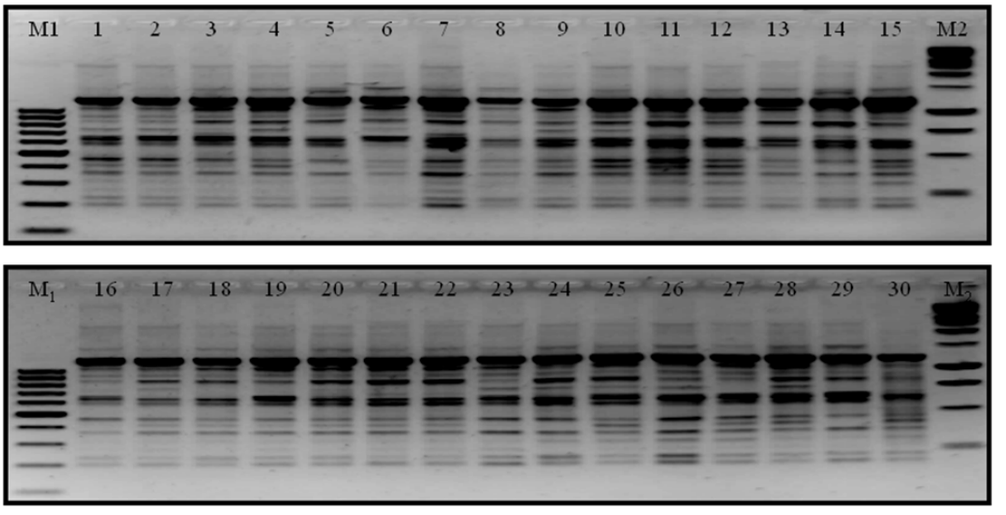

Figure 1: RAPD profile of ES 17 (M_1= 100 bp DNA ladder, M_2 = 1 kb DNA ladder)

and documented using gel documentation system. Each experiment was repeated two times with each primer and those primers gave reproducible bands were considered for data analysis.

Data analysis

The RAPD and ISSR bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. By comparing the banding patterns of genotypes for a specific primer, genotype-specific bands were identified. Faint or unclear bands were not considered. The binary data generated were used to estimate levels of polymorphism by dividing the polymorphic bands by the total number of scored bands. The polymorphism information content (PIC) was calculated by the formula: PIC = 2Pi(1−Pi) (Bhat, 2002) where, Pi is the frequency of occurrence of polymorphic bands in different primers. Pairwise similarity matrices were generated by Jaccard's coefficient of similarity (Jaccard, 1908) by using the SIMQUAL format of NTSYSpc (Rohlf, 1993). A dendrogram was constructed by using the unweighted pair group method with arithmetic average (UPGMA) with the SAHN module of NTSYSpc to show a phenetic representation of genetic relationships as revealed by the similarity coefficient (Sneath and Sokal, 1973).

Results and Discussion

Qualitative and quantitative analysis of genomic DNA

The concentration of DNA was obtained between 1000 to 3000 ng/µl and A260/A280 was 1.84 to 2.00, as stated by Sachdev *et al*., (2003). None of the DNA samples analyzed were of poor quality.

RAPD analysis

Total 80 RAPD primers were screened, of which 29 primers amplified ten or more scorable bands. PCR amplification of DNA, using these 29 primers generated total 5118 scorable bands with 308 loci, among them

