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RESEARCH ARTICLE

Candidate screening of blast resistance donors for rice breeding

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Abstract. Rice blast is one of the most serious diseases in the world. The use of resistant cultivars is the most preferred means to control this disease. Resistance often breaks down due to emergence of new races; hence identification of novel resistance donors is indispensable. In this study, a panel of 80 released varieties from National Rice Research Institute, Cuttack was genotyped with 36 molecular markers that were linked to 36 different blast resistance genes, to investigate the varietal genetic diversity and molecular marker-trait association with blast resistance. The polymorphism information content of 36 loci varied from 0.11 to 0.37 with an average of 0.34. The cluster analysis and population structure categorized the 80 National Rice Research Institute released varieties (NRVs) into three major genetic groups. The principal co-ordinate analysis displays the distribution of resistant and moderately resistant NRVs into different groups. Analysis of molecular variance result demonstrated maximum (97%) diversity within populations and minimum (3%) diversity between populations. Among tested markers, two markers (RM7364 and pi21_79-3) corresponding to the blast resistance genes (*Pi56(t)* and *pi21*) were significantly associated and explained a phenotypic variance of 4.9 to 5.1% with the blast resistance. These associated genes could be introgressed through marker-assisted to develop durable blast resistant rice varieties. The selected resistant NRVs could be good donors for the blast resistance in rice crop improvement research.

Keywords. rice blast; resistance gene; genetic diversity; association mapping; population structure.

Introduction

Rice (*Oryza sativa* L.) is the staple food crop for more than half of the world's human population and cultivated in diverse agro-climatic conditions. Rice blast caused by the fungus, *Magnaporthe oryzae* is one of the most devastating and one of the top 10 fungal diseases which is major threat to global food security (Dean *et al.* 2005). In India, it has caused a considerable yield losses over the past few decades ranging between 20–100% (Sharma *et al.* 2012). Majority of the plant pathogens mutated rapidly and resulted in

the breakdown of resistance causing epidemics, further it is aggravated by conducive weather conditions, high disease pressure, and genome stability of the pathogen. The genome of *M. oryzae* is rich in retrotransposons and repetitive segments (Dean *et al.* 2005) which helps the fungus to change its virulence to overcome the resistance conferred by *R*-genes (Vasudevan *et al.* 2014). The rice blast system follows classical gene for gene model where a blast resistance (*R*) gene products prevent its infection by the races of *M. oryzae* carrying the corresponding avirulence (*Avr*) gene (Silue *et al.* 1992; Sahu *et al.* 2018). This disease can be managed by maintaining adequate flood depth, suitable planting dates, recommended dose of nitrogen fertilizer, chemical fungicides and use of resistant varieties (Bonman

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1992). Among these, use of resistant varieties is the most economical and environment friendly method to manage this disease (Panda *et al.* 2017).

Until now, more than 100 race-specific *R* genes, and more than 350 QTLs for resistance to *M. oryzae* have been identified, and 27 have been cloned and characterized and most of them encode nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins except *pi21* and *Pid2*, which encodes proline-containing protein and receptor kinase (Fukuoka *et al.* 2009; Kouzai *et al.* 2013; Zheng *et al.* 2016; Zhu *et al.* 2016; Yadav *et al.* 2019). Among them, eight genes have been located in two gene clusters; *Pi2*, *Pi9* and *Piz-t* in *Pi2* locus and *Pik*, *Pik-m*, *Pik-p*, *Pi1* and *Pi-ke* in *Pik* locus (Wang *et al.* 2016). Most of the blast *R* genes are dominant except the recessive genes *pi21*, *Pid*-2 and *pi66(t)* (Liang *et al.* 2016). Most of the *R*-genes were identified in landraces, cultivars, or wild rice collections because of differential physiological races of *M. oryzae* (Tanksley *et al.* 1997). The existing phenotypic screening technique for blast resistance is time-consuming, laborious, and entailed specific procedures. Many PCR based molecular markers have been developed for fine mapped and cloned blast *R* genes for mining and identification of different *R* genes.

In the current scenario, molecular markers have a significant contribution in increasing the efficiency and precision to incorporate blast resistance genes in cultivars (Wang *et al.* 2014). Marker-assisted selection (MAS) is an advanced molecular tool in rice breeding for improvement of resistance to rice blast and with the aid of MAS many rice cultivars resistant to biotic stress have been developed and widely accepted by farmers (Xu and Crouch 2008).

Association mapping (AM) is a molecular approach used for identifying target genes governing important traits in a natural population including diverse germplasm. AM can be categorized into candidate-gene association and genomewide association mapping (Zhang *et al.* 2017). In addition, AM utilizes a natural population as compared to biparental mapping population, thus it saves the time required to constitute the population and hasten the identification of gene in crops and hence the AM is a powerful means of genetic dissection and identification of a gene of interest.

National Rice Research Institute released varieties (NRVs) can be studied as donor source of favourable genes for biotic stresses which are grown in blast endemic areas in diverse agro-ecological zones in India. During the previous study, NRVs were genotyped for 12 major blast resistant genes using 17 molecular markers (Yadav *et al.* 2017). In continuation of the previous study, the present study was undertaken to investigate the genetic association of 36-mapped resistance genes in 80 NRVs using linked/functional markers. The objective of the current study was to identify the candidate *R* genes which confer blast resistance to these NRVs and that could be used for identification of novel donor source (*R* genes/alleles) for blast resistance, and genomic studies.

Material and methods

Plant material and disease reaction in uniform blast nursery

A set of 80 NRVs originated from eight different agro-ecologies (table 1) was collected from the National Gene

Table 1. The NRRI released varieties (NRVs) of different ecologies.

	Ecology	Number of lines	NRRI variety
1	Irrigated	30	Satya Krishna*, Improved Lalat, Radhi, Phalguni, Improved Tapaswini, Saket-4, HUE, Maudamani, Geetanjali, Chandrama*, Kalinga-II, Naveen, Shaktiman, Tapaswini, Abhishek*, Kalinga-I, Ratna, CR Dhan-303, CR Dhan-304, CR Dhan-305, CR Dhan-306, Khitish, Sarasa*, Indira, Udaya, Supriya, CR Dhan 300*, CR Dhan 907, Rajalaxmi and Ajay
2	Shallow low land	15	Poorna Bhog, Nua Chinikamini, Sumit*, Nua Dhusara, Nua Kalajeera, CR Dhan-701, Ketekijoha, Dharitri, Moti, Padmini, Savitri*, Reeta*, Samalei*, Pooja and Swarna sub-1
3	Upland	10	Kamesh, Satyabhama*,Virender, Anjali, Sattari, Heera, Sahbhagidhan*, Hazaridhan, Kalyani-II, and Annada
4	Medium deep water logged	9	Hanseswari, CR Dhan-501, Panidhan*, CR 1014, Sarala, Gayatri, Varshadhan, Durga and Utkalprabha
5	Coastal saline	6	Luna Sankhi, Luna Sampad, Sonamani, Luna Barial, Luna Suvarna and Lunishree*
6	Aerobic	5	Pyari, CR Dhan 205*, CR Dhan 202*, CR Dhan 201 and CR Dhan 204*
7	Deep water	3	Jalamani*, Jayanti Dhan* and CR Dhan 500
8	Boro	2	Chandan* and CR Dhan 601
9	Total	80	

*Resistant NRVs in each ecology.

