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STUDIES ON MOLECULAR CHARACTERIZATION OF RAGI BLAST PATHOGENS FOR VARIABILITY AMONG *PYRICULARIA GRISEA* (CKE) SACC. ISOLATED FROM VARIOUS REGIONS OF TAMIL NADU BY RAPD MARKERS ANALYSIS

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Abstract- *Pyricularia grisea* (Cke) Sacc incites blast disease in finger millet and rice. An r-DNA region was amplified from the 12 isolates of *P. grisea* (B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11 and B12) isolated from finger millet and rice, using the universal primers to form a phylogenetic tree revealed that the isolates fall in 3 different clusters. Where in, ITS1-5.8S – ITS5 sequences and Randomly Amplified Polymorphic DNA profiling of *P.grisea* isolates used in the study clustered into two main groups, in which the isolates B1, B2, B3, B4, B5, B6, B7, B8, B9, B10 and B11 formed one group with approximately 12 per cent similarity coefficient between them and B12 was grouped in separate cluster. Molecular characterization of ragi blast pathogens in the study by RAPD markers analysis segregated the isolates into different clusters confirmed that the rice and finger millet-infecting *P. grisea* populations of India are clearly distinct.

Keywords- Ragi blast, *Pyricularia grisea*, Tamil Nadu, Randomly Amplified Polymorphic DNA

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Introduction

Finger millet (*Eleucinecoracana* L) commonly known as 'ragi' is one of the important millet crop in India and widely cultivated in several parts of Tamil Nadu, Andhra Pradesh, Karnataka and Maharastra. Ragi blast caused by a filamentous heterothallic ascomycete, *P.grisea* (Cke) Sacc. is a major disease resulting in excessive crop damage starting from seedling to ear head stage. The disease known to occur in all the growing seasons and almost all the varieties cultivated. Under epidemic circumstances, the yield loss may go beyond 50%. The genus *Magnapor the* collectively paratisizes more than 50 hosts, individual isolates have limited host range and cross-infectivity is relatively rare. *Pyricularia oryzae* is composed of host-specific subgroups such as *Oryza* isolates pathogenic on rice, *Setaria* isolates pathogenic on foxtail millet, *Triticum* isolates pathogenic on wheat, *Eleusine* isolates pathogenic on finger millet. Detection of phytopathogenic fungi can be difficult, especially at low infestation levels. Moreover, correct identification of the pathogen is not possible at the field level. In such cases, suitable diagnostic assay is must in order to avoid cross-reactions with other fungi [1].

Though serological techniques have been widely used to detect viral diseases, not much employed for the diagnosis of fungal pathogens. Also it is generally difficult to obtain antibodies with the required specificity for fungi [2]. In this context, molecular based tools such as polymerase chain reaction (PCR)-based detection of *P. grisea* may be available alternative to the existing methods. PCR-based detection assays aid in rapid diagnosis with high levels of sensitivity[3]. Several rice workers have used PCR technique to characterize *P. grisea* populations using Random Amplified Polymorphic DNA (RAPD) from different regions [4]. The objective of this research is to develop a PCR-based assay for the characterization of *P. grisea* in ragi using RAPD and ITS primer among rice and ragi.

MaterialsandMethods

The experiments were conducted at Lab 20, Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

Isolation of *P. grisea* from finger millet and rice

Blast-infected leaf samples of finger millet and rice were collected from different parts of Tamil Nadu. The monoconidial isolates of *P. grisea* were obtained by placing the sterile bits Potato Dextrose Agar (PDA) and incubated at room temperature (28±2 °C).The isolated fungi were purified by single spore isolation technique of Riker and Riker (1936) [5] and maintained in slants. One-week to three-month-old cultures were used for inoculation experiments.