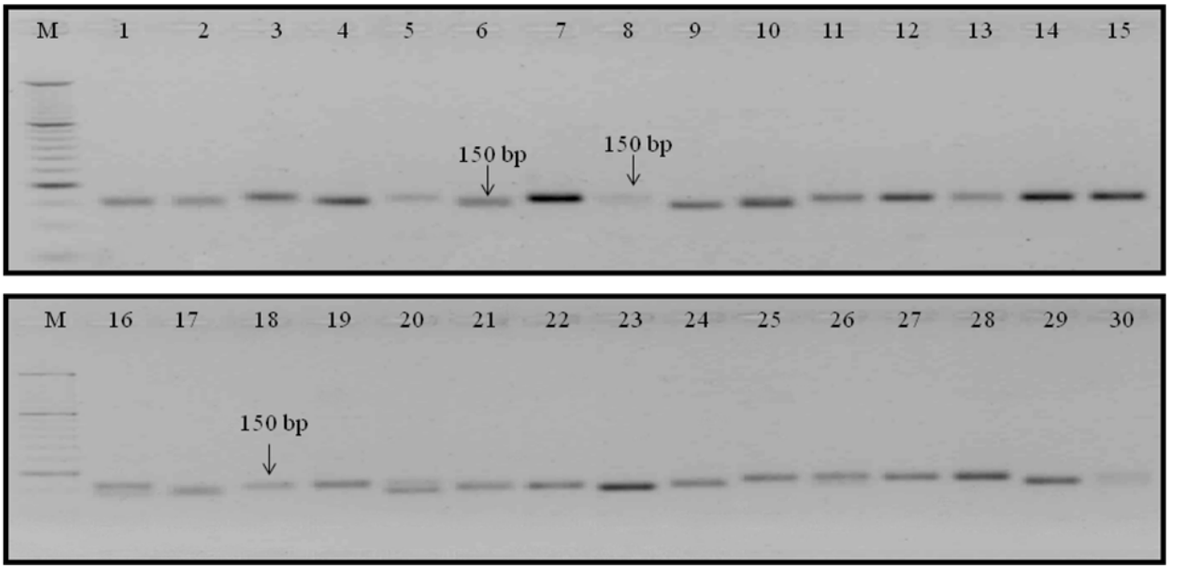

Figure-2: SSR profile of RM 31 (Expected product size-150 bp for *xa5* gene) (M= 50 bp DNA ladder)

1) IR-64	2) IET-20667	3) IET-20668	4) IET-20669	5) SWARNA	6) IET-20670
7) IET-20671	8) IET-20672	9) LALAT	10) IET-21063	11) IET-21064	12) IET-21065
13) IET-21066	14) IET-21068	15) TAPASWINI	16) IET-21067	17) IET-21069	18) IET-21070
19) IET-21071	20) IET-21072	21) IRBB-60	22) GR-11	23) SAMBA MASURI	24) IET-1904
25) PUSA BASMATI	26) IET-18990	27) GR-4	28) JAYA	29) GR-3	30) TN-1

