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Research Article

MOLECULAR VARIABILITY OF *Pyricularia grisea* ISOLATES FROM PRAKASAM AND SRI POTTI SREERAMULU NELLORE DISTRICT OF ANDHRA PRADESH

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Abstract: The molecular variability of twelve isolates of *Pyricularia grisea Sacc.* causing blast of rice was studied by using Random Amplified Polymorphic DNA (RAPD) analysis. The twelve isolates were divided into two major clusters A and B based on their Similarity Degree (SD) value higher than 0.25. The cluster A is divided into A1 and A2. The sub group A2 comprised PKM 3 and sub group A1 divided into A3 and A4 comprised NLR 6 and PKM 5 isolates. The cluster B was further divided into groups B1 and B2. The cluster B2 comprised PKM 2 isolate, the B1 group was further divided into sub groups B3 and B4. The sub group B4 comprised PKM 6 and sub group B3 was divided into B 5 and B6. The B5 included PKM 1 and PKM 4 and NLR 1, NLR 5, NLR 2, NLR 3 and NLR 4 were grouped in B6.

Keywords: *Pyricularia grisea*, Random Amplified Polymorphic DNA (RAPD) analysis, Dendrogram, Similarity Degree

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Introduction

Rice (*Oryza sativa* L.) is an important food crop in supplying approximately 23% per capita energy for Six billion people worldwide. Rice blast caused by *Pyricularia grisea* Sacc. is an important biotic constraint for rice production across the world where paddy is being cultivated [1]. The fungus has ability to overcome the resistance within a short time after release and spread of a resistant cultivar [2]. In recent times, rice blast has become one of the prevalent and major disease on rice in Prakasam and Sri Potti Sreeramulu Nellore districts of Andhra Pradesh, causing heavy losses to the rice growing farmers. The objective of the present investigation was to study the variation among the isolates of *P. grisea* collected from Prakasam and Sri Potti Sreeramulu Nellore districts of Andhra Pradesh by using Random Amplified Polymorphic DNA (RAPD) analysis.

Material and Methods

Twelve monoconidial blast isolates of *Pyricularia grisea* collected from different rice growing tracts of Prakasam and Potti Sreeramulu Nellore districts were used in this study [Table-1].

Table-1 *Description of isolates of P. grisea*

SN	Location	District	Isolate Designation
1	Darsi	Prakasam	PKM 1
2	Giddaluru	Prakasam	PKM 2
3	Mopadu	Prakasam	PKM 3
4	Chimakurthy	Prakasam	PKM 4
5	Pulipadu	Prakasam	PKM 5
6	Kandukur	Prakasam	PKM 6
7	Nellore	SPSR Nellore	NLR 1
8	Podalakuru	SPSR Nellore	NLR 2
9	Kavali	SPSR Nellore	NLR 3
10	Kovuru	SPSR Nellore	NLR 4
11	Atmakuru	SPSR Nellore	NLR 5
12	Bitragunta	SPSR Nellore	NLR 6

Sampling

All the monoconidial isolates of *P. grisea* were grown in 100 ml flasks containing 30 ml oat meal broth for 10 days at 27 ± 1°C. The fungal mats were placed on the Whatman No. 1 filter paper to drain excess of medium for 30 min and kept in the deep freeze for overnight before homogenizing it. Later, fungal mat was ground in extraction buffer with pre cooled mortar and pestle and then total genomic DNA was isolated.

Extraction buffer preparation

Extraction buffer (50 mM Tris- Hcl,150 mM NaCl' and 50 mM EDTA) prepared by using 1 M Tris – Hcl (pH 8.0), 0.2 M EDTA (pH 8.0), 5M NaCl, 10% sodium dodecyl sulphate (SDS), 10% CTAB in 0.7 M NaCl solution, Chloroform, Iso-amyl alcohol (24 : 1) mixture, 2 – isopropanol and 70% Ethyl alcohol.

TE buffer
10 mM Tris-HCl,
1.0 mM EDTA
pH – 8.0
10X assay buffer
200 m M Tris-HCl
500mM KCl
pH 8.4

DNA extraction

The total genomic DNA was extracted by adopting CTAB [3] as described below:

Protocol

The harvested mycelia (25 mg) were freeze-dried, lyophilized and macerated in liquid nitrogen using extraction buffer with the help of pre-cooled mortar and pestle.

