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Research Article

Path towards genetic diversity analysis and evaluation of blast resistant genes in popular varieties of paddy

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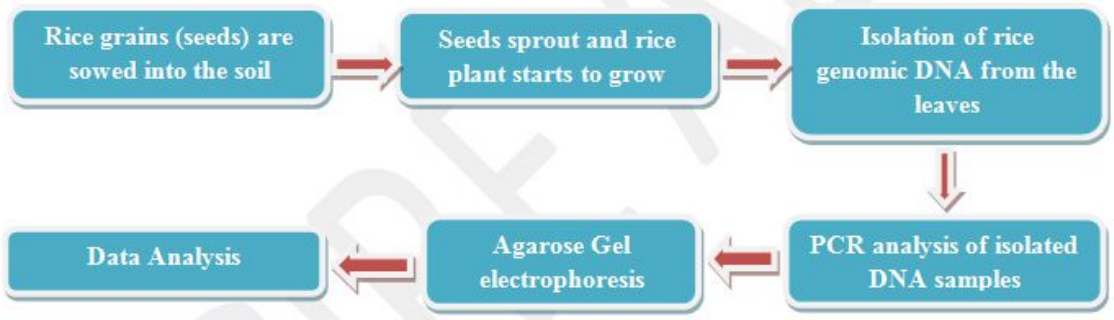
Objective: DNA isolation and quantification of selected rice varieties. Identification of blast resistance genes in rice varieties through genotypic method. Diversity analysis for rice varieties through set of SSR markers

Methodology:



Duration taken for the research: 3 months

Conclusion: A total of 44 alleles, an average PIC value of 0.41 and cluster analysis performed by using UPGMA resolved 19 advanced breeding lines into two major clusters with 24% dissimilarity so the set of markers used were highly informative and useful for genetic diversity studies as well as most of the lines might have maximum number of shared genetic loci which reflects the closeness.

Applicable Industries: Agriculture and Horticulture

Applicable geographical area: Karnataka, India

Expected outcome: The major difficulty in controlling rice blast is the durability of genetic resistance so enhancing the host plant resistance, international cooperation, completion of the rice genome project and candidate gene identification through rice functional genomics are the best approaches for handling the rice blast disease.

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Path towards genetic diversity analysis and evaluation of blast resistant genes in popular varieties of paddy

Sanjana et al.

Abstract

Rice being one of the most important cereal crops holds the second prominent position in global agriculture.With everyday increase in population, adverse climatic changes and new pathogen and pest activities, the rice breeders are facing great challenges to meet the demands. Worldwide rice is affected by most devastating blast disease caused by *Magnaporthe grisea.* Although chemical control is often found to be successful, this adds to the cost of cultivation and also contaminates the environment. To address this problem the genetic diversity analysis and evaluation of blast resistant genes in rice with the help of SSR molecular markers was conducted. The use of rice genetic resources available at the Rice Research Laboratory can be used to incorporate the genetic variability in rice breeding programme. This will allow new cultivars with broadened genetic basis to create new and useful allelic combination. Development of crosses can be used to broaden the genetic basis of rice and promote the preservation of rare alleles which can be incorporated into elite germplasm. Domestication of crops restricts the crop improvement by limiting the range of valuable traits used in modern plant breeding. The assessment of genetic diversity allows germplasm characterization and conservation. Wild species can improve germplasm but create problems due to reproductive isolation. Rice crop suffers from yield plateau due to narrow genetic base as well. Genetic variability is used in crop improvement program .In this present study 19 advanced breeding lines were selected for diversity analysis among rice varieties.

Keywords: Blast disease, Genetic diversity analysis, Magnaporthe grisea, SSR (simple sequence repeat) molecular markers.

Introduction

Various pathogens such as viruses, nematodes, bacteria, fungi and insects attack plants. Resistance (R) genes present in plant identifies the Avirulence (Avr) genes in insects and thus protect the plants from these attacks. Rice blast caused by the fungi Magnaporthe grisea is one of the most devastating diseases occurring in rice plant (Ashikawa *et al.*, 2008).Cloning of blast resistance genes, fine mapping techniqueswith aid of PCR-based markers help in identifying different blast resistance genes. Marker assisted selection (MAS) can be effectively employed when DNA markers are closely linked to blast R gene, conferring resistance towards a particular variety of paddy against the blast disease. Recently more than 100 blast resistant genes are identified from aponica (45%), indica (51%) and other (4%) genotypes and many of them have been cloned. It is expected that MAS of blast resistant genes shall help breeding programmers by the production of multi-disease resistant rice. Even though MAS has an advantage of identifying resistance, the main power depends on the markers used. PCR-