Bank, NRRI, Cuttack (table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet). These NRVs were phenotyped for leaf blast under natural conditions in the uniform blast nursery (UBN). The screening was done in two replications during dry and wet seasons, 2015–2016, at the research farm of NRRI, Cuttack (85°55′48″E longitudes and 20°26′35″N latitude). Thirty seeds of each NRVs were grown in a 50-cm long row with a 10 cm row spacing. The highly susceptible varieties HR12 and CO39 were used as a spreader row to ensure the uniform spread of the disease. The disease scoring was recorded from 25 to 40 days after sowing at 5-day intervals when the spreaders row showed more than 85% infection. Disease reaction was scored using the standard evaluation system (SES), IRRI, Philippines (2002) on a 0–9 scale as: resistant (0–3), moderately resistant (4–5), and susceptible (6–9). The higher disease score was considered for evaluation, whenever there were different blast disease score between replications as well as season.

Genomic DNA isolation

Young leaves from 3-week-old seedlings were collected and stored in –80°C freezer. The genomic DNA was isolated following Doyle and Doyle (1990) method with slight modification. In brief, the 200 mg leaf sample was grinded with liquid nitrogen; powder was immediately transferred to 1ml CTAB isolation buffer and incubated at 65°C in a recirculating water bath. After one hour, equal volume of PCI (Phenol:Chloroform:Isoamyl alcohol; 25:24:1) was added, and centrifuged at 10,000 rpm for 10 min. The aqueous phase was transferred to new 2ml tube and mixed with Chloroform:Isoamyl alcohol (24:1) and again centrifuged as above. The aqueous phase was pipetted out to a new 2ml tube and absolute alcohol was added twice the volume followed by $1/10^{th}$ of sodium acetate (3.5 M) and mixed properly. The samples were kept for 2 h in −20°C followed by centrifuged of 10,000 rpm for 10 min at room temperature (RT). The white pellet was wash with 70% ethanol by centrifuging at 7000 rpm for 7 min at RT followed by air dry. The completely dried pellet was dissolved in nuclease free water for further quantification and used for PCR amplification. The quantity and quality of nuclear DNA were assessed by 0.8% agarose gel electrophoresis and Nano-drop ND-1000 Spectrophotometer (Thermofisher Scientific, Waltham, USA). Nuclease-free water was used to dilute the DNA samples to the concentration of 20 ng/μL for PCR amplification.

PCR amplification and visualization

The polymerase chain reactions (PCR) were executed in 20 μL reaction volume containing 25 ng template DNA, 1x*Taq* buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 0.2 μM of each of dNTP, 0.2 μM of each forward and reverse primers, and 1 U of *Taq* DNA polymerase (DreamTaq, Thermo Scientific, USA). The PCR cycle was set up as follows: initial denaturation of 5 min at 94°C; 35 cycles at 94°C for 45 s, primers annealing for 45 s at varied temperature (table 2), extension at 72°C for 45 s, and a final extension for 10 min at 72°C. The PCR products were separated by electrophoresis in 3.5% agarose gels and visualized using a gel documentation system (Alpha Imager, USA). The amplified PCR products were scored as presence (1) or absence (0). The PCR reaction was repeated twice for each marker to cross-check the scoring data.

Statistical data analysis

A total of 36 linked/functional markers were used to score the presence or absence of the resistance genes in the 80 NRVs. Jaccard's coefficient similarity matrix was assessed using binary data. The polymorphism information content (PIC) value, allele number and allele frequency were estimated for each marker using the PowerMarker v3.25 (Liu and Muse 2005). The genetic distance matrix among the NRVs was assessed through principal co-ordinate analysis (PCoA) in GenAlEx 6.5 software. Similarly, analysis of molecular variance (AMOVA) between and within the populations and population assignment was estimated using GenAIEx 6.5.0 (Peakall and Smouse 2012). An unweighted neighbour-joining (NJ) unrooted tree was constructed in the DARwin 5 program (Perrier and Jacquemound-Collect 2006, DARwin Software, http://darwn.cirad.fr/darwin). The dissimilarity index was estimated using NEI coefficient (Nei 1973) with a bootstrap value of 1000. The general linear model (GLM) function in TASSEL5 software was used to understand the genetic association of blast resistance genes with the disease (Bradbury *et al.* 2007). The GLM model of Tassel 5 software was run with permutations of 1000. The population structure analysis was performed using the Bayesian model-based approach employed in Structure v 2.3.4 software (Pritchard *et al.* 2000). The number of subgroups (K) in the population varied from 1 to 10. The population structure was run using the admixture model, correlated allele frequencies and five independent iterations per K with a burn-in period length of 200,000 and 200,000 Markov chain Monte Carlo (MCMC). The optimal K was determined from the peak value of ΔK (Evanno *et al.* 2005) using Structure Harvester 0.6.93 (Earl 2012).

Results

Phenotyping and genetic diversity

The NRVs panels of 80 varieties were phenotyped for resistance to leaf blast. Nineteen NRVs (24.69%) were found to be resistant, 21 (26.25%) were moderately resistant, and 40 (50%) were susceptible. The disease score ranged from

Table 2. List of markers used for genetic association of blast resistance in rice varieties.

