ORIGINAL RESEARCH ARTICLE OPEN ACCESS

Genetic diversity analysis of rice germplasm in gujarat state of india using simple sequence repeat markers

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Received: 10 August 2019; Accepted: 12 December 2019

Abstract

The present investigation was undertaken to detect the polymorphism among blast resistant and susceptible rice genotypes employing twenty three simple sequence repeat (SSR) markers,which targeted nine blast resistance genes. The clustering patterns of 2D and 3D of principal component analysis (PCA) were in accordance with the dendrogram clustering pattern. The dendrogram obtained from the pooled SSR analysis showed significant differences between all genotypes. The cophenetic correlation analysis revealed that the dendrograms generated by pooled SSR data were good to fit. The highest similarity index value of 0.71 was found in Ajaya and IET-20006, while the least similarity index value of 0.14 was found in GR-7 and IET-21094, GR-7 and IET-21070. The average similarity coefficient among genotypes was 0.46. The SSR markers linked to the blast resistance genes showed a high level of polymorphism among the genotypes.

Keywords: *Oryza sativa*, Blast resistance, *Magnaporthe grisea*, simple sequence repeat marker

Introduction

Rice is the major staple food of nearly half of the world population (Khush *et al*., 2005). It is planted on about 165 million hectares or on about 12% of the world's cultivated land with an annual production of 744 million tons (FAO, 2014) and 43.5 million hectares land is under rice cultivation in India. The demand for production is increasing steadily for meeting the requirement of a growing population in India. For achieving this increasing demand, rice production will have to increase by at least 40% by the year 2030. To meet this challenge, there is an imminent need to develop high yielding rice varieties with durable resistance to both biotic and abiotic stresses (Leung *et al*., 2004). The rice blast disease caused by fungus *Magnaporthe grisea* (anamorph *Pyricularia oryzae*), is one of the most devastating diseases affecting the rice crop throughout the world and has been responsible for 35-50% world yield loss in rice (Variar *et al*., 2007). Till now more than 100 blast rice genes have been identified (Khanna *et al*., 2015) and 26 genes have been cloned. Most of the known blast-resistance genes are located on chromosomes 6, 11 and 12 (Sallaud *et al*., 2003). Among the blast resistance genes, *Pi54*, *Pi1*, *Pi2*, *Pi9* and *Pizt*, etc. are considered to be highly useful under Indian conditions. It is possible to increase rice production without expansion of cultivation area by using molecular markers and breeding methodology. Molecular markers play an important role in plant genome analysis and crop improvement. Of the many types of molecular markers, simple sequence repeats (SSR) or microsatellite markers are widely used due to their abundance in the genome. SSR markers are co-dominant, easily assayed by PCR, and exhibit variation based on the motif repeats. Due to these features, SSRs are being widely used for the estimation of genetic diversity (Freeg *et al*., 2016), establishment of varietal identity, analysis of genetic structure with in the cultivated rice (Garris *et al*., 2005), construction of molecular genetic maps and marker assisted selection.

Genetic diversity is a pre-requisite for any crop improvement program as it helps in development of superior recombinants. It is a source of variation, which is raw material for any improvement work. Genetic diversity

analysis is very useful for estimating and establishing genetic relationship in collection of different germplasm, for identifying the different parental combinations to create segregating progenies with maximum genetic variability for further selection and introgression of desirable genes into elite genotypes (Islam *et al*., 2012). Analysis of genetic diversity also helps breeder to enhance the progress of breeding program.

Therefore, the current study was carried out for the assessment of genetic variability at molecular level among 35 rice genotypes differing in their resistance to blast. Twenty three SSR markers were used for developing unique finger print for each genotype. This may also be used to identify the best genotype as donor for blast resistance in breeding program for development of new blast resistance varieties.

Materials and methods

Plant materials

The seeds of 35 rice genotypes were collected from the Main Rice Research Station, Nawagam, Gujarat. Names of these genotypes and their response against blast disease are mentioned in Table 1.