based markers and SSR molecular markers are effective in screening of resistance genes (Rau *et al.*, 2000). Pi-ta and Pi-ta2 genes identified by Kiyosawa and colleagues have been found to be tightly linked to the centromere on chromosome 12 which are linked to cluster of R genes. AVR-Pita is avirulent alleles of Magnaporthe grisea. The development of R genes for effectively controlling genetically occurring rice blast disease was aided by cloning the AVR-Pita and Pi-ta genes (Bryan *et al*., 2000).

Materials and methods

This project was carried out during 2017 at the Dept of Plant biotechnology, University Agricultural Sciences, GKVK, and Bangalore, India. The experimental material for this investigation comprised of 19 genotypes and a total of four simple sequence repeat (SSR) markers, i.e., 30 markers were used for studying molecular diversity (Table 1 and Table 2).

Isolation of rice genomic DNA Sample Preparation

Already collected and stored tender, fully expanded leaves (20-25 days old) were initially surface sterilized with ethanol (70%) before extraction.

Extraction of genomic DNA

Two gram of leaf samples were cut frozen using liquid nitrogen and ground into a fine powder in a pre chilled mortar. The fine powder was thawed in 1.5 ml of pre-heated extraction buffer (CTAB-Cetyl Trimethyl Ammonium Bromide) in 2 ml of polypropylene centrifuge tubes and incubated for 30 minutes at 65°C in water bath with occasional mixing. Following this equal volume of chloroform isoamylalcohol mixture was added and mixed by inversion for 15 minutes and centrifuged at 10,000 rpm for 20 minutes at room temperature. The clear aqueous phase was transferred to a new sterile tube to which equal volume of ice cold isopropanol was added and mixed gently by inversion and then kept in the -20 ^{o}C freezer to precipitate DNA for whole night. The tubes were then centrifuged at 8000rpm for 20 minutes and sedimentation of DNA as a hard pellet was seen. The pellet was washed twice by suspending in 1 mL of 70 percent ethanol for 5 to 10 minutes and the DNA was centrifuged at 6000 rpm for five minutes. Ethanol was drained off slowly and the pellet was air dried then dissolved in 100 µl of TE buffer by flicking the tubes. (Sterile H_2O /TEbuffer = 0.1 mM Tris + 0.05 mM EDTA) and stored at -20°C. The extracted DNA was subjected for checking quality and quantification using both Biospec-nano and by 0.8 per cent agarose gel electrophoresis.

Table 1: Improved Varieties of rice selected to identify the blast resistant genes at molecular level and genetic diversity analysis

CODING NO.	SAMPLE'S NAME	CODING NO.	SAMPLE'S NAME
1	IR64	11	JAYA
2	MTU1001	12	THANU
3	GSONA	13	RASI
4	RAJMUDI	14	JAYACROSS
5	RATNACHOODI	15	ASD
6	BR2655	16	MSONA
7	BPT	17	ANAGHA
8	MTU1010	18	KCP1
9	KMP175	19	IET30864
10	KMP153		

DNA purity and quantification done by nano spectrophotometer

To estimate the DNA purity advanced automated DNA quantifier Biospec-nano was used and ratios between 260 nm and 280 nm was estimated. Pure DNA samples will have a ratio of 1.8 to 2.0. If the sample is contaminated by protein the ratio will be significantly less than 1.8. Ratio of 2.0 or more indicates a high proportion of RNA in the sample. With this informationpure DNA was prepared (ratios of 1.8 to 2.0) and further dilution was decided. All the samples were diluted to a final concentration of 40ng/µl. Loading dye consisted of Glycerol 50% (V/V); Bromophenol blue 0.5% (W/V) and 10X TBE (Tris Boric EDTA buffer) .Volume was made up to 1000 ml with 8.0 pH. Agarose 0.8 % (0.8g/100 ml) was added to 1x TBE, boiled until the agarose dissolved completely and then allowed to cool. Ethidium bromide (5 µl/100 ml) was added to fluorescence the DNA under UV light. Then it was poured into the gel mould and allowed to solidify. The gel was placed in the electrophoresis unit with wells towards the cathode and submerged with 1XTBE to a depth of about 1cm. About 2 µl of the DNA sample mixed with 2 µl of loading dye was loaded. The electrophoresis was carried out at 100V for 10-15 min. Bands were visualized and documented using a gel documentation system (Gene flash).