	Gene	Chromosome number	Marker used	Forward (5′–3′)	Reverse (5′–3′)	Type of marker	Annealing temperature (°C)	Reference
1	*Pit*	1	tk59-1	atgataacctcatcctcaataagt	gttggagctacggttgttcag	FM	54	Hayashi et al. (2010)
			tk59-2	atgataacctcatcctcaataagt	ccaaggattaggtcctagtg			
2	*Pi27(t)*	1	RM151	ggctgctcatcagctgcatgcg	tcggcagtggtagagtttgatctgc	LM	55	Zhu et al. (2004)
			RM259	tggagtttgagaggaggg	ctgttgtcatggtgcatgt	LM	55	Zhu et al. (2004)
3	*Pitp(t)*	1	RM246	gagctccatcagccattcag	ctgagtgctgctgcgact	LM	55	Barman et al. (2004)
4	*Pi35(t)*	1	RM1216	ttcccaatggaacagtgac	agggtctaccacccgatctc	LM	55	Nguyen et al. (2006)
5	*Pish*	1	RM6648	gatcgatcatggccagagag	acagcaggttgatgaggacc	LM	55	Koide et al. (2010)
6	*Pi37(t)*	1	RM212	ccactttcagctactaccag	caccatttgtctctcattatg	LM	55	Chen et al. (2005)
7	*Pi64(t)*	1	RM11787	gtcaccctccttctacatagagc	tacataacgcaacagacagacg	LM	55	Ma et al. (2015)
8	*Pi-Da(t)*	2	RM5529	agccgaaactacattcggtg	ttgtgtagttggcacgcttc	LM	55	Shi et al. (2012)
9	*Pid1(t)*	2	RM262	cattccgtccgctcaact	cagagcaaggtggcttgc	LM	55	Chen et al. (2004)
10	*Pig(t)*	2	RM208	tctgcaagccttgtctgatg	taagtcgatcattgtgtggacc	LM	55	Zhou et al. (2004)
11	*pi21*	4	pi21-79-3	gatcctcatcgtcgacgtctggc	agggtacggcaccagcttg	InDel	55	Fukuoka et al. (2009)
12	*Pi46(t)*	4	RM5473	acacggagataagacacgag	cgagattaacgtcgtcctc	LM	54	Matsushita et al. (2011)
13	*Pikhahe-1(t)*	4	RM17496	taaacggtgtgcagcttctg	tattatgggcggtcgctaac	LM	54	Xu et al. (2008)
14	*Pi63(t)*	4	RM6629	taaacggtgtgcagcttctg	tattatgggcggtcgctaac	LM	54	Xu et al. (2014)
15	*Pi45(t)*	4	RM5709	ctgaatttattataggacgggaag	catagtattggattggacacg	LM	55	Kim et al. (2013)
16	*Pi59(t)*	6	RM7311	agtggtcgttgaactcggag	tcgtggcgccttaatctc	LM	55	Koide et al. (2013)
17	*Pi36*	8	RM5647	actccgactgcagttttgc	aacttggtcgtggacagtgc	LM	55	Liu et al. (2005)
18	*Pi33*	8	RM72	ccggcataaaacaatgag	gcatccgtctaactaaggg	LM	55	Berruyer et al. (2003)
19	*pi55(t)*	8	RM3452	ggcagcccatcaactagatc	ttgcaaaccctagtccaagc	LM	55	He et al. (2012)
20	*Pi56(t)*	9	RM7364	ttcgtgatggagggagtac	agcgtttgtaggagtgccac	LM	55	Liu et al. (2013)
21	*Pia*	11	CRG4-2	cctgtcagtctttccgagag	gaatccggtagctcaaggtg	Gene specific	55	Liu et al. (2013)
			Pia-STS	ctttgagctgattggtctgc	ctattgcaccagaggaccag	InDel	54	Okuyama et al. (2011)
22	*Pi60(t)*	11	Y10	cgttcccgtgaaatattgggcaat	gcaaagacctggctgagcaactgaagaa	InDel	52	Lei et al. (2013)
23	*Pi38*	11	RM206	cccatgcgtttaactattct	cgttccatcgatccgtatgg	LM	55	Gowda et al. (2006)
24	*Pi47*	11						Huang et al. (2011)
25	*Pb1*	11	RM26998	acgcacgcacatcctcttcc	cggttctccatctgaaatccctagc	LM	55	Hayashi et al. (2010)
26	*Pi1*	11	RM224	atcgatgctcttcacgagg	tgctataaaaggcattcggg	LM	55	Jiang et al. (2012)
27	*Pi-jnw1*	11	RM27273	atgcgtttgccgtgaagaaagg	gtctgggcccacatgtcaatagc	LM	55	Wang et al. (2016)
28	*Pi65(t)*	11	SNP_3	tgccaccagccatcttcaacat	accaatcactcatcgccatcc	InDel	54	Zheng et al. (2016)
29	*Pi49*	11	RM6094	tgcttgatcgtgttcgtcc	tagcagcaccagcagcatgaaag	LM	55	Sun et al. (2013)
30	*Pi39(t)*	12	RM247	tagtgccgatcgatgtaacg	catatgtttttgacaaagcg	LM	55	Liu et al. (2007)
31	*Pi41*	12	RM28130	cagcagacgttccggttctactcg	aggacggtggttggtgatctgg	LM	55	Yang et al. (2009)
32	*Pi58(t)*	12	RM6905	gcactcatcgatggtagcag	agaacggacctgaattgtc	LM	55	Koide et al. (2013)
33	*Pi61(t)*	12	RM101	gtgaattggtcaagtgacttaggtggc	acacaacatgttccctcccatgc	LM	55	Lei et al. (2013)
34	*Pib*	12						Fjellstrom et al. (2004)
35	*Pi48*	12	RM5364	gtattacgctcgatagcggc	gtatcctttctcgcaatcgc	LM	55	Huang et al. (2011)
36	*Pi51(t)*	12						Wang et al. (2012)

LM, linked marker; FM, functional marker; InDel, insertion-deletion marker.
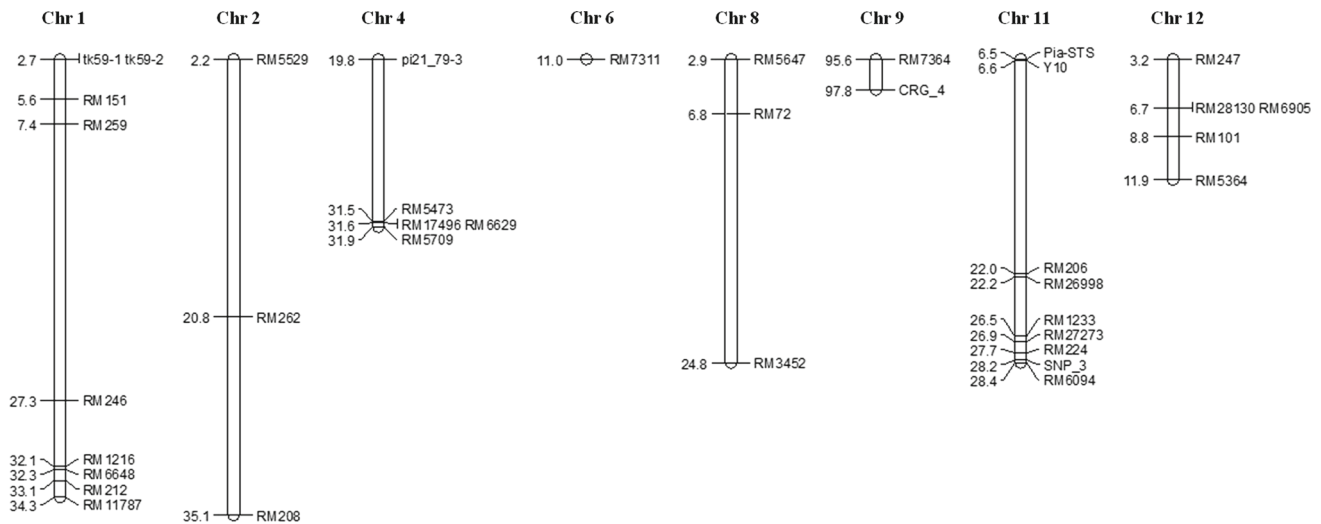
Figure 1. Position of marker loci used in this study.