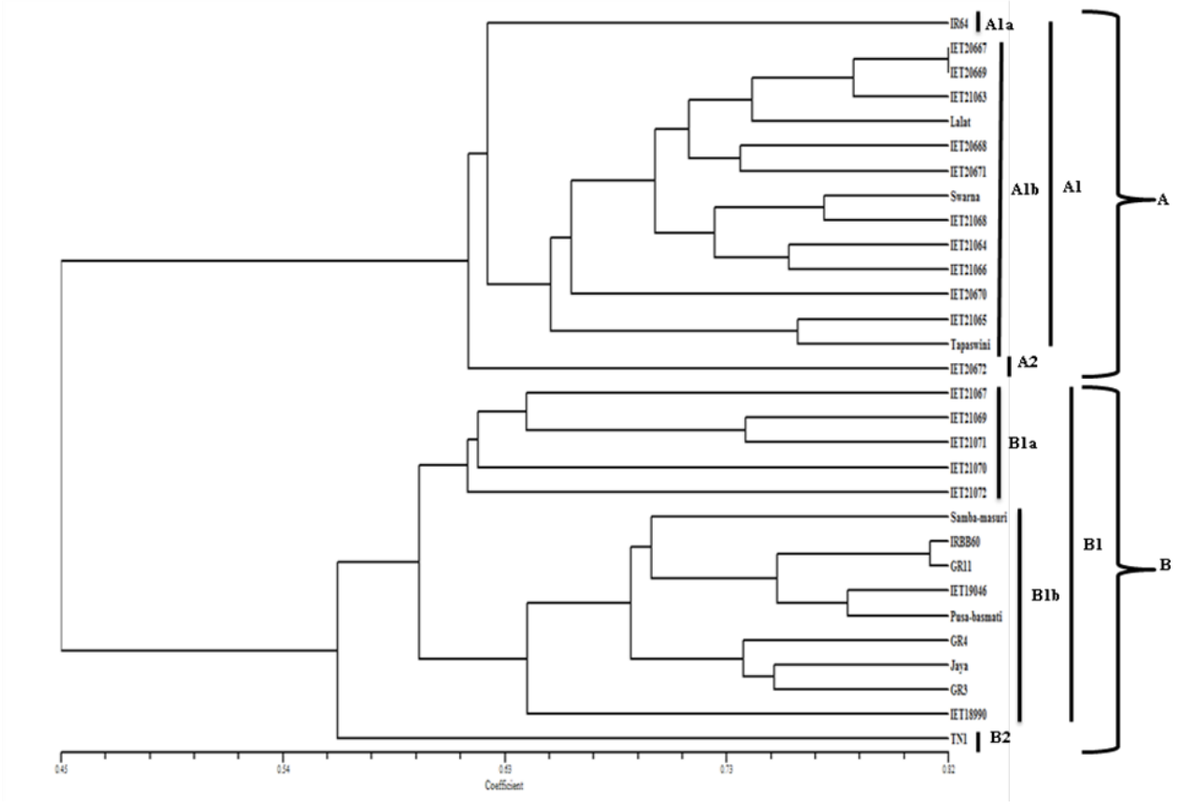

Figure 3: Dendrogram showing clustering of 30 rice genotypes constructed using UPGMA based on Jacquard's similarity coefficient obtained from RAPD analysis.

273 loci were found polymorphic, showing 86.68% polymorphism. The PIC values ranged from 0.72 (OPC 11) to 0.94 (OPP-06) (Table-2) with an average of 0.86. The RAPD primers produced fragments of different sizes. The minimum sized fragment (95 bp) was amplified by primer OPP 01, whereas maximum sized fragment (5713 bp) was amplified by primer OPP 06. The highest (100%) polymorphism were exhibited by primers, OPA 05, OPA 09, OPA 12 (Rao *et al.,* 2003), OPAB 09, OPAE 03, OPB 17, OPH 04, OPP 03, OPP 17 and ES 19, while the lowest polymorphism (40%) was observed with primer ES 22. The Jaccard's similarity coefficient values ranged from 0.35 (between IET 20667 and IET 21067) to 0.82 (between IET 20667 and IET 20669) (Table-3). Out of three NILs of IR 64, IET 20667 showed maximum genetic similarity (0.74) with the recurrent parent IR 64; while for Swarna, all three lines exhibited moderate degree of genetic similarity ranging from 0.63 to 0.67. IET 21063 (NIL of Lalat) recorded maximum similarity (0.75) with its recurrent parent Lalat. All the NILs of Tapaswini showed low values of similarity coefficient (≤ 5.0). IET 19046 (NIL of Samba Masuri) and IET 18990 (NIL of Pusa Basmati-1) registered 0.71 and 0.63 similarity coefficient, respectively, with their respective recurrent parents. IET 21067 (NIL of Tapaswini) showed least similarity values with all other NILs. Similarly, IRBB 60 also exhibited lower values. A dendrogram (Figure-1) based on UPGMA analysis, produced by pooled RAPD data showed two major clusters A and B. Cluster A included recurrent parents IR 64, Swarna, Lalat and their respective NILs and Tapaswini and its near isogenic line (IET 20672). Cluster B comprised recurrent parents Samba Masuri, Pusa Basmati-1 and their respective NILs, NILs of Tapaswini and susceptible check cultivars. Cluster A was divided into two sub clusters A1 and A2. Sub-cluster A1 was further divided into A1a and A1b.