Table 2: Finalized Polymorphic Markers for identification of blast resistant genes

Sl. No	Marker	*Gene*	Forward primer	Reverse primer	Res. Allele Size	References
1	RM3825	*Pish*	AAAGCCCCCAAAAGCAGTAC	GTGAAACTCTGGGGTGTTCG	230	Berruyer *et al*., 2003
2	RM72	*Pi-33*	CCGGCGATAAAACAATGAG	GCATCGGTCCTAACTAAGGG	290	Wang *et al*., 2009.
3	RM1896	*Pii,Pi3(t), Pi-5(t)*	GGACAGGGTAAAGTGTTAGA	CCTAAGACCTATCAACTCCA	168	Sharma *et al*. 2002
4	RM206	*Pi-38*	CCCATGCGTTTAACTATTCT	CGTTCCATCGATCCGTATGG	256	Fjellstrom *et al*. 2004
5	RM224	*Pik*	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG	291	Li *et al*. 2008
6	Pi54 Indel	*Pi54*	CAATCTCCAAAGTTTTCAGG	GCTTCAATCACTGCTAGACC	210	Hayashi *et al.*, 2006
7	Nmsm	*Pi9*	CGAGAAGGACATCTGGTACG	GAGATGCTTGGATTTAGAAGAC	290	Fjellstrom *et al*. 2004
8	AP5659-5	*Pi2/Piz*	CTCCTTCAGCTGCTCCTC	TGATGACTTCCAAACGGTAG	310	Li *et al*. 2008

PCR analysis

Thirty microsatellites were used to easily amplify by the PCR (Polymerase Chain reaction) using unique flanking sequences as forward and reverse primers. The reaction mixture was given a momentary spin for thorough mixing of the cocktail components. Then 0.20 ml PCR tubes were loaded in a thermal cycler (Table 3 and Table 4).

Table 3: PCR components

PCR components

PCR components	Concentration	Quantity
Primers		
Forward	10 µM	1.0 µl
Reverse	10 µM	1.0 µl
Template DNA	40 ηg/µl	1.0 µl
Sterile water		6.1 µl
Taq pol	3u	0.1 µl
DNTps	2.5 mM	0.8µl
Total		**10 µl**

Table 4: Temperature profile used for PCR amplification using SSR primers

Temperature profile used for PCR amplification using SSR primers

Profile	Activity	Temperature (°C)	Duration	Cycles
1.	Initial denaturation	95	5 mins	1
2.	Denaturation	95	30 secs	34
3.	Annealing	55	30 secs	
4.	Extension	72	1 mins	1
5.	Final extension	72	10 mins	1
6.	Storage	4	∞	

Data analysis

The amplified fragments were scored as absence (0) or presence (1) of amplicon linked to each blast resistance gene DNA fragment. Using Jaccard's coefficient, genetic similarities were analyzed from the binary data matrix (Sneath and Sokal, 1973). The similarity coefficient was used for cluster

analysis of traditional rice varieties utilizing UPGMA (unweighted pair group method with arithmetic averages).

Results and discussion

Rice crop suffers from yield plateau because of narrow genetic base. To overcome plateau, there is a need to broaden the genetic base or select the diverse set of lines in the breeding populations. The variety Samba mahsuri is extremely popular among rice farmers and consumers because of its high yield, medium-slender, fine grain type and excellent cooking and eating quality. This variety is being targeted for improvement of multiple traits like biotic and abiotic stress tolerance or resistance. Creation of genetic variability is a pre-requisite for any crop improvement program. Keeping these points in view, in present study 19 advanced breeding lines which were selected and diversity analysis was carried out among those to determine the diversity (Figure 1).

Molecular markers analysis

Number of alleles

In present study, a total of 44 alleles were detected across 19 advanced breeding lines of rice. The numbers of alleles generated per locus by each marker were ranged from 2 to 3. This was comparable to the report of Prabakaran *et al*., 2010 for genetic divergence of rice land races where in they reported average alleles of 2.2 per locus The results obtained in present study were lower than the observations made by (Li *et al.*, 2012, Rabey *et al.*, 2013) average number of alleles detected were 3.5, 3.83 and 2.75 in Taiwan rice germplasms, genetic diversity of eight rice cultivars, diversity within the aromatic and non-aromatic rice varieties respectively. However, in the present study, less number of alleles was detected since the works was carried among the advanced breeding lines of the same region, where the variations are expected in the small regions of the genome as generally due to lesser recombination.