0 to 9. Interestingly, resistant NRVs were observed across all the ecologies. Highest resistant varieties proportion was observed in irrigated (5), whereas lowest proportion was in Boro, on the other hand medium deep water and coastal saline ecologies with one each (table 1). The NRVs were genotypes for 36 markers corresponding to 36 blast resistance genes (figure 1). The genetic diversity parameters of 36 marker loci measured during current study are presented in table 3. The major allele frequency varied from 0.52 to 0.93 with a mean value of 0.75. Similarly, the genetic diversity of 36 markers had a mean value of 0.34 and varied from 0.11 to 0.49. The PIC was used to measure the informativeness of a genetic marker. The PIC value for 36 markers ranged from 0.11 (RM101 and RM 11787) to 0.37 (RM72 and Pia-STS) with an average of 0.27. The PIC value of two markers, RM72 and Pia-STS, corresponding to the Pi33 and Pia genes showed the highest value of 0.37 which can be used effectively for genetic diversity study (table 3).

Genetic relatedness through cluster analysis, population structure and PCoA

The cluster analysis was analysed with UPGMA and NJ methods using Darwin software based on 36 markers linked to 36 blast resistance genes. The cluster analysis categorized the NRVs into three major clusters (I, II and III) (figure 2). Cluster I consisted of 43 NRVs, was further categorized into two subclusters IA and IB. Subcluster IA included 38 NRVs, with 11 (28.94%) resistant genotypes. Subcluster IB included only five NRVs with no resistant genotype. Similarly, cluster II possessed 32 NRVs, further divided into two subclusters, IIA and IIB. Subclusters IIA consisted of 27 NRVs, having six resistant genotypes (22.22%). Conversely, subclusters IIB consisted of only five NRVs with only one resistant genotype. Cluster III

Table 3. Genetic diversity indices of 36 marker loci for NRVs.

| Marker	Major allele frequency	Gene diversity	PIC
RM151	0.8375	0.2722	0.2351
RM259	0.8375	0.2722	0.2351
RM72	0.5250	0.4988	0.3744
RM1216	0.7750	0.3488	0.2879
RM5647	0.6125	0.4747	0.3620
RM212	0.6625	0.4472	0.3472
RM206	0.6875	0.4297	0.3374
RM247	0.8750	0.2188	0.1948
RM28130	0.7250	0.3988	0.3192
RM5709	0.7875	0.3347	0.2787
RM5473	0.6125	0.4747	0.3620
RM5364	0.6250	0.4688	0.3589
RM6094	0.7000	0.4200	0.3318
RM3452	0.8625	0.2372	0.2091
RM7364	0.8750	0.2188	0.1948
CRG_4	0.7375	0.3872	0.3122
RM6905	0.7000	0.4200	0.3318
RM7311	0.7875	0.3347	0.2787
Y10	0.8625	0.2372	0.2091
RM101	0.9375	0.1172	0.1103
RM6629	0.8250	0.2888	0.2471
RM11787	0.9375	0.1172	0.1103
SNP_3	0.6750	0.4388	0.3425
RM27273	0.6250	0.4688	0.3589
RM246	0.5500	0.4950	0.3725
RM262	0.7250	0.3988	0.3192
RM5529	0.5500	0.4950	0.3725
RM208	0.7875	0.3347	0.2787
RM26998	0.5750	0.4888	0.3693
tk59-1	0.9250	0.1388	0.1291
tk59-2	0.9250	0.1388	0.1291
RM6648	0.8875	0.1997	0.1797
pi21_79-3	0.8500	0.2550	0.2225
Pia-STS	0.5250	0.4988	0.3744
RM17496	0.8875	0.1997	0.1797
RM224	0.7375	0.3872	0.3122
Mean	0.7510	0.3432	0.2769
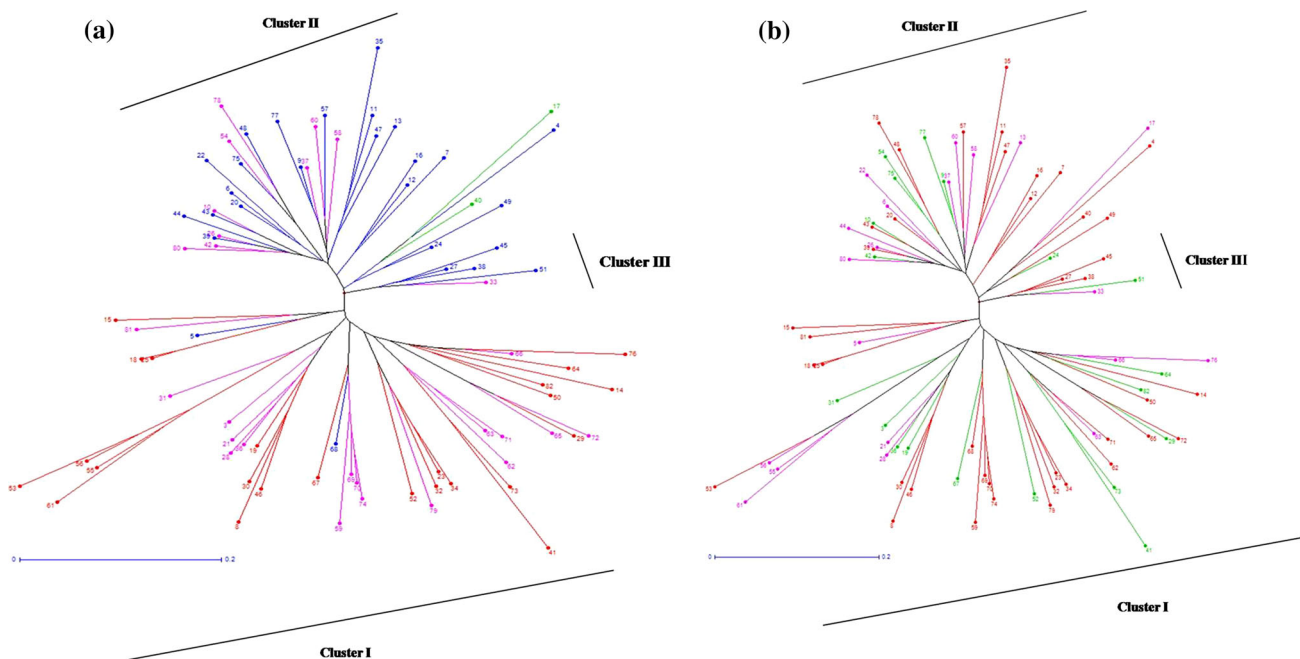
Figure 2. Unweighted–NJ tree based on molecular markers linked to blast resistance in 80 NRVs. These NRVs are represented corresponding to (a) subpopulations determined from structure analysis (SG1, red; SG2, pink; SP3, blue; admixture, green); (b) disease reaction (resistant, green; moderately resistant, pink; susceptible, red).
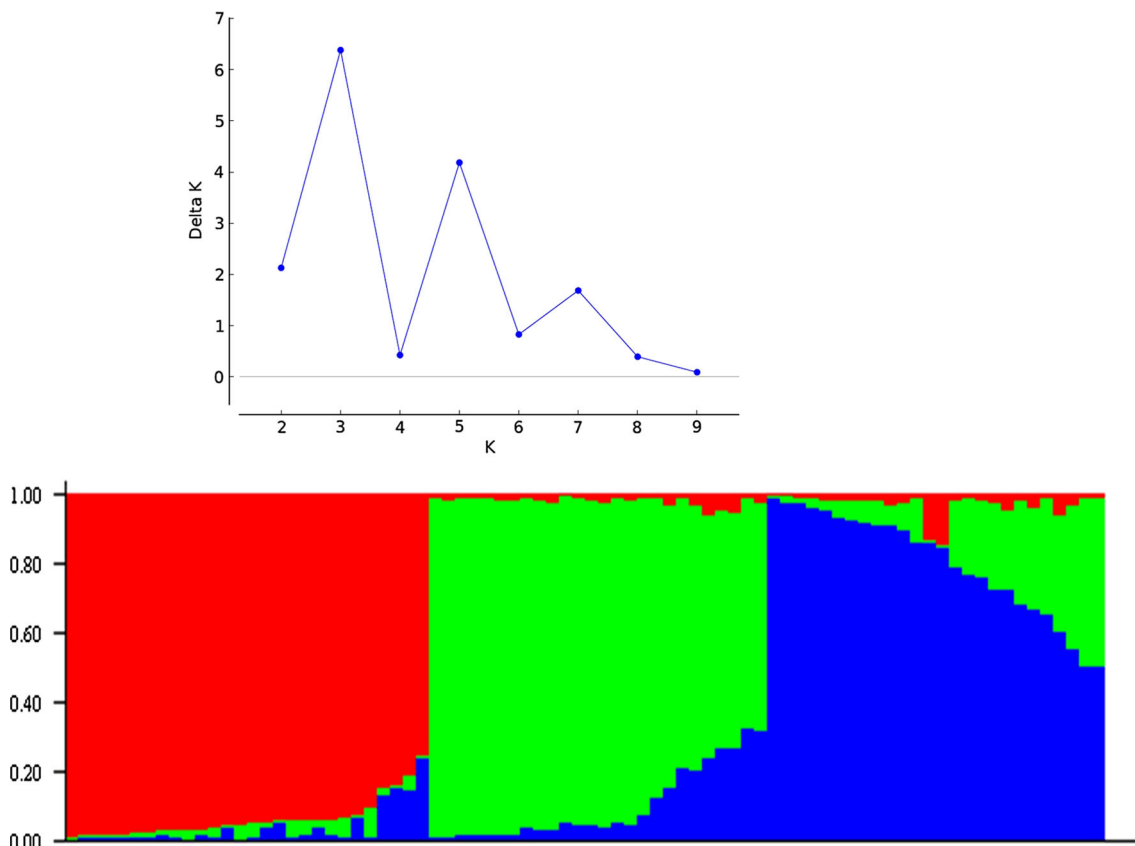