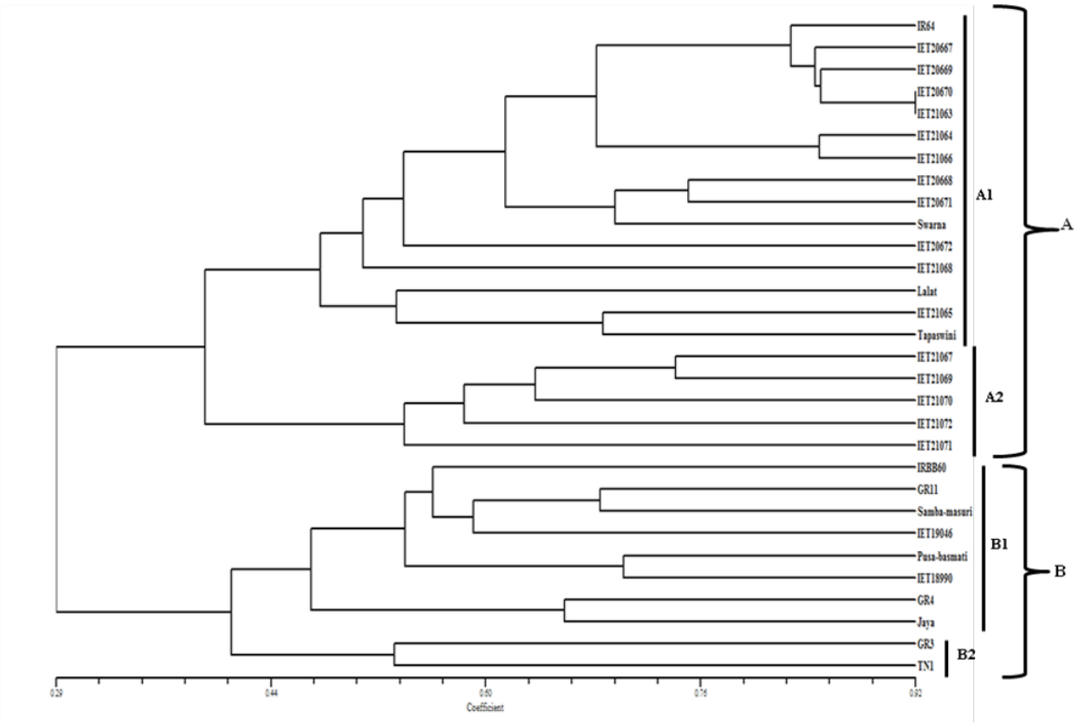

Figure-4: Dendrogram showing clustering of 30 rice genotypes constructed using UPGMA based on Jacquard's similarity coefficient obtained from SSR analysis.

Solitary genotype IR 64 formed group A1a, whereas Lalat, Swarna and their NILs, Tapaswini and NILs of IR 64 were in group A1b. Group A1b showed the maximum genetic proximity between NILs of Lalat to its recurrent parent and NILs of Swarna to its recurrent parent. Groups A1a and A2b suggested that all the three NILs of IR 64 were genetically different from their recurrent parent IR 64. Sub-cluster A2 consists of single genotype IET 20672 (NIL of Swarna). Cluster B was divided into two sub clusters B1 and B2. Sub-cluster B1 was further divided into group B1a and B1b. NILs of Tapaswini were in group B1a, whereas group B1b comprised of Samba Masuri, Pusa Basmati-1 and their near isogenic lines, IRBB 60, GR 11, GR 3, GR 4 and Jaya. Sub-cluster B2 consisted of susceptible variety TN 1. More diverse NILs (to their respective parents) can be used as parents, to bypass marker assisted selection. Since, these NILs have three common R genes viz., *xa5, xa13* and *Xa21.* There will be no segregation between NILs. Hence, they can be used as potential parents in breeding programmes.