Polymorphism of SSR markers

In present study, a total of 30 SSR markers (approx 2 to 3 markers per chromosomes) were selected to assess the extent of genetic diversity across 19 advanced breeding lines of rice. Of 30 SSR markers, 8 were found to be polymorphic and remaining 3 were not amplified and rests were monomorphic.

Polymorphism information content (PIC) values

Polymorphism Information Content (PIC) value is the reflection of allelic diversity and frequency among the varieties or lines. To measure the in formativeness of each SSR marker, PIC values were calculated. In the present study, PIC values varied widely among SSR loci tested and it ranged from

0.36 (RM 1007 chromosome 1) to 0.48 (RM 24914 of chromosome 10), with an average of 0.41 per marker.In the present study, the hyper variable SSR markers was selected which covered uniformly across the genome, hence resulted a fair amount of PIC values for these markers. Among polymorphic SSR markers, RM 26213 of chromosome 11 (with repeat motive of (TA) 46) showed maximum number of alleles 3 as well as high PIC value 0.816. Markers with PIC values of 0.5 or above are highly informative for genetic studies since they are extremely useful in distinguishing the polymorphism rate of a specific locus (DeWoody *et al*., 1995; Akkaya and Buyukunal Bal, 2004).Hence in present study, set of markers showed an average PIC value 0.41, hence these could considered as highly informative and useful for genetic diversity studies.

Genetic diversity pattern by cluster analysis (cluster analysis performed by using UPGMA based on similarity co-efficient values).

It resolved 19 advanced breeding lines into two major clusters with 24% dissimilarity. While cluster-I was major cluster with 18 lines and it was divided into two sub clusters i.e. IA and IB and they showed 25% dissimilarity. Cluster II consisted of only one breeding line i.e. MTU 1001. Cluster IA was a major sub cluster which had 16 lines, where as sub cluster IB consisted of two breeding line. For clarity, again divided the Cluster- IA in to two sub clusters viz I-AB and I-AC and they showed 14% dissimilarity. The sub cluster I-AB comprised of 14 lines where as I-AC comprised of 2 lines only. This analysis revealed that most of the lines might have maximum number of shared genetic loci. Upadhyay *et al.,* (2011) reported clustering of 29 rice genotypes in to major clusters while studying the development of molecular tags for rice lines. Rajendran *et al.*, 2013 studied clustering of maintainer and restorer groups into two different clusters in DNA fingerprinting and estimation of genetic diversity among hybrid rice parental lines (*Oryza sativa* L.). This showed that the diversity pattern observed among 19 advanced breeding lines in the present study based on cluster analysis using UPGMA software reflects the closeness (Figure 2 and Table 5).

Conclusion

Rice an important cereal crop has is diploid (2n=24) and has a relatively small genome (430Mb). Rice productivity is adversely impacted by Blast disease of rice caused by *Magnaporthe grisea*. The fungus attacks aerial parts of the rice plant at any stage of plant growth through the production of lesions on the leaves, nodes and panicles. Although chemical control is successful, it adds to the cost of cultivation and also contaminates the environment. Molecular markers used to point the position of a particular gene or the inheritance of a particular trait. In this project of four simple sequences repeat SSR (Simple sequence repeats), *i.e.,* 30 markers were used.DNA of total 19 rice varieties were

extracted from the frozen leaf sample. The extracted DNA was subjected for checking quality and quantification using both Biospec-nano and by 0.8 per cent agarose gel electrophoresis.