Table 1: List of genotypes

Sr. No.	Genotypes	Susceptibility/Resistance to Blast disease	Sr. No.	Genotypes	Susceptibility/Resistance to Blast disease
1	IR-64	Resistant	19	IET-20669	Resistant
2	GR-11	Susceptible	20	IET-20862	Resistant
3	Pankhali-203	Susceptible	21	IET-20866	Resistant
4	GR-7	Resistant	22	IET-20868	Resistant
5	GAR-1	Susceptible	23	IET-20872	Resistant
6	GR-12	Resistant	24	IET-20874	Resistant
7	GAR-13	Resistant	25	IET-20892	Resistant
8	Gurjari	Resistant	26	IET-20894	Resistant
9	NWGR-2006	Resistant	27	IET-20929	Resistant
10	Azucena	Susceptible	28	IET-21000	Resistant
11	Ajaya	Susceptible	29	IET-21070	Resistant
12	IET-20006	Resistant	30	IET-21094	Resistant
13	IET-20082	Resistant	31	IET-21190	Resistant
14	IET-20214	Resistant	32	IET-21200	Resistant
15	IET-20235	Resistant	33	IET-21216	Resistant
16	IET-20375	Resistant	34	IET-21299	Resistant
17	IET-20667	Resistant	35	IET-21649	Resistant
18	IET-20668	Resistant			

DNA isolation

A modified mini preparation procedure (Zaidani *et al*., 2005) for extraction of total genomic DNA from leaf samples of 35 genotypes was used. The quality and quantity of the isolated DNA samples were checked by spectrophotometer (Thermo electronic corporation, UV1) and 0.8% agarose gel electrophoresis with lambda (λ) *Hind* III restriction digested DNA as molecular weight standard. The concentration and quality of DNA in individual samples were determined based on the intensity and thickness of the genomic DNA bands, compared to *Hind* III digested lambda (λ) DNA.

Simple sequence repeats (SSR) amplification

A total number of 23 SSR markers which are related to nine blast resistance traits/QTLs were selected for the genetic analysis based on the Gramene Marker database (http:// www. gramene.org/markers) and previously published reports of Temnykh *et al*., 2001 and McCouch*et al*., 2002 (Table 2). The PCR reaction mixture contained 50ng template DNA,5 pM of each of the forward and reverse primers, 200µM dNTPs, 1X PCR buffer (10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM of $MgCl_2$ and 0.01mg/ml gelatin) and 0.5U of Taq DNA polymerase (JONAKI) in a volume of 10µl. Amplification cycling was performed in a gradient 96 well programmable master cycler (Veriti™, Applied Biosystems). The PCR was carried out with one cycle of denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 45s, 55°C for 45s and 72°C for 1 min, with a final extension of 72°C for 10 minutes. The final PCR products were then mixed with Bromophenol blue and run in 3% agarose gels along with 50-bp ladder for an hour in 0.5x Tris-Acetic and EDTA (TAE) buffer. The resolved PCR bands were documented using gel documentation system (BIO-RAD Molecular Imager Gel Doc XR system) and images were stored for further scoring and permanent records.

Data analysis: The amplicons were scored individually as (1) and (0) for the presence or absence of an allele, respectively. Data entry was done in a binary data matrix as discrete variables PIC values were calculated by using the formula PIC=1 -$\sum$ pi2, where pi indicates frequency of the allele of each locus (Botstein *et al*., 1980). The matrices were used to calculate pair wise genetic similarity based on Jaccard's coefficient. Dendrogram displaying relationships among 35 rice genotypes was constructed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) by NTSYSpc version 2.02 (Rohlf, 1994).The allele frequency was determined by the use of GenAlEx version 6.3 (Peakall and Smouse 2006). The expected heterozygocity and observed heterozygocity were also determined as described in the following formula by the use of same software.

Ho = Observed Heterozygocity = No. of Hets / N

He = Expected Heterozygocity = 1 - Sum pi^2 Where pi is the frequency of the i^{th} allele for the population and Sum pi^2 is the sum of the squared population allele frequencies.