Figure 3. Estimated population structure of the rice NRVs which is partitioned into coloured segments that represent the estimated membership for K=3. The maximum of ad hoc measure ΔK was observed to be K=3, which indicated that the entire population can be grouped into three subgroups.

is the smallest cluster comprised of five NRVs, with only one resistant variety. Interestingly, the majority of resistant genotypes were clustered together in major cluster I and few in major cluster II. Genetically similar NRVs were clustered together in the same group, on the contrary NRVs of same ecologies clustered in different groups.

The population structure of 80 NRVs were explored using the model-based population structure based on 36 markers corresponded to 36 blast resistance genes. The peak plateau of ad hoc statistic ΔK was observed to be K=3 (figure 3), which indicated the presence of three subgroups (SG1, SG2 and SG3) in the NRVs. The threshold value of >55%, classified the entire NRVs into three subgroups with two admixture (table 4). The SG1 consisted of 24 NRVs, of which eight (33.33%) were highly resistant. The SG2 comprised of 27 NRVs, of which six (22.22%) were highly resistant. Similarly, SG3 included 27 NRVs with five (18.51%) highly resistant NRVs. Interestingly, resistant NRVs were distributed in all the three subgroups with maximum percentage observed in SG1. The moderately resistant genotypes were mostly in SG1 and SG1 whereas SG3 was dominated by susceptible genotypes. Further, high resistant variety, Sarasa belonged to SG2. Accordingly, structure analysis could not differentiate resistant genotypes but partially categorized moderately resistant and susceptible genotypes.

The molecular markers genotypic data were used to calculate the PCoA to estimate the genetic relationship among NRVs. Based on disease reaction, NRVs exhibited uniform distribution across the two axes. According to the PCoA analysis, the first two axes explained 12.71% and 8.93% of the total variance (table 5). In PCoA, resistant landraces were observed to be distributed mostly in the first quadrant, moderately resistant genotypes in third and fourth quadrants, whereas, susceptible genotypes were distributed in all the four quadrants (figure 4). The population assignment test was estimated using GenAlex which partly distinguished resistant populations from moderately resistant or susceptible populations (figure 5, a&b). Similarly, it was partly able to distinguish between moderately resistant from susceptible populations (figure 5c).

AMOVA

AMOVA is a statistical method to detect molecular variation using molecular markers. In AMOVA analysis, 80 NRVs were categorized into three groups based on their disease reaction: resistant (19), moderately resistant (21) and susceptible (40). Through AMOVA analysis, greater variance (97%) was observed within the population, whereas less (3%) between population (figure 6; table 6a). The highest pairwise fixation indices (F_{ST}) value of 0.040 was observed between the resistant and susceptible, while the lowest was observed between the resistant and moderately resistant populations. The inbreeding coefficient F_{IS}

Table 4. Population structure group of NRVs based on inferred ancestry values.