PCR detection of *P. grisea*

Extraction of DNA

Cetyltrimethyl ammonium bromide (CTAB) method was adopted to extract the total DNA from the mycelial mat of *M. grisea*. 5 mm hyphal plug from the edge of an actively growing cultures were grown in 250 ml conical flasks containing 100 ml potato dextrose broth at room temperature (28 ± 2 °C) for 5-7 days. The culture filtrate was removed and the mycelia were blot dried. 0.1 g mycelium was ground in a pestle and mortar using liquid nitrogen. Powdered mycelia were mixed in pre-warmed (65°C) extraction buffer (500 µl) consisting of 100M Tris (pH 8.5), 250 mM NaCl, 0.5 mM EDTA and 0.5 per cent SDS. The samples were incubated at 60°C for 30 min. with occasional vortexing for 5 seconds for complete mixing of the buffer and spores. After incubation, the tubes were spun at 10000 rpm for 3 min. and the supernatant was collected. The supernatant containing the pellets was mixed again with fresh extraction buffer to a final volume of 500 µl, mixed by vortexing and incubated for 10 min. To this equal volume of chloroform and

isoamyl alcohol (24: 1) (v/v) was added and incubated for 30 min in a horizontal position. Then the tubes were centrifuged at 10000 rpm for 10 min and the aqueous phase was transferred to new eppendorf tube and the above step was repeated to extract the entire DNA. The pellet was washed with 70 per cent ethanol (Centrifuge for 3 min at 10000 rpm) and the pellet was dried and finally dissolved in 50 µl of the sterile distilled water. The final concentration of DNA in the extract was tested for the amount of DNA by 1.5 per cent agarose gel electrophoresis [6].

Variability of *P. grisea* among the ITS regions

PCR amplification of ITS region

To analyze molecular variability among the isolates of *P. grisea* ITS1-5.8S-ITS5 region was amplified with the primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS1 (5'-TCCGTAGGTGAACCTGCGG- 3') [7]. The reaction mixture for the PCR amplification consists of 20 µl vol. (0.25 mM each of primer pair, 0.25 mMdNTP, 1.5 mM $MgCl_2$, 50-80 ng of template DNA, 2µl of Taq DNA polymerase and 1x PCR buffer mix (Genei, Bangalore, India) and PCR was undertaken using a Master cycler gradient (Eppendorf, Germany). The conditions followed in the PCR amplification were 2 min preheating step at 95 ºC followed by 35 cycles consisting of denaturation at 95 ºC for 1 min, 50 ºC annealing for 30 sec, extension at 72 ºC for 2 min and with a final extension at 70 ºC for 7 min [7]. PCR amplified products (Approx. 580bp) were visualized by staining with 0.5 µg of ethidium bromide after size fractionation by electrophoresis on 1.5 per cent agarose gel and photographed in the gel documentation system (Alpha InfoTech, California, USA).

Gel extraction and sequencing

Amplified PCR products were excised and purified using QIA quick gel extraction kit (Qiagen Inc., Chats wroth, CA, USA). After elution, the eluted fragments were confirmed through 2 per cent agarose gel electrophoresis. All the above-eluted fragments were partially sequenced using an automated DNA sequencer (Bioserve Biotechnologies (I) Pvt. Ltd. Hyderabad, India).

Construction of a phylogenic tree from nucleotide sequences of the internal transcribed spacer 1 (ITS1)- 5.8S-ITS5 region of the nuclear ribosomal RNA gene(rDNA)

A total of twelve sequenced ITS samples and ten ITS sequences of *M. grisea* (Mg1 to Mg10) collected from GenBank were used for comparison. Multiple sequence alignments were made using CLUSTAL X (1.8). Sequence phylograms were constructed using PHYLIP package (Bootstrap analysis software), and uprooted trees were made using TREEVIEW software (Page, 1996). Sequence identities of the GenBank database were performed with BLAST analysis [8].