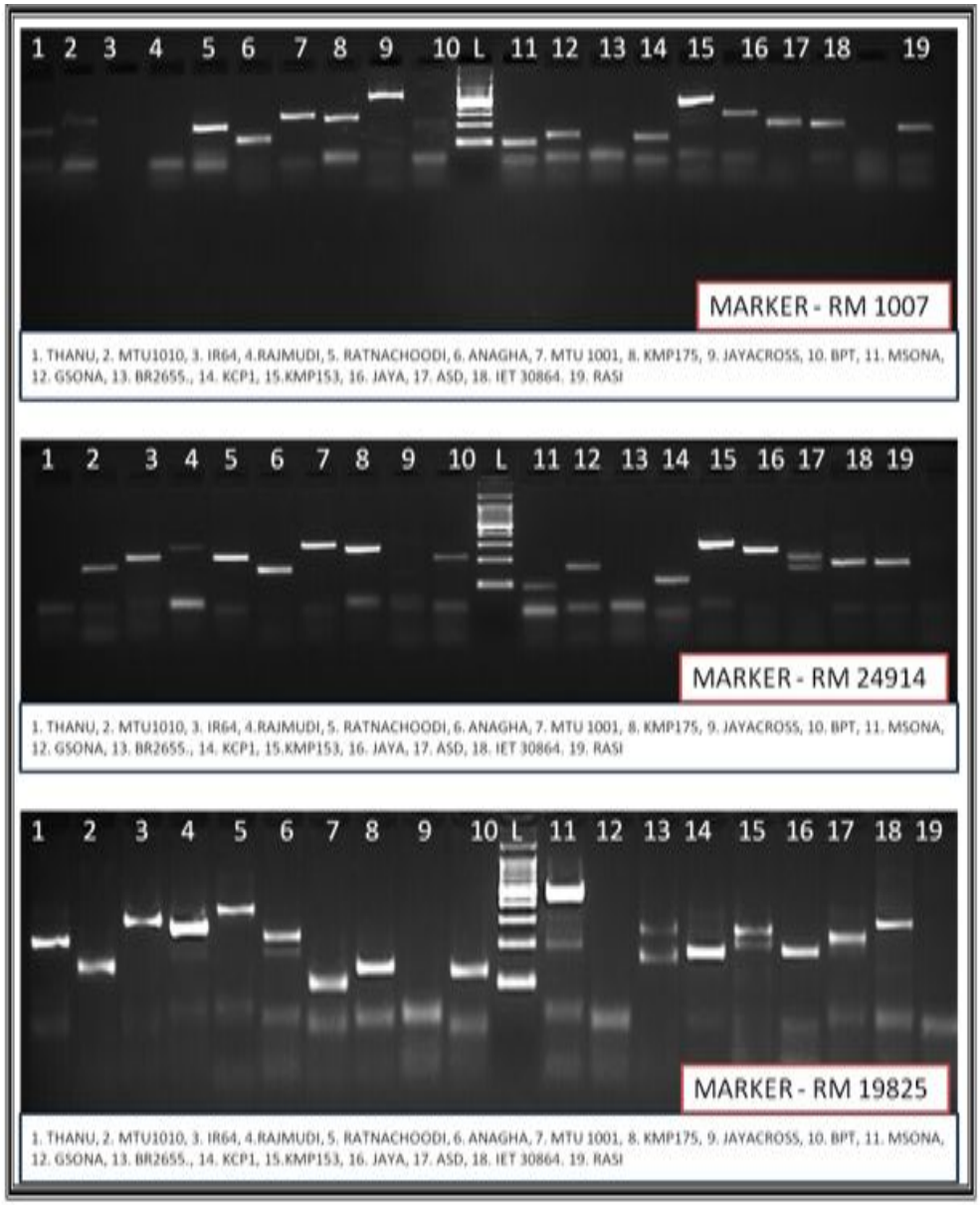

Figure 1: Gel profiles of different markers used in this study

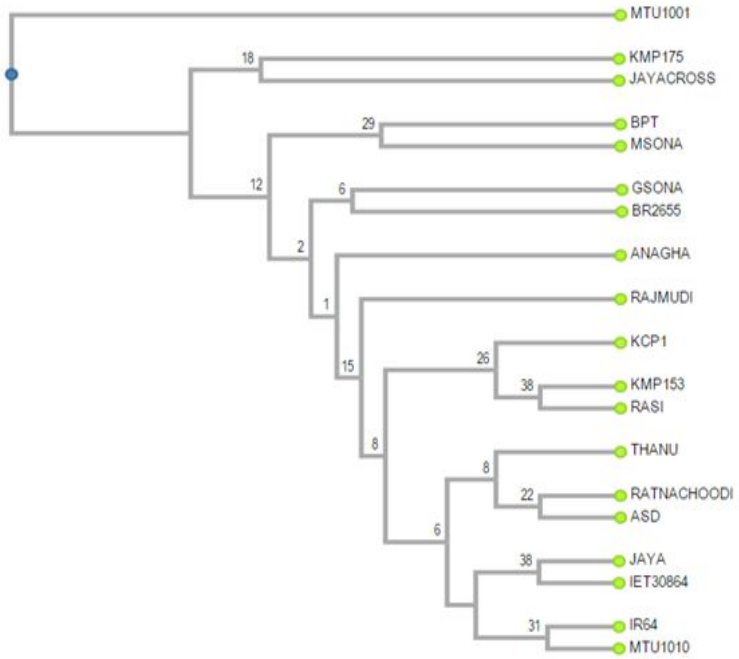

Figure 2: Genetic diversity analysis through UPGMA software

Table 5: Similarity matrix computed with Jaccard's coefficient

	IR64	MTU1001	GSONA	RAJMUDI	RATNACHOODI	BR2655	BPT	MTU1010	KMP175	KMP153	JAYA	THANU	RASI	JAYACROSS	ASD	MSONA	ANAGHA	KCP1	IET30864
IR64	1	0.2	0.7	0.7	0.8	0.6	0.4	0.9	0.4	0.8	0.9	0.7	0.9	0.5	0.9	0.6	0.7	0.7	0.8
MTU1001		1	0.125	0.286	0.25	0.143	0	0.222	0	0.111	0.222	0.286	0.1	0.167	0.222	0	0	0.125	0.25
GSONA			1	0.556	0.667	0.625	0.375	0.6	0.222	0.5	0.6	0.556	0.6	0.333	0.778	0.625	0.556	0.4	0.667
RAJMUDI				1	0.667	0.625	0.375	0.6	0.222	0.667	0.6	0.556	0.6	0.5	0.778	0.444	0.4	0.556	0.667
RATNACHOODI					1	0.556	0.5	0.7	0.333	0.6	0.7	0.875	0.7	0.444	0.889	0.75	0.5	0.667	0.778
BR2655						1	0.25	0.667	0.429	0.4	0.5	0.625	0.5	0.571	0.667	0.5	0.444	0.3	0.556
BPT							1	0.3	0.333	0.5	0.444	0.375	0.444	0.286	0.444	0.667	0.571	0.571	0.5
MTU1010								1	0.444	0.7	0.8	0.778	0.8	0.556	0.8	0.5	0.6	0.6	0.7
KMP175									1	0.333	0.444	0.375	0.444	0.5	0.3	0.429	0.571	0.375	0.333
KMP153										1	0.7	0.5	0.889	0.444	0.7	0.556	0.667	0.875	0.6
JAYA											1	0.6	0.8	0.4	0.8	0.5	0.778	0.6	0.889
THANU												1	0.6	0.5	0.778	0.625	0.4	0.556	0.667
RASI													1	0.4	0.8	0.667	0.778	0.778	0.7
JAYACROSS														1	0.4	0.375	0.333	0.5	0.3
ASD															1	0.667	0.6	0.6	0.889
MSONA																1	0.625	0.625	0.556
ANAGHA																	1	0.556	0.667
KCP1																		1	0.5
IET30864																			1