Place the tissue suspended in extraction buffer in 1.5 ml Eppendorf tube and vortexed until evenly suspended. 50 µl of 10% SDS was added and gently shaken at 370C for 1 hr.75 µl of 5 M NaCl was added and mixed thoroughly. To the same mixture, 65 µl of CTAB (cetyltrimethyl ammonium bromide) solution was added and mixed thoroughly. The mixture was incubated at 650C for 10 - 20 min. The mixture was emulsified with equal volume of chloroform iso - amyl alcohol (24 : 1) for 5 min. The mixture was centrifuged at 10,000 rpm for 12 min with REMI centrifuge. DNA was precipitated in 200 µl of cold isopropanol, washed with 70% EtOH solution and dried under vacuum. The supernatant was left inside was kept at -20C freezer for overnight. The precipitated DNA was resuspended in TE buffer containing 10mg/ml and incubated at 370C for 30 min. The DNA concentration was estimated by fluorimeter ad adjusted to 10 ng /lm.

DNA quantity and quality estimation

To test the quality, DNA samples were run on 0.8% agarose gel in 1x TAE buffer and stained with ethidium bromide and checked for contamination by RNA. The DNA was evaluated by comparing it with a standard undigested DNA sample. Serial dilutions were carried out to get desired quantity of DNA for Polymerase Chain Reaction (PCR).

RAPD – PCR amplification

The PCR amplification for RAPD analysis was performed as described by Williams *et al*., (1990) [4]. The materials i.e., template DNA, random primers, dNTP's, Taq DNA polymerase and thermocycler used in the present study.

1) Template DNA

The purified genomic DNA extracts (10 ng) of monoconidial isolates of *Pyricularia grisea* were used as template DNA for amplification reaction.

2) Random primers

Commercial kit OPB and OPF of decamer DNA primers were used and which were obtained from Integrated DNA technologies supplied by Sigma Industrial and Laboratory Equipment Inc., Bangalore, India.

3) dNTP's

The four individual dNTPs such as dATP, dGTP, dCTP and dTTP were obtained from M/s Bangalore Genei, Pvt. LTd. Bangalore.

4) Taq DNA polymerase

Taq DNA polymerase and 10 x Taq buffer were obtained from M/s Bangalore Genei, Pvt. Ltd. Bangalore.

5) Thermo cycler

Eppendorf Master cycler gradient supplied by Eppendorf gradient-2231 was used for cyclic amplification of DNA, the master mix was distributed to PCR tubes (19 µl / tube) and later 1 µl of template DNA from the respective isolates was added making the final volume of 20 µl.

Stock solutions

100 µm Random primer.

25 ng ml-1 Template DNA

3.0 µml-1 Taq DNA polymerase. A total 18 random primers were used in the following sequences were shown in the [Table-2].

Master mix for PCR

Amplification reaction mixture was prepared in 0.2 ml thin walled PCR tubes containing following components. The total volume of each reaction mixture was 20µl. The following reaction mixture was found to be optimum for PCR amplification.

1.	10 X assay buffer	2.50 µl
2.	dNTPs mix (10 mM each)	0.50 µl
3.	Primer (100 mM)	1.25 µl
4.	Template DNA (10 ng/µl)	5.00 µl
5.	Sterile distilled water	10.25 µl
6.	Taq DNA polymerase (1.0 U µl-1)	0.5 µl

Thermo profile for PCR

The enzymatic amplification was performed in a thermocycler programmed for 40 cycles. The optimum conditions for DNA amplifications were used as follows.

Step	Temp (°C)	Duration (Min)	No. of cycles
Denaturation	94	2	1
Denaturation	94	1	35
Annealing	36	1	40
Extension	72	2	40
Final Extension	72	5	1

Separation of amplified products by agarose gel electrophoresis

After amplification, the products were separated in 1.4 % Sodium dodecyl sulphate gel electrophoresis by using electrophoretic unit with gel combs, spacers, glass plates and power pack V – Trans illuminator, Agarose (1.4 %), Bromo phenol blue, Ethidium bromide (10 µg / ml), 20 x TAE (stock) Tris – base – 19.3 g, Glacial acetic acid – 4.568 ml, 0.2 M EDTA – Make up the volume to 20 ml, pH 8 and Working solution (1 x TAE) by adding Five ml of 20 x TAE and made up to 100 ml by using distilled water.