Studying the variability of *P. grisea* by RAPD markers analysis

RAPD analysis of *M. grisea*

RAPD profiles were produced by the method of Zakaria *et al*. (2005) [9]. All the RAPD primers were purchased from Bangalore Genei Pvt. Ltd., Bangalore, India. PCR reaction was set for 20 µl volume consisting of 5 mM each dNTPs, 10 pmol of primer, 0.5 U of Taq DNA polymerase and 50 ng of template. PCR was performed in a Master cycler gradient (Eppendorf, Germany) under the following conditions; 1 cycle of 94˚C for 3 min; 40 cycles at 94˚C for 1 min, 37˚C for 2 min and 72˚C for 2 min; and a final extension cycle at 72˚C for 10 min. 10 µl of each PCR product was electrophoresed on 2 per cent agarose gel in 10X Tris–acetic acid–EDTA (TAE) buffer. The DNA fragments in the gels were stained with ethidium bromide, visualized under ultraviolet light (UVL) and recorded with an AlphaImager 2000 (Alpha Innotech, San Leandro, CA, USA). Selected RAPD amplifications were repeated to ensure reproducibility.

Construction of dendrograms for RAPD-PCR cluster analysis

The banding patterns of *P. grisea* isolates were scored for RAPD starting from the small size fragment to large sized one. Presence and absence of each band in each isolate was coded as 1 and 0 respectively. The scores were used to create a data matrix to analyze genetic relationship using the NTSYS-pc program version 2.02 (Numerical Taxonomy System Applied Biostatistics, Setauket, New York, USA) described by Rohlf (1993) [10]. Similarity matrix was developed using the Jaccard's coefficient of similarity with the data matrix. A phenogram was reproduced by the unweighted pair group method for arithmetic average (UPGMA) in the SAHN procedure. Finally, twelve isolates of *P. grisea* were grouped into different clusters using Jaccard's coefficient of similarity [11].

Result and Discussion

The blast fungus *Pyriculariagrisea* (Cke) Sacchas a wide host range and is known to infect almost 40 species of *Gramineae* and previous studies have given discrepant results concerning the host range of this pathogen [12] and the extent of variation in the fungus was based on physical characteristics such as spore and appressorial morphologies and germ tube branching. In the present study, results revealed that *P. grisea* isolates of ragi of failed to infect rice. This investigation supported the overall suggestion that rice and finger millet-infecting isolates of *P. grisea* exist as genetically isolated and distinctly different host-limited groups.

Isolation of *P. grisea* causing blast disease of finger millet and rice

A total of six isolates of *P. grisea* from finger millet and six isolates from rice were isolated from the blast infected samples collected from different geographical regions of Tamil Nadu, India [Table-1]; the pathogenicity was done and the Koch's postulates were proved successfully. . Several workers have reported that the pathogenicity of the blast fungus is largely restricted to its host species of origin [13].

Table-1 *Pyricularia grisea isolates used in cross infectivity and molecular characterization studies*

S. No	Isolate	Species	Host	Place
1	B1	*P. grisea*	Ragi	Coimbatore
2	B2	*P. grisea*	Ragi	Hosur
3	B3	*P. grisea*	Ragi	Trichy
4	B4	*P. grisea*	Ragi	Salem
5	B5	*P. grisea*	Ragi	Orissa
6	B6	*P. grisea*	Ragi	Krinagiri
7	B7	*P. grisea*	Rice	Killikulam
8	B8	*P. grisea*	Rice	Coimbatore
9	B9	*P. grisea*	Rice	Cuddalore
10	B10	*P. grisea*	Rice	Gudalore
11	B11	*P. grisea*	Rice	Sathiyamangalam
12	B12	*P. grisea*	Rice	Trichy