SSR analysis

PCR amplification of genomic DNA of 30 rice genotypes, using 20 SSR primers generated 798 scorable bands with average of 40 bands per primer (Table 4). The size of the bands ranged from 65 bp to 1289 bp. On an average, four alleles were generated per primer. The minimum sized fragment (65 bp) and the maximum sized fragment (1289 bp) were amplified by primer RM 139. The PIC values ranged from 0.07 (SR 6) to 0.86 (RM 139). The highest PIC value of RM 139 indicated that it would be very useful SSR marker for diversity analysis of rice genotypes. Out of 20 microsatellite markers studied, seven gene specific SSR markers (RM 13, RM 31, RM 122, RM 164, RM 390, SR 11 and RM 21) were very informative as they readily distinguished BLB resistant near isogenic lines from their recurrent parents and also from susceptible check cultivars (Blair and McCouch, 1997; Rao *et al.,* 2003; Chu *et al.,* 2006; Davierwala *et al.,* 2001). Genetic similarity values (Table-5) and dendrogram (Figure-2) revealed genetic relationship among the genotypes. The

Jaccard's similarity coefficient values ranged from 0.17 (between IET 20670 and GR 4) to 0.92 (between IET 20670 and IET 21063) (Table 4.2.7). Among NILs of IR 64, IET 20667 (0.84), followed by IET 20669 (0.78) showed genetic proximity with its recurrent parent IR 64 whereas, IET 20671 recorded genetic similarity to the extent 0.69 with its recurrent parent Swarna. Among five NILs of Lalat, only one NIL IET 21065 showed moderate similarity (0.61). All the near isogenic lines of Tapaswini exhibited low degree of similarity (< 0.5). Solitary NIL of Samba Masuri (IET 19046) and Pusa Basmati-1 (IET 18990) registered similarity coefficient of 0.62 and 0.7, respectively. Low to moderate values for similarity coefficient were expected when either less recovery of recurrent parent or less number of molecular markers used. The low similarity coefficient values such as, 0.17, 0.18, 0.20, 0.22 etc indicated that these are less similar genotypes and will be useful for getting most diverse recombinants in segregating generations, when they are used as parents. Clustering pattern of dendrogram generated by pooled SSR data showed two major clusters A and B. Cluster A included recurrent parents IR 64, Swarna, Tapaswini, Lalat and their respective NILs. Cluster B comprised Samba Masuri, Pusa Basmati-1 and their respective NILs, IRBB 60 and susceptible check cultivars. Cluster A was divided into two sub clusters A1 and A2. IR 64 and its NILs, Lalat and its NILs, Swarna and its NILs and Tapaswini were in sub-cluster A1. NILs of Tapaswini were in sub-cluster A2. Some SSR markers were found to be in linkage with BLB resistance gene along with some events of recombination. Since they were found to be linked to the resistance gene, they were found separated the near isogenic lines from their recurrent parent. In this study, Tapaswini was included in sub-cluster A1 which was separated from its NILs included in sub-cluster A2. Sub-cluster A1 exhibited the maximum genetic proximity between NILs of IR 64, Lalat and Swarna to their respective recurrent parents. Cluster B was divided into two sub clusters B1 and B2. Sub-cluster B1 was formed by Samba Masuri, Pusa Basmati-1 and their respective NILs, IRBB 60, GR 11, GR 4 and Jaya. Sub-cluster B2 was formed by GR 3 and TN 1. Sub-cluster B1 showed the maximum genetic similarity between recurrent parents Samba Masuri and Pusa Basmati-1 to their respective NILs.

Conclusions

A defence response against bacterial leaf blight disease was found mediated by resistance genes. Near isogenic lines of rice, used in this study, have been confirmed for the presence of bacterial leaf blight resistance genes (*xa5*, *xa13, Xa4, Xa21*), using molecular markers, therefore, to bypass marker assisted selection and extensive field screening, IET 21072 (near isogenic line of Tapaswini), IET 20668 (near isogenic line of IR 64), IRBB 60 and some of the near isogenic lines among them, could be utilized as potential parents in hybridization programmes aimed at developing bacterial leaf blight resistant varieties. The DNA markers, RAPD and SSR were also found useful for genetic diversity studies between near isogenic lines and their recurrent parents.

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