	Varieties	Q1	Q2	Q3	Subgroup
1	Satya Krishna	0.011	0.976	0.013	SG2
2	Kamesh	0.195	0.013	0.791	SG3
3	Improved Lalat	0.196	0.041	0.763	SG3
4	Radhi	0.028	0.022	0.951	SG3
5	Luna Sankhi	0.174	0.01	0.816	SG3
6	Phalguni	0.902	0.03	0.068	SG1
7	Satyabhama	0.011	0.023	0.966	SG3
8	Chandan	0.011	0.933	0.056	SG2
9	Improved-Tapaswini	0.047	0.016	0.937	SG3
10	Saket-4	0.068	0.017	0.915	SG3
11	HUE	0.047	0.007	0.946	SG3
12	Luna Sampad	0.935	0.012	0.053	SG1
13	Maudamani	0.872	0.029	0.099	SG1
14	Poorna Bhog	0.094	0.035	0.87	SG3
15	Geetanjali	0.484	0.01	0.507	AD
16	Virender	0.613	0.041	0.346	SG1
17	Chandrama	0.908	0.02	0.071	SG1
18	Kalinga-II	0.02	0.019	0.962	SG3
19	Naveen	0.149	0.833	0.018	SG2
20	Shaktiman	0.034	0.022	0.944	SG3
21	Tapaswini	0.838	0.021	0.141	SG1
22	Abhishek	0.039	0.014	0.947	SG3
23	Anjali	0.661	0.025	0.314	SG
24	Kalinga-I	0.013	0.865	0.123	SG2
25	Ratna	0.057	0.022	0.921	SG3
26	Pyari	0.014	0.975	0.012	SG2
27	CR Dhan 205	0.97	0.018	0.012	SG1
28	CR Dhan 303	0.785	0.022	0.193	SG1
29	CR Dhan 202	0.128	0.823	0.049	SG2
30	CR Dhan 304	0.742	0.014	0.244	SG1
31	CR Dhan 305	0.018	0.963	0.019	SG2
32	CR Dhan 306	0.871	0.013	0.116	SG1
33	CR Dhan 201	0.076	0.013	0.911	SG3
34	CR Dhan 204	0.019	0.968	0.013	SG2
35	CR Dhan 601	0.007	0.931	0.062	SG2
36	Khitish	0.02	0.017	0.963	SG3
37	Sattari	0.012	0.929	0.059	SG3
38	Heera	0.495	0.012	0.493	AD
39	Sahbhagidhan	0.886	0.027	0.087	SG1
40	Sarasa	0.021	0.926	0.053	SG2
41	Indira	0.017	0.011	0.972	SG3
42	Hazaridhan	0.015	0.011	0.974	SG3
43	Kalyani-II	0.241	0.023	0.735	SG3
44	Udaya	0.932	0.024	0.044	SG1
45	Supriya	0.056	0.021	0.923	SG3
46	Annada	0.013	0.012	0.975	SG3
47	Sonamani	0.341	0.019	0.64	SG3
48	Hanseswari	0.911	0.014	0.076	SG1
49	CR Dhan 300	0.141	0.065	0.794	SG3
50	Jalamani	0.573	0.059	0.369	SG1
51	Nua Chinikamini	0.984	0.006	0.01	SG1
52	Sumit	0.021	0.916	0.063	SG2
53	Nua Dhusara	0.823	0.168	0.009	SG1
54	Nua Kalajeera	0.969	0.009	0.022	SG1
55	CR Dhan 907	0.286	0.039	0.675	SG3
56	CR Dhan 701	0.009	0.962	0.029	SG2
57	Moti	0.014	0.979	0.007	SG2
58	Rajalaxmi	0.009	0.956	0.035	SG2
59	Ketekijoha	0.822	0.168	0.01	SG1
60	Dharitri	0.043	0.945	0.012	SG2

Table 4 (*contd*)

	Varieties	Q1	Q2	Q3	Subgroup
61	Luna Barial	0.046	0.941	0.013	SG2
62	Jayanti Dhan	0.936	0.025	0.039	SG1
63	CR Dhan 501	0.098	0.896	0.007	SG2
64	CR Dhan 500	0.376	0.612	0.012	SG2
65	Panidhan	0.599	0.028	0.374	SG1
66	Padmini	0.28	0.054	0.665	SG3
67	CR 1014	0.01	0.978	0.012	SG2
68	Sarala	0.01	0.981	0.01	SG2
69	Gayatri	0.028	0.962	0.01	SG2
70	Varshadhan	0.218	0.772	0.01	SG2
71	Savitri	0.681	0.057	0.263	SG1
72	Durga	0.012	0.979	0.009	SG2
73	Reeta	0.051	0.027	0.921	SG3
74	Luna Suvarna	0.956	0.013	0.031	SG1
75	Samalei	0.014	0.011	0.975	SG3
76	Utkalprabha	0.01	0.95	0.04	SG2
77	Pooja	0.031	0.951	0.018	SG2
78	Ajay	0.011	0.93	0.059	SG2
79	Swarna sub-1	0.039	0.934	0.028	SG2
80	Lunishree	0.893	0.02	0.086	SG1

Table 5. Percentage of variation explained by the first three axes using blast resistance gene in PCoA.

Axis (%)	1-axis	2-axis	3-axis
Variation of individual axis	12.71	8.93	7.42
Cumulative variation	12.71	21.65	29.06

and F_{IT} were observed to be 1.000. This suggested that individuals from different populations are weakly isolated and genetically more closely related. The pairwise Nei's

genetic distance ranged from 0.34 (between resistant and moderately resistant population) to 0.043 (resistant and susceptible population) (table 6b).

Genetic association of blast resistant genes

The genetic relatedness between disease score and molecular markers was investigated using the GLM to know any significance relatedness. Among the 36 markers corresponding to the 36 blast resistant genes, only two markers (RM7364, and pi21_79-3) corresponding to the blast resistant genes (*Pi56(t)* and *pi21*) were observed to be significantly associated with blast disease resistance. The phenotypic variance of the two markers varied from 4.9 to 5.1% (table 7). Among these markers, pi21_79-03 exhibited the highest phenotypic variance (5.1%) followed by RM7364 (4.9), whereas remaining markers did not exhibit significant association at $P < 0.1$.