Phylogenic analysis using r-DNA ITS1-5.8S-ITS5 sequences

Molecular-based detection techniques have facilitated diagnosis of many plant pathogens in the past. Anr-DNA region was amplified from the 12 isolates of *P. grisea* isolated from finger millet and rice, using the universal primers. Nucleotide sequences of the ITS1-5.8S-ITS5 region (580bp) in these amp icons were aligned with those of the 10 isolates of nucleotide sequences from various hosts, which had been obtained from the GenBank. The phylogenetic NJ tree of *P. grisea* isolates constructed from nucleotide sequences of r-DNA ITS 1- 5.8S-ITS 5 region formed three clusters I, II and III with high boot strap values in which group I was separated among them [Fig-1]. Comparative studies of the nucleotide sequences of ribosomal RNA (rRNA) genes provide a means for analyzing phylogenetic relationships over a wide range of taxonomic levels [14]. The cluster I included all Genbank sequences (Mg1 to Mg10) and few isolates such as B2, B5, B7, B8, B9 and B11 while the other cluster II included the isolates B1, B3, B4, B6, B10 and B12 of *P. grisea*. The rDNA ITS 1- 5.8S-ITS 5 region of B10 formed a separate cluster III. In one group of *P. grisea* isolates B3 with B12 shared 99 percent identical sequence. In other group of *P. grisea* designated B6 and B4 shared a 93 percent identical sequence. Per cent nucleotide sequences identity matrix between 12 isolates of *P.grisea* from India and ITS *P. grisea* were also illustrated [Table-4]. One group of *P. grisea* isolates shared a 99 per cent identical sequence B1 with B3. The gene coding for nuclear ribosomal DNA (rDNA) contains both conserved and variable regions that are suitable to generate probes for various taxonomic levels such as genus, species, family or subphyla [15].

Molecular diversity of isolates of *P. grisea* using RAPD

A suitable diagnostic assay needs to be both sensitive and specific in order to avoid cross-reactions with other fungi [16]. An attempt was made to assess the genetic variability among *P. grisea* isolates and to establish possible genetic relationships among the Indian isolates of ragi and rice blast fungus. The selected primers were used to study the RAPD patterns among the 12 isolates [Table-2]. The relationships among the isolates were represented as molecular genetic similarity coefficient matrix [Table-3] and dentogram constructed from RAPD data [Fig-2]. In the present study, among the isolates, 11 isolates were grouped under group I and one isolate was grouped under group II. In group I, the isolates B1, B2, B3, B4, B5, B6, B7, B8, B9, B10 and B11 were fall in one group with approximately 12 per cent similarity coefficient between them and B12 was grouped in another group.

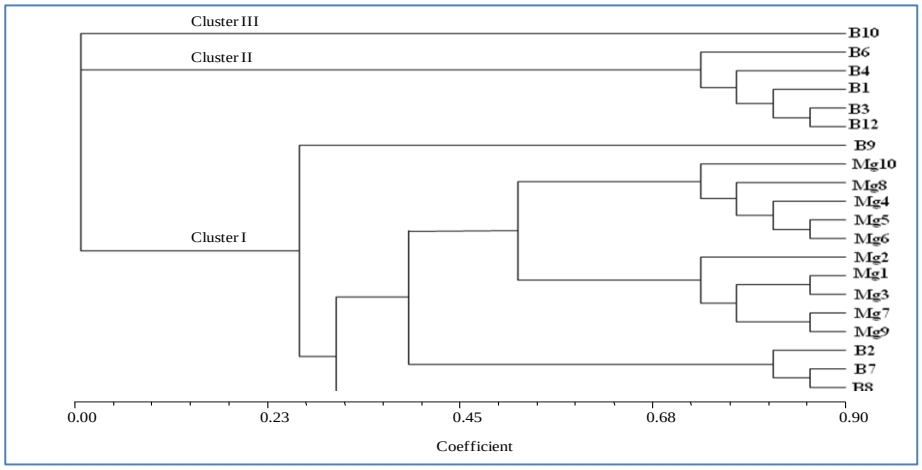

Fig-1 Neighbor-joining phylogenic tree from nucleotide sequences of the internal transcribed spacer 1 (ITS1) - 5.8S-ITS5 region of the nuclear ribosomal RNA gene (rDNA) of *Pyricularia* isolates (B1 to B12) and ten ITS sequences of *M. grisea* (Mg1 to Mg10) collected from GenBank.