Results and Discussion

Allelic diversity of microsatellite markers

For the present study, the seeds of 35 rice genotypes were analyzed (Table 1). Among them, IR-64 was selected as the resistant check. The susceptible checks used for the present study were GR-11 and Pankhali-203. The remaining 32 genotypes were used for comparison with the above mentioned checks for blast resistance and for analysis with SSR markers. A total of 81alleles were detected of which 71 (87.65%) were polymorphic (Table 3). The Polymorphic Information Content (PIC) values ranged from 0.28 (RM339) to 1.00 (RM 28050) with an average of 0.59. The number of alleles per SSR locus detected in this study corresponded well with Sajib *et al*., (2012) and Singh *et al*., (2016) and was lower than the average number of alleles (11.9) reported by Zhou *et al*., (2004) and Lin *et al*., (2007). The wide variation in the number of alleles detected was due to the different sets of germplasm, number of genotypes, number of selective SSR markers and method of gel electrophoresis detection in different studies. The low number of alleles was usually obtained from closely related cultivars (IR64, IET 20667-IET20669 and Ajay and Azucena). High number of alleles was expected when a large number of landraces were chosen from different geographical origins (Masuduzzaman *et al.*, 2016).

Table 2: List of blast resistance genes, markers and the chromosome details

Sr. No.	Gene	Marker	Chromosome	Primer sequences (5'-3')		Reference
1.1	*Pi25*	RM110	2	F	TCGAAGCCATCCACCAACGAAG	Temnykh *et al*., 2001
				R	TCCGTACGCCGACGAGGTCGAG	
1.2		RM485	2	F	CACACTTTCCAGTCCTCTCC	Temnykh *et al*., 2001
				R	CATCTTCCTCTCTTCGGCAC	
2.1	*Pi27*	RM549	6	F	ACGAACTGATCATATCCGCC	Temnykh *et al*., 2001
				R	CTGTGGTTGATCCCTGAACC	
2.2		RM538	6	F	GGTCGTTGAAGCTTACCAGC	Temnykh *et al*., 2001
				R	ACAAGCTCTCAAAACTCGCC	
2.3		RM276	6	F	CTCAACGTTGACACCTCGTG	Lopez-Gerena *et al*., 2004
				R	TCCTCCATCGAGCAGTATCA	
3.1	*Pi29*	RM339	8	F	GTAATCGATGCTGTGGGAAG	Lopez-Gerena *et al*., 2004
				R	GAGTCATGTGATAGCCGATATG	
3.2		RM325	8	F	GACGATGAATCAGGAGAACG	Temnykh *et al*., 2000
				R	GGCATGCATCTGAGTAATGG	
3.3		RM483	8	F	CTTCCACCATAAAACCGGAG	Temnykh *et al*., 2001
				R	ACACCGGTGATCTTGTAGCC	
4.1	*Pi30*	RM441	11	F	ACACCAGAGAGAGAGAGAGAGAG	Zeng *et al*., 2011
				R	TCTGCAACGGCTGATAGATG	
4.2		RM120	11	F	CACACAAGCCCTGTCTCACGACC	Lopez-Gerena *et al*., 2004
				R	CGCTGCGTCATGAGTATGTA	
5.1	*Pi31*	RM83	12	F	ACTCGATGACAAGTTGAGG	Chen *et al*., 1997
				R	CACCTAGACACGATCGAG	
5.2		RM277	12	F	CGGTCAAATCATCACCTGAC	Temnykh *et al*., 2000
				R	CAAGGCTTGCAAGGGAAG	
5.3		RM463	12	F	TTCCCCTCCTTTTATGGTGC	Temnykh *et al*., 2001
				R	TGTTCTCCTCAGTCACTGCG	
5.4		RM260	12	F	ACTCCACTATGACCCAGAG	Lopez-Gerena *et al*., 2004
				R	GAACAATCCCTTCTACGATCG	
6.1	*Pi33*	Pi33-53	8	F	ACAGGATCTTTACCGGCATTTA	Raboin *et al*., 2016
				R	ACGCAAGGAGATTGTTGAGATT	
6.2		RM5647	8	F	ACTCCGACTGCAGTTTTTGC	McCouch *et al*., 2002
				R	AACTTGGTCGTGGACAGTGC	
6.3		RM3507	8	F	ACCCCTATCGATCAACCCTC	McCouch *et al.*, 2002
				R	TTCGTTTGGTGTTAGGGGC	
6.4		RM3374	8	F	ATGAACTAGTGAACCCCCCC	McCouch *et al.*, 2002
				R	GTAGCGGTAGCTGCAAAAGC	
6.5		RM44	8	F	CGGGCAATCCGAACAACC	Lopez-Gerena *et al*., 2004
				R	TCGGGAAAACCTACCCTACC	
7.1	*Pita*	YL100-102	12	F	CAATGCCGAGTGTGCAAAGG	Eizenga *et al.*, 2002
				R	TCAGGTTGAAGATGCATAGC	
7.2		YL183-87	12	F	AGCAGGTTATAAGCTAGCTAT	Eizenga *et al.*, 2002
				R	CTACCAACAAGTTCATCAAA	
8.1	*Pib*	RM535	2	F	ACTACATACACGGCCCTTGC	Kim *et al*., 2005
				R	CTACGTGGACACCGTCACAC	
9.1	*Pi20*	RM28050	12	F	GATAAGACTTGGGTGGACATCACG	Li *et al*., 2008
				R	CTTCTATGGTCGCAATTCAGATGC	