Discussion

Genetic diversity of crop plants has been eroded due to replacement of landraces and traditional local varieties with improved and high yielding varieties (Tanksley *et al.* 1997). The emergence of new and virulent races imposed a constant threat to sustainable rice production and global food security. To keep pace with the pathogen, it is necessary to identify the potential donor for novel resistance genes/alleles to combat the nuisance caused by this disease. In this study, we performed the candidate gene-based screening of blast resistance donors (NRVs) in rice breeding distributed over eight ecologies using 36 known blast resistance genes.
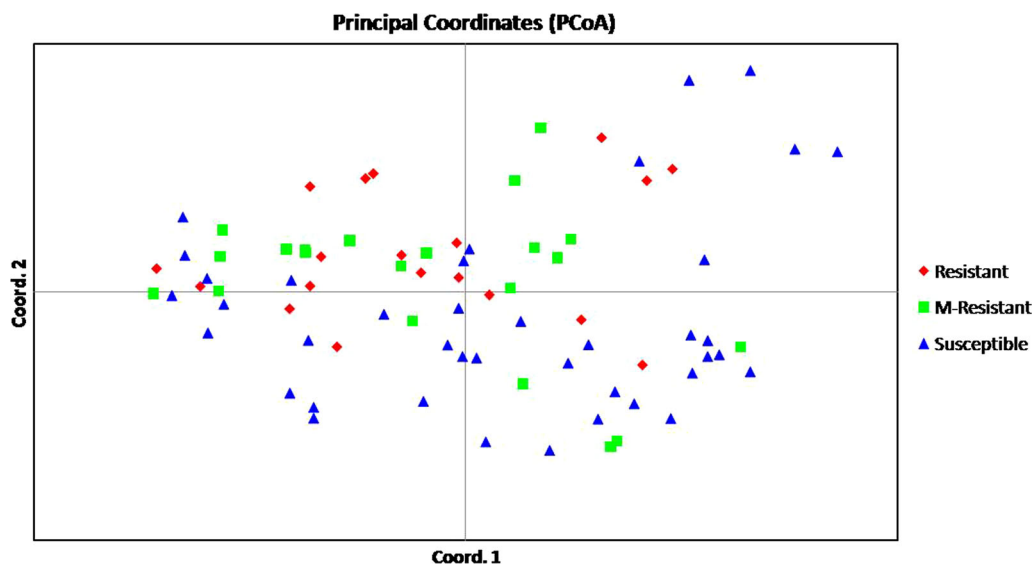
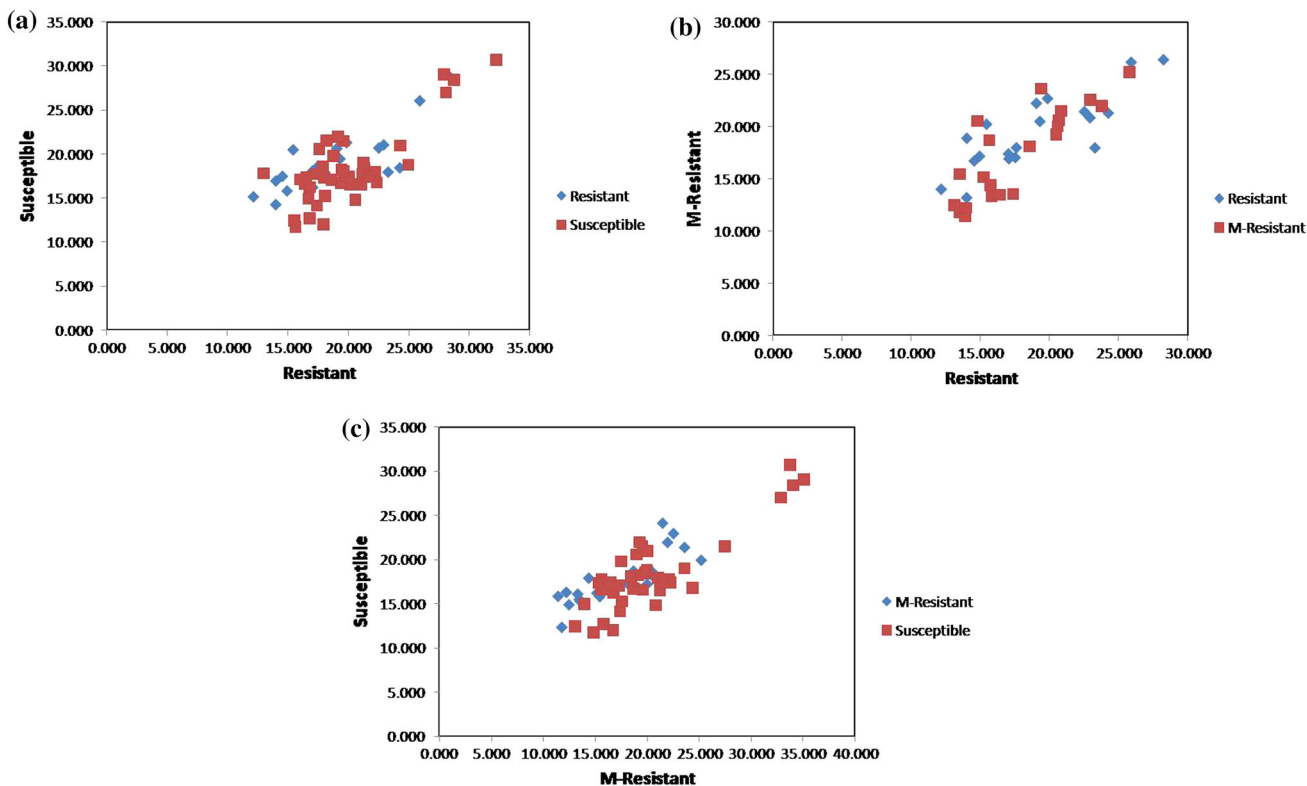**Figure 4.** PCoA of 36 molecular markers linked to blast resistance in 80 NRVs.

Figure 5. Population assignment of NRVs signifying the log likelihood assignment of each NRVs using disease reaction: (a) resistant and susceptible populations, (b) resistant and moderately resistant populations, (c) moderately resistant and susceptible populations.
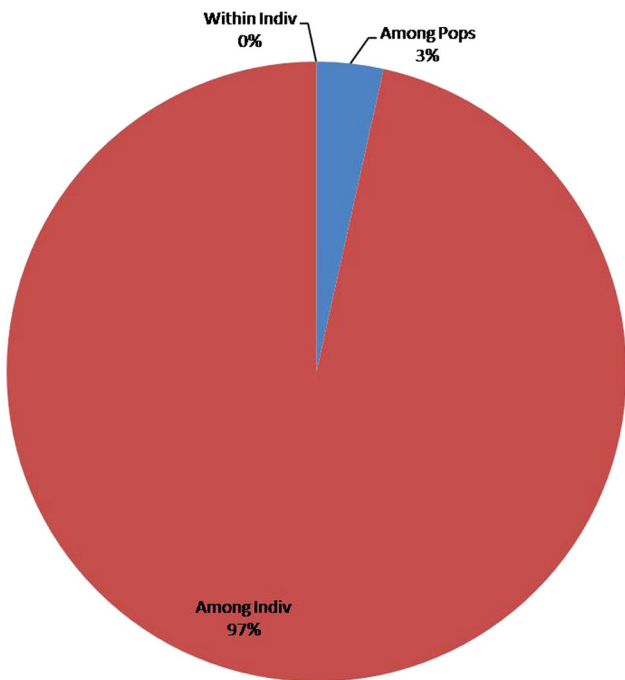

Figure 6. AMOVA analysis of NRVs.

Among the NRVs screened for blast disease resistance in the uniform blast nursery under natural screening at NRRI Cuttack, 19 NRVs were found to be resistant to the leaf blast disease. Among 19 NRVs, seven were reported to be released as resistant to *M. oryzae* (Yadav *et al.* 2017). These seven NRVs belonged to three different agro-ecologies, namely Satya Krishna, Chandrama, and Abhishek (irrigated), Sahbhagidhan (upland), Sumit, Reeta and Samalei (shallow low land). Interestingly, all these NRVs were released after 2006 except Samalei. Similarly, Zhu *et al.* (2016) reported 40 cultivars as highly resistant in China and 20 were previously reported to be resistant. During the breeding programme, identification of the individual resistance gene is often difficult through phenotype-based screening, as it is influenced by the developmental stage and environmental conditions. Instead, DNA markers linked to *R* genes are the easy and quickest way to identify and select several blast resistance genes without performing phenotype-based screening (Hayashi *et al.* 2006).

The average gene diversity was found to be 0.34, ranging from 0.11 to 0.49, whereas the major allele frequency had a mean value of 0.75 and varied from 0.52 to 0.93. The gene diversity was observed to be 0.67 in 107 NE collections, 0.25 in 80 NRVs, 0.227 in 288 landraces, and 0.32 in 167 landraces (Roy *et al.* 2016; Yadav *et al.* 2017; Susan *et al.* 2019; Yadav *et al.* 2019). The degree of polymorphism was detected by calculating PIC values that varied from 0.11 (RM101 and RM11787) to 0.37 (RM72 and Pia-STS) with an average of 0.27. It was higher than that observed by Yadav *et al.* (2017) in 80 NRVs (0.18) and Susan *et al.*

Table 6a. AMOVA among and within populations.