Table-2 *Random primers with following sequences were used in RAPD*

Primer	Sequence
OPB-2	5 TGA TCC CTG G-3
OPB-3	5 CAT CCC CCT G-3
OPB-4	5 GGA CTG GAG T-3
OPB-5	5 TGC GCC CTT C-3
OPB-6	5 TGC TCT GCC C-3
OPB-7	5 GGT GAC GCA G-3
OPB-8	5 GTC CAC ACG G-3
OPB-9	5 TGG CTG ACT C-3
OPB-10	5 GTG CTG GGA C-3
OPB-11	5 TGA TCC CTG G-3
OPB-12	5 CCT TGA CGC T-3
OPB-13	5 TTC GCT CGC T-3
OPB-14	5 TCC GCT CTG G-3
OPF-1	5 ACG GAT CCT G-3
OPF-2	5 GAG GAT CCC T-3
OPF-3	5 CCA AGC TTC C-3
OPF-4	5 GGT GAT CAG G-3
OPF-16	5 GGA GTA CTG G-3

Table-3 *Banding profile of different primers for different isolates of P. grisea*

SN	Primer	Total Bands	Polymorphic Band	Percent polymorphism
1	OPB 2	4	4	94.44
2	OPB 3	2	2	100
3	OPB 4	8	4	50
4	OPB 5	18	18	100
5	OPB 6	6	4	66.66
6	OPB 7	6	5	83.33
7	OPB 8	12	10	66.66
8	OPB 9	8	8	100
9	OPB 10	6	6	100
10	OPB 11	11	10	90.9
11	OPB 12	13	11	80
12	OPB 13	10	10	100
13	OPB 14	4	4	83.33
14	OPF 1	5	4	80
15	OPF 2	6	6	100
16	OPF 3	7	7	100
17	OPF 4	7	6	85.71
18	OPF 16	3	3	100

Procedure followed for electrophoresis

Three gram of Agarose was weighed and added to a conical flask containing 250 ml of 1x TAE buffer.