Nine genes were used for analysis in the present study *viz.Pi25, Pi27, Pi29, Pi30,Pi31, Pi33, Pita, Pib* and *Pi20*. These genes are located on different chromosomes. The *Pi27* is located on chromosome No. 6, *Pi29* and *Pi33* on chromosome number 8, *Pita, Pi20 and Pi31* on chromosome 12, *Pib* and *Pi25* on chromosome 2 and *Pi30* on chromosome 11. The markers linked to these genes were selected (Table 2) for the study.

The nine different resistance imparting genes were targeted for the SSR study in which twenty three SSR markers were used. The twenty three SSR primers amplified a total of 81 alleles with an average of 3.52 alleles per marker. Based on the SSR data, cluster analysis was performed using genetic similarity values and a dendrogram was generated showing genetic relationships among these genotypes. The highest similarity index value of 0.71 was found between Ajaya and IET-20006, while the least similarity index value of 0.14 was found between GR-7 and IET-21094 and also between GR-7 and IET-21070. The average similarity coefficient among genotypes was 0.46. The SSR marker RM83, specific for *Pi31* gene amplified one allele, while RM463 for the same gene amplified5 alleles. The SSR marker RM441 for *Pi30* gene produced maximum number of alleles (7) and RM538 for *Pi27* gene, RM83 for *Pi31* gene, YL183-87 for *Pita* gene and RM28050 for *Pi20* gene produced only single allele. The average number of alleles amplified by 23 markers was 3.52. The highest PIC (Polymorphic Information Content) value obtained was 1.0 in RM538 for *Pi27* gene, YL183-87 for *Pita* gene, and RM28050 for *Pi20* gene and lowest PIC value was 0.12 for YL100-102 for *Pita* gene. The allele frequency, expected hetero zygosity and observed hetero zygosity of each marker were also determined in the present study through GenAl Ex software (version 6.3) (Peakall and Smouse., 2006). The SSR gel results of Pi-33-53 marker for gene *Pi33*is shown in Figure 1. The details of amplification products are given in Table 3.

Table 3: Results of candidate genes and respective molecular markers in SSR analysis

Sr. No.	Gene Name	Marker	Chromosome location	No. of alleles amplified	Molecular Weight Range	Total No. of bands	PIC value
1.1	*Pi25*	RM110	2	5	74-200	5	0.69
1.2		RM485	2	6	270-393	5	0.78
2.1	*Pi27*	RM549	6	3	145-813	3	0.40
2.2		RM538	6	1	290	1	1.0
2.3		RM276	6	4	98-146	4	0.59
3.1	*Pi29*	RM339	8	4	73-155	4	0.28
3.2		RM325	8	2	62-189	2	0.35
3.3		RM483	8	3	263-316	3	0.53
4.1	*Pi30*	RM441	11	7	49-194	7	0.78
4.2		RM120	11	4	144-658	3	0.50
5.1	*Pi31*	RM83	12	1	307	2	1.0
5.2		RM277	12	3	114-128	2	0.41
5.3		RM463	12	5	176-214	5	0.65
5.4		RM260	12	5	70-165	5	0.58
6.1	*Pi33*	Pi33-53	8	3	252-304	6	0.41
6.2		RM5647	8	6	61-144	3	0.70
6.3		RM3507	8	4	86-124	2	0.64
6.4		RM3374	8	3	63-115	1	0.53
6.5		RM44	8	5	104-138	2	0.58
7.1	*Pita*	YL100-102	12	2	366-419	3	0.12
7.2		YL183-87	12	1	308	2	1.0
8.1	*Pib*	RM535	2	3	132-429	3	0.54
9.1	*Pi20*	RM28050	12	1	174	1	1.0
Total	-	-	-	**81**	-	**74**	-
Average	-	-	-	**3.52**	-	**3.22**	**0.59**