Source	df	Sum of squares	Mean squares	Estimated variance	Percentage of variation
Among populations	2	50.307	25.154	0.238	3
Among individuals within populations	77	1020.418	13.252	6.626	97
Within individuals	80	0.000	0.000	0.000	0
Total	159	1070.725		6.864	100

Table 6b. Pairwise population matrix of Nei's genetic distance among the three populations of NRVs.

Population	Resistant	Moderately resistant	Susceptible
Resistant	0.000		
Moderately resistant	0.034	0.000	
Susceptible	0.043	0.037	0.000

F_{st} values below diagonal between populations based on gene specific markers.

(2019) and slightly lower than Roy *et al.* (2016) with 0.62 in NE Himalayan landraces. In the present study, low gene diversity and PIC values were observed as compared to landraces. These NRVs are developed as a result of strong artificial selection pressure, whereas it does not strongly operate in case of landraces.

Based on marker genotype data, distance based clustering categorized the entire NRVs into three major clusters. Most of the resistant NRVs were clustered in major cluster I followed by major cluster II. Our results are in accordance with the previous studies where resistant accessions were clustered in one group and susceptible in another group (Yadav *et al.* 2017; Susan *et al.* 2019). Interestingly, NRVs of same ecologies were not grouped together whereas genetically similar NRVs were clustered together.

The genetic architecture of NRVs was investigated using the model-based structure software based on 36 molecular markers. Population structure differentiated 80 NRVs into three subgroups (SG1, SG2 and SG3) with two admixtures. Most of the resistant genotypes (eight) belonged to SG1 whereas, SG2 and SG3 included six (22.22%) and five (18.51%) resistant NRVs, respectively. Consequently, a weak association for blast reaction was observed through structure analysis. Yadav *et al.* (2017) categorized 80 NRVs into three subgroups through structure analysis using 17 markers linked to blast resistance. Similarly, Roy *et al.* (2016) and Susan *et al.* (2019) divided the NE landraces into three and two subpopulation, respectively. The result of cluster analysis is in accordance with the structure analysis of the NRVs. The corresponding NRVs in SG1 and SG2 were found concurrent with the cluster I, NRVs of the cluster III is harmonized with SG3 whereas most of the NRVs of subcluster III belonged to SG3.

Table 7. Genetic association of rice blast resistant genes with blast disease in 80 NRVs.

Marker	*F* value	*P* value	Phenotypic variance (%)
SNP_3	0.42225	0.51773	0.00538
RM151	1.64612	0.20329	0.02067
RM259	0.06501	0.79941	$8.33E{-}04$
RM72	0.50908	0.47767	0.00648
RM1216	0.61272	0.43614	0.00779
RM5647	1.56626	0.21449	0.01968
RM212	0.72454	0.39727	0.0092
RM206	1.72785	0.19254	0.02167
RM247	0.04329	0.83572	$5.55E{-}04$
RM28130	0.28505	0.59493	0.00364
RM5709	0.01413	0.90569	$1.81E{-}04$
RM5473	0.08775	0.76785	0.00112
RM5364	0.76565	0.38425	0.00972
RM6094	0.53782	0.46554	0.00685
RM3452	0.00751	0.93115	$9.63E{-}05$
RM7364	4.07908	0.04685	0.0497*
CRG_4	1.81314	0.18203	0.02272
RM6905	1.45582	0.23124	0.01832
RM7311	0.36287	0.54866	0.00463
Y10	1.45733	0.231	0.01834
RM101	0.05403	0.8168	$6.92E{-}04$
RM6629	0.0619	0.80418	$7.93E{-}04$
RM11787	1.80805	0.18264	0.02266
RM27273	1.07613	0.30277	0.01361
RM246	0.20882	0.64897	0.00267
RM262	$6.59E{-}06$	0.99796	$8.45E{-}08$
RM5529	0.54091	0.46426	0.00689
RM208	0.27423	0.60199	0.0035
RM26998	1.80737	0.18272	0.02265
tk59-1	0.04189	0.83837	$5.37E{-}04$
tk59-2	0.04189	0.83837	$5.37E{-}04$
RM6648	0.22146	0.63924	0.00283
pi21_79-3	4.26175	0.0423	0.05181*
Pia-STS	0.04178	0.83857	$5.35E{-}04$
RM17496	0.97557	0.32635	0.01235
RM224	0.18421	0.66896	0.00236

*Significance level at $P < 0.01$ and 0.05 respectively.

The PCoA through the scatter plots partitioned the resistant and susceptible NRVs into different quadrant. Likewise, previous study also showed the portioning of resistant and susceptible genotypes into different groups (Yadav *et al.* 2017; Susan *et al.* 2019). However, population assignment partially differentiated resistant and moderately resistant populations.

AMOVA is a method of estimating molecular variance within the species. Based on AMOVA, variation within the population was higher (97%) as compared with between populations (3%). The highest F_{ST} was observed between the resistant and susceptible populations (0.040) while, the minimum was observed between the resistant and moderately resistant populations. The inbreeding coefficient F_{IS} and F_{IT} were observed to be 1.0. The lower F_{ST} value indicated that the lower divergence between subgroups. Yadav *et al.* (2017) observed more variance within the population (96%), whereas less between populations (4%) and F_{ST} was in accordance with our result. Similarly, Susan *et al.* (2019) reported higher (96%) variance within the population and lower (4%) between the populations in the NE landraces. The pairwise Nei's genetic distance ranged from 0.034 (between resistant and moderately resistant population) to 0.043 (resistant and susceptible population).

Candidate gene-based association mapping is an approach to dissect trait of interest that investigate individual genes for genetic association with a phenotype (Neale and Savolainen 2004). Through GLM, among 36 markers tested, two markers corresponding to two blast resistance genes, *Pi56(t)* and *pi21* were identified to be significantly associated with phenotypic variance varied from 4.9 to 5.1%. Interestingly, *Pi56(t)* and *pi21* genes were reported to be a broad spectrum in nature and individual markers can be used for identification of rice blast resistant genes in the diverse rice germplasm. The genetic association of blast resistance in 80 NRVs, 167 landraces and 288 NE landraces against rice blast varied from 6.5 to 7.7% and explained its implication in markers identification associated with the blast resistance (Yadav *et al.* 2017; Susan *et al.* 2019; Yadav *et al.* 2019). However, the present study did not completely explain the genetic differences between the resistance gene(s) and the disease reaction. Similarly, Yadav *et al.* (2017) was not able to explain the resistance spectrum using 17 markers corresponding to the 12 blast resistance genes. The resistance spectrum of these NRVs could be explained through the identification of new blast resistance genes, their allelic variant or QTLs. In addition, these varieties had been released with multiple stress tolerance for different ecologies, which could be tested for other biotic and abiotic stresses. These resistant NRVs identified in the present study could be used as a potential donor for the breeding of blast resistance as well as genetic material for identification cloning and characterization of new blast resistant genes.

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