Then thirty microsatellites were used to easily amplify by the PCR (Polymerase Chain reaction) using unique flanking sequences as forward and reverse primers. The Resolution of amplified products on agarose gel electrophoresis was done. Using Jaccard's coefficient, genetic similarities were analyzed from the binary data matrix (Sneath and Sokal, 1973).The similarity coefficient was used for cluster analysis of traditional rice varieties utilizing UPGMA. Of 30 SSR markers, 8 were found to be polymorphic and remaining 3 were not amplified and rests were monomorphic. In present study, a total of 44 alleles were detected across 19 advanced breeding lines of rice and set of markers showed an average PIC value 0.41, hence these could considered as highly informative and useful for genetic diversity studies. Genetic diversity pattern, cluster analysis performed by using UPGMA based on similarity co-efficient values resolved 19 advanced breeding lines into two major clusters with 24% dissimilarity and this analysis revealed that most of the lines might have maximum number of shared genetic loci which reflects the closeness.

Social relevance, expected outcome and limitations

During the evolution and artificial selection processes a significant portion of beneficial alleles was left behind in the landraces which could have been used for the development of better rice varieties. The major difficulty in controlling rice blast is the durability of genetic resistance so enhancing the host plant resistance, international cooperation, completion of the rice genome project and candidate gene identification through rice functional genomics are the best approaches for handling the rice blast disease.

Applicable industries

Agriculture and Horticulture

Acknowledgement

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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