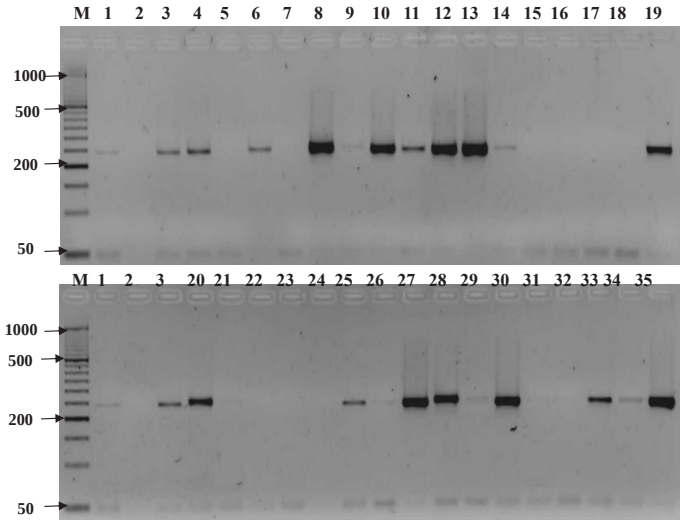

Figure 1: SSR Profile of Pi-33-53 marker for gene *Pi33*, M = 50 bp DNA ladder

1. IR-64	8. Gurjari	15. IET-20235	22. IET-20868	29. IET-21070
2. GR-11	9. NWGR-2006	16. IET-20275	23. IET-20872	30. IET-21094
3. Pankhali- 203	10. Azucena	17. IET-20667	24. IET-20874	31. IET-21190
4. GR-7	11. Ajay	18. IET-20668	25. IET-20892	32. IET-21200
5. GAR-1	12. IET-20006	19. IET-20669	26. IET-20894	33. IET-21216
6. GR-12	13. IET-20082	20. IET-20862	27. IET-20929	34. IET-21299
7. GAR-13	14. IET-20214	21. IET-20866	28. IET-21000	35. IET-21649

Clustering pattern of dendrogram generated by pooled molecular data of 23 markers of nine genes produced two main clusters namely A and B (Figure 1). Cluster A included IR64 and GR7. Cluster B was divided into two sub clusters B1 and B2. Sub cluster B1was again divided into two clusters *viz*. B1a and B1b. The cluster B1a consisted of GR-11, NWGR-2006, IET-20375, IET-20667, IET-20668 and IET-20669 and B1b comprised of only single genotype *i.e.* GAR-13. Sub cluster B2 was divided into B2a and B2b. The cluster B2b had only two genotypes namely IET-20235 and IET-21000. Subcluster B2a was again divided into B2ac and B2ad. The cluster B2ac consisted of Pankhali-203, IET-20892, IET-20868, IET-21190, IET-20894, GAR-1, IET-20866, IET-20872, IET-20874, IET-21070 and IET-21200 whereas cluster B2ad comprised of GR-12, Gurjari, Ajay, IET-20006, Azucena, IET-20862, IET-20082, IET-20214, IET-20929, IET-21216, IET-21299, IET-21094 and IET-21649.