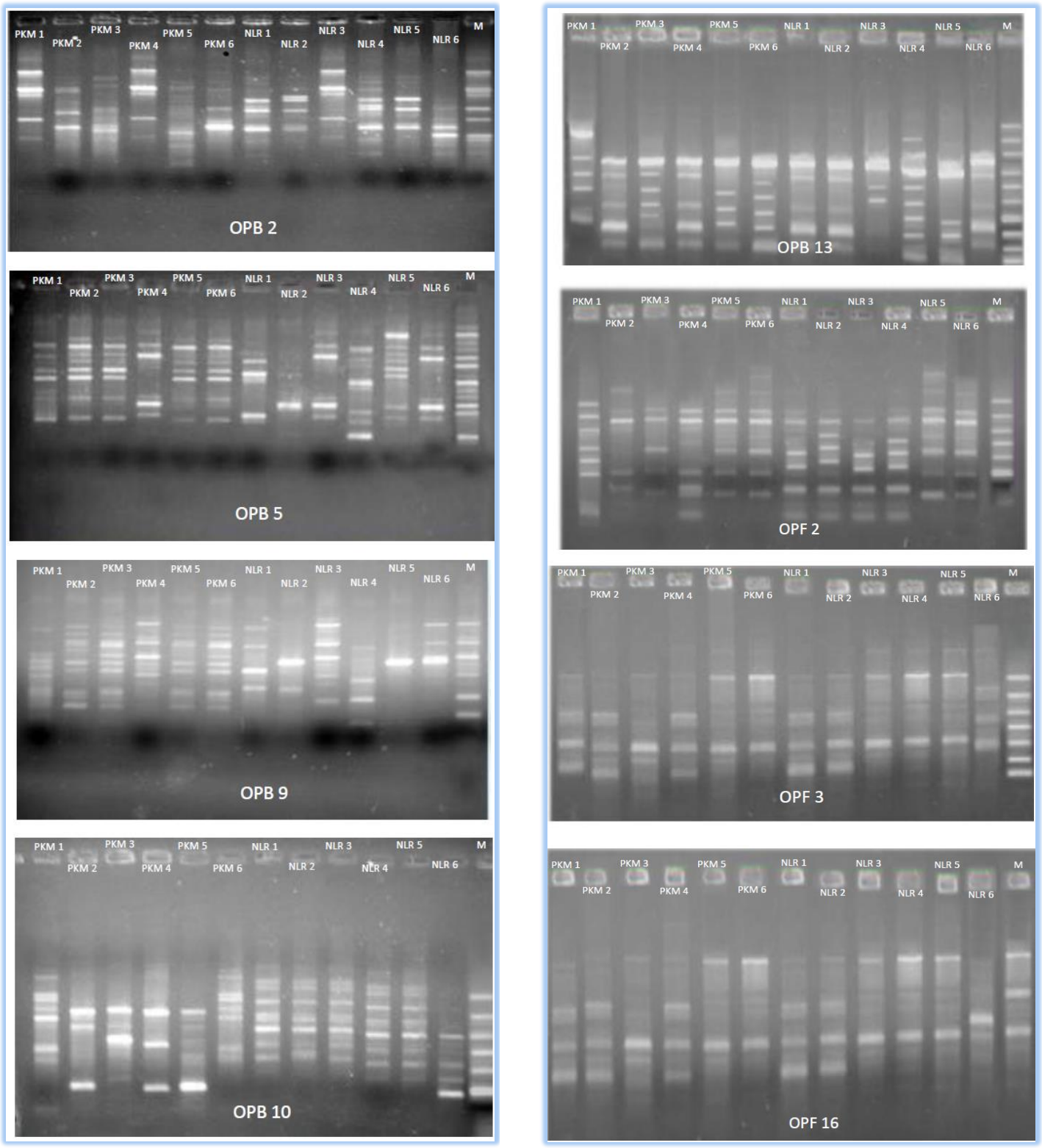

Fig-2 Banding pattern of different isolates of *Pyricularia grisea* in RAPD analysis

The agarose was melted by heating the solution on an oven and the solution was stirred to ensure even mixing and complete dissolution of agarose. The solution was then cooled 250C.Two to three drops of ethidium bromide (10 µg/ ml) was added. The solution was mixed and poured into the gel casting platform after inserting the comb in the tough. While pouring sufficient care was taken for not allowing the air bubbles to trap in the gel. The gel was allowed to solidify and the comb was removed after placing the solidified gel into the electrophoresis apparatus containing sufficient buffer (1 X TAE) so as to cover the wells completely. The amplified products (20 ml) to be analyzed were carefully loaded into the sample wells, after adding bromophenol blue with the help of micropipette. Electrophoresis was carried out at 100 volts, until the tracking dye migrated to the end of the gel. The gel was taken out from electrophoretic apparatus and stained by placing it in distilled water containing ethidium bromide (10 µm/ml) for 10 min. Ethidium bromide stained DNA bands were viewed under UV- transilluminator and photographed for documentation.

Scoring the amplified fragments

The amplified profiles for all the primers were compared with each other and the bands of DNA fragment were scored as '1' for the presence and '0' for the absence of a band generating the '0','1' matrices.

Table-4 Similarity Degree of *Pyricularia grisea* isolates

Isolates	PKM 1	PKM 2	PKM 3	PKM 4	PKM 5	PKM 6	NLR 1	NLR 2	NLR 3	NLR 4	NLR 5	NLR 6
PKM 1	1											
PKM 2	0.38	1										
PKM 3	0.27	0.42	1									
PKM 4	0.87	0.58	0.33	1								
PKM 5	0.26	0.52	0.36	0.28	1							
PKM 6	0.58	0.49	0.34	0.42	0.3	1						
NLR 1	0.36	0.42	0.28	0.35	0.32	0.52	1					
NLR 2	0.38	0.36	0.26	0.36	0.82	0.49	0.83	1				
NLR 3	0.49	0.35	0.26	0.51	0.36	0.47	0.64	0.67	1			
NLR 4	0.43	0.4	0.37	0.34	0.29	0.52	0.73	0.62	0.88	1		
NLR 5	0.46	0.35	0.34	0.27	0.26	0.46	0.85	0.58	0.54	0.85	1	
NLR 6	0.18	0.32	0.37	0.21	0.86	0.35	0.44	0.33	0.37	0.52	0.39	1

Percent polymorphism was calculated by using the following formula.

$$\text{Percent polymorphism} = \frac{\text{No. of polymorphic bands}}{\text{Total number of bands}} \times 100$$

Analysis of the profile of the amplified fragments

Pair wise genetic similarities between isolates were estimated by DICE similarity coefficient. Clustering was done using the symmetric matrix of similarity co-efficient and cluster obtained based on un weighted pair group arithmetic mean (UPGMA) using SHAN model of NTSYS – PC version 2.0 [5].