The genotype IR-64 was selected as a resistant genotype in the present study which showed entirely different cluster *i.e.* Cluster A along with GR-7. The genotypes Ajay and Azucena came into the same cluster of B2ad. The genotype IET-20667, IET-20668 and IET-20669 were the pyramided lines of IR64 and were present in the same cluster B1a. (Figure 2) The principle component analysis (PCA) carried out with 35 genotypes almost coincided with the results of cluster analysis. The clustering pattern of 2D and 3D of PCA analysis were in accordance with the dendrogram clustering pattern. The 2D (Figure 3) plot showed that the genotypes IET-20375, IET-20667, IET-20668 and IET-20669 were nearer to each other. Similarly, genotypes IET-20892, IET-20868, IET-21190, IET-20894, GAR-1, IET-20866, IET-20872, IET-20874, IET-21070 and IET-21200 were found to be nearer to each other. In the 2D plot, GR-12, Gurjari, Ajay, Azucena, IET-20006, IET-20862 and IET-20214 were found in close proximity to each other. The genotypes IET-20082, IET-20929, IET-21216, IET-21299, IET-21094 and IET-21649 were also located nearer. The genotypes, Azucena and Ajay overlapped on one another. The principle component analysis (PCA) of 3D (Figure 4) plot coincided with the results of cluster analysis and 2D plot analysis. In 3D plot also genotypes IET-20892, IET-20868, IET-21190, IET-20894, GAR-1 came together. However, IET-20866, IET-20872, IET-20874, IET-21070 were found nearer but slightly away from it. The Ajaya and Azucena were nearer to each other. All other genotypes came in same group like in 2D and cluster analysis.

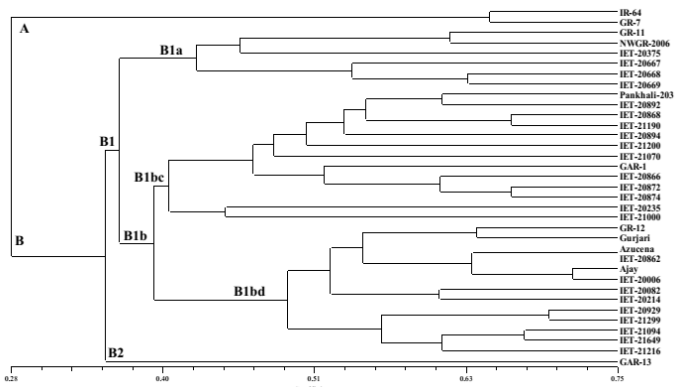

Figure 2: Dendrogram showing clustering of 35 rice genotypes for blast resistance genes constructed using UPGMA based on Jaccard's coefficient obtained from SSR analysis

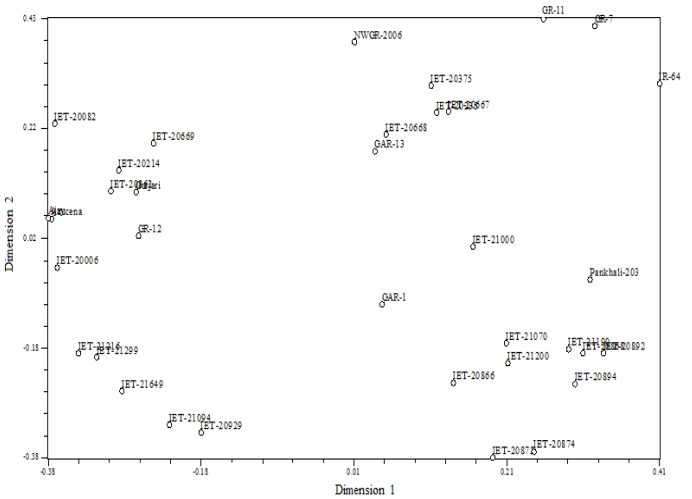

Figure 3: Two dimensional plot by PCA using SSR primers

Correlation study was carried out to compare the correlation of original similarity matrix of SSR results with the dendrogram clustering pattern. Using the COPH module of NTSYSpc version 2.02, 'r' value was calculated and results were also expressed graphically. High correlation between the similarity matrix and dendrogram pattern was justified by the 'r' value which was found to be 0.77 and was found good to fit. The genotypes IR 64 and GR-7 were found to be in the same cluster. The genotypes IET-20667, IET-20668 and IET-20669 showed similar allelic pattern in most of the markers. The Azucena and Ajaya were found to be closely related to each other as they showed similar banding pattern in maximum number of the markers studied. The diversity of alleles in blast resistant genotypes using 32 improved varieties with three micro-satellite marker including RM483 and a similarity matrix based on pair wise comparison of pooled data showed 60% similarity and three allelic conditions of the plants were produced (Lang *et al*., 2009). In the present study, 46% of similarity was observed and up to seven allelic conditions were also found using 23 markers including RM483. This variation may be due to the difference in number of markers and genotypes used in the present study and also may be due to the difference in varieties used.

The productivity and yield of cultivated rice, is severely affected by many diseases. Among them blast is the most destructive. Rice blast and gray leaf spot of grasses are caused by *Magnaporthe oryzae*. The disease often causes a significant yield loss, as high as 70-80% during an epidemic. Rice blast has been found in over 85 countries across the world. The disease has never been eradicated from the regions of rice cultivation.

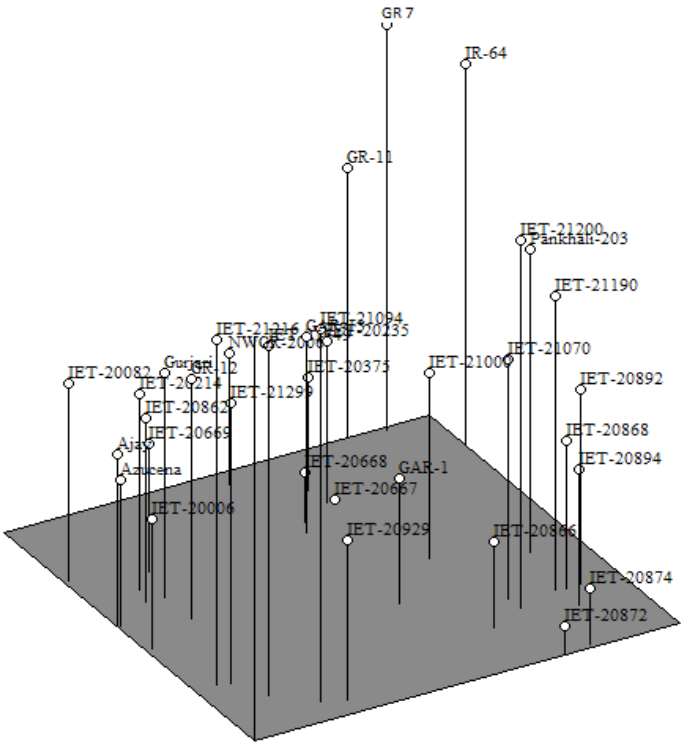

Figure 4: Three dimensional plot by PCA using SSR primers

To negate the effect of blast disease to some extent some has resorted to the use of fungicides and others to development of resistant cultivars. Utilization of genetic resistance is the most effective and economical way of controlling blast disease. In the past few decades, more than 40 major blast resistance genes have been mapped through molecular marker technology. However, because of either the rapid evolution of new pathogen races, or the selection of a rare component of the pathogen population that is already virulent the resistance has been short lived.

Molecular analysis of genotypes prior to crosses can increase genetic diversity among parental genotypes. This helps in maximizing genetic variation and minimizing the efforts in the screening, for direct selection in traditional breeding. Hence, the present investigation was undertaken to detect the polymorphism among blast resistant and susceptible rice genotypes. With the aid of information obtained from microsatellite marker study, assessment of linkage between specific marker and its gene could be done which will help in breeding and development of pyramided lines harboring different blast resistant genes leading to the development of much efficient genotypes for fighting the malady of blast.

Acknowledgments

Authors are thankful to the Department of Agricultural Botany, Department of Agricultural Biotechnology, Anand Agricultural University, Anand, Gujarat for providing basic laboratory facilities for the molecular research. We are also grateful to Late Dr. Atul Mehta, Main Rice Research Station, Nawagam, Gujarat,for valuable suggestions and providing seeds of rice genotypes. We are grateful to Dr. G.C. Jadeja, Former Principal, B.A. College of Agriculture,AnandAgricultural University, Anand, for his consistent guidance with constructive suggestions, deliberative discussions and active persuasion.

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