Results and Discussion

The analysis of genetic variation in plant pathogen populations is an important prerequisite for understanding the evolution of pathogen in the plant pathosystem. In the present investigation Random Amplified Polymorphic DNA (RAPD) analysis were used to detect the variation among the isolates of *P. grisea*. The primers of OPB and OPF series were used to determine genetic diversity between the isolates and to construct a dendrogram. Banding profiles of different primers for the isolates of *P. grisea* were given [Table-3] and [Fig-2]. Of the 18 primers used for amplification, OPB3, OPB5, OPB9, OPB10, OPB13, OPF 2, OPF 3 AND OPF 16 showed hundred percent polymorphism [Table-3]. Since out of a total of 135 bands, 122 polymorphic bands were obtained. The isolates exhibited over all polymorphism of about 87.8%. A dendrogram was generated based on Similarity Co-efficient. The similarity coefficient estimated by the Similarity Degree (SD) values. The SD value for the isolates ranged from 0.33 to 0.86 [Table-4] and [Fig-1]. Taking the SD value 0.33 as the basis, the twelve isolates were divided into two major clusters A and B. The cluster A is divided into A1 and A2. The sub group A2 comprised PKM 3 and sub group A1 divided into A3 and A4 comprised NLR 6 and PKM 5 isolates. The cluster B was further divided into groups B1 and B2. The cluster B2 comprised PKM 2 isolate, the B1 group was further divided into sub groups B3 and B4. The sub group B4 comprised PKM 6 and sub group B3 was divided into B 5 and B6. The B5 included PKM 1 and PKM 4 and NLR 1, NLR 5, NLR 2, NLR 3 and NLR 4 are grouped in B6 [Fig-2]. The number of clusters and their composition largely depend on rice varieties grown in that region. On the basis of the present study, it was concluded that the isolates of *P. grisea* in Prakasam and Potti Sree Ramulu Nellore districts are genetically heterogeneous. The isolates belonged to one ecosystem may possesses same genetic diversity and the genetic diversity varies according to the geographical areas. Genetic information on the population structure is essential for understanding the virulence dynamics of the pathogen and to develop strategies for reducing the impact of rice blast. The genetic mechanisms like simple mutations meiotic recombination and parasexual recombination are responsible for variation in *P. grisea*. Genetic variations observed in the collected isolates of *P. grisea* due to simple mutations, meiotic recombination and parasexual recombination [6]. The results are in agreement with Kumar, *et al*., (1999), Viji, *et al*., (2000), Shrinivasachary, *et al*., (2002) [7-9].

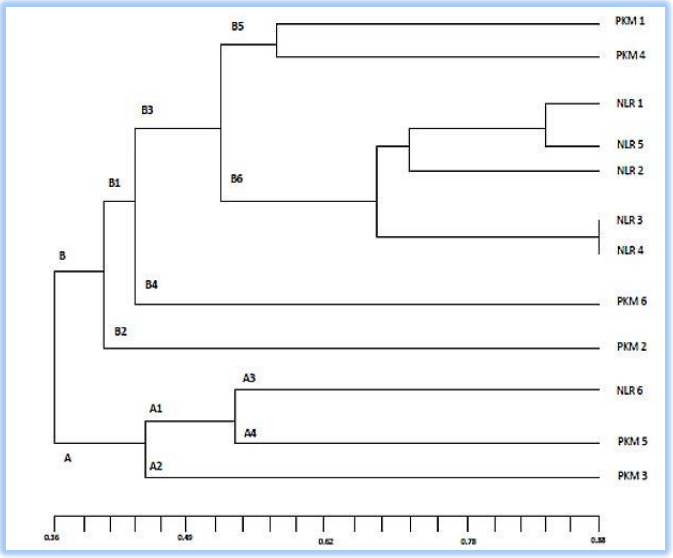

Fig-1 Dendrogram based on RAPD analysis of 12 isolates of *P. grisea*

Chadha and Gopalkrishna, (2005) conducted genetic diversity studies of isolates of M. *grisea* from Andhra Pradesh, Maharashtra and Karnataka through RAPD analysis and stated that most of the isolates were more that 15-20% different from each other, indicated that existence of local and geographical polymorphism and a high genetic diversity in Indian rice blast fungus [10]. A significant amount of diversity within the isolates could be correlated with different crop seasons. Park, *et al*., (2008) tested eighty-three isolates of *P. oryzae* with respect to genetic diversity and the possibility of race differentiation by PAGE-electrophoresis [11]. They reported the fungus was genetically very heterogeneous and the isolates were differentiated into races by pathogenicity on race differential varieties. Sonah, *et al*., (2009) observed high level genetic variability of *P. grisea* through PCR based RAPD analysis [12]. A total of 131 polymorphic markers were scored using 16 selected random decamer primers. The similarity degree value for the isolates ranged from 0.51 to 0.89 and the cluster analysis showed isolates from same location grouped together and isolates from different locations differ in their cluster group.

Application of research: Rice blast is a major disease in Andhra Pradesh where rice is growing in large area. The two districts Prakasam and Sri Potti Sreeramulu Nellore districts are facing severe rice blast problem in every year. In view of this the variability was studied to take better management practices to specific race

Research Category: Plant Pathology

Abbreviations:

RAPD: Random Amplified Polymorphic DNA

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Author statement: All authors read, reviewed, agree and approved the final manuscript

Conflict of Interest: None declared

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

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