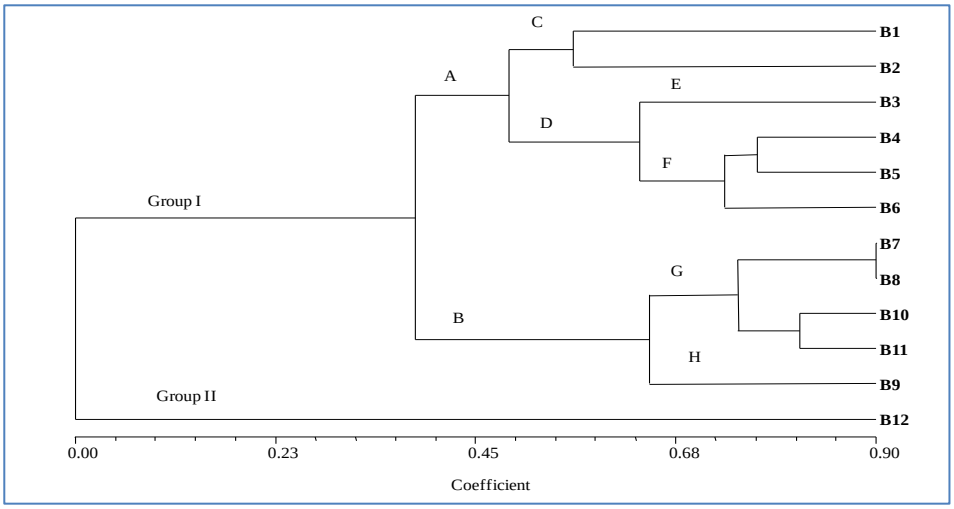

Fig-2 Un-weighted pair group method arithmetic average dendrogram constructed from RAPD data indicating the relationship among the isolates of *Pyriculari agrisea* from ragi (B1–B5) and Rice (B7–B12)

Table-2 *Sequences of RAPD primers used to study the genetic variability among isolates of Pyriculariagrisea from Ragi and Rice*

Primer	Nucleotide sequence
R3	5'TGCCGAGCTG3'
OPF 5	5'CCGAATTCCC3'
OPF 7	5'CCGATATCCC3'
OPF 8	5'GGGATATCGG3'
OPF 10	5'GGAAGCTTGG3'
OPF 11	5'ACGGTACCAG3'
OPF 12	5'GGCTGCAGAA3'
OPF-14	5'TGCTGCAGGT3'

Group I was subdivided into clusters A and B with similarity of approximately 36 per cent. Cluster A was divided into sub cluster C and D with approximately 38 per cent similarity. Similarly, seventy-six strains of *Penicillium roqueforti* used as starter cultures for mould ripened blue cheeses have been analysed for their RAPD genotype by using three different primers. A comparison of the RAPD patterns within each primer group revealed that the genetic constitution of the strains was similar, as most of the strains showed very similar overall patterns [17]. Depending on the type of random primer used, the resulting RAPD pattern can be species specific or can differentiate between various RAPD genotypes within a species or even between strains [18]. Molecular characterization based on the RAPD markers among 10 *Penicillium* species was reported by Pereira *et al.* (2002) [19] indicated that RAPD has the potential to detect polymorphism throughout the entire genome as compared to other PCR-based techniques. The present study was in accordance with several successful works for the establishment of phylogenetic relationships between the isolates of a morphological species of fungal pathogens such as *Verticillium* spp.[20], *Rhizoctonia solani* Kuhn [23], *Coprinus cinereus* (Schaeff.: Fr) [21]. More generally, the use of RAPDs in systematics has several limitations and the relevance and taxonomic meaning of RAPD groupings always need careful comparison with that of other sources data. Although RAPD markers can serve in distinguishing taxa by producing taxon-specific amplified fragments or grouping closely related taxa in numerical or cladistic analyses [22], the utility of RAPDs as systematic characters is limited because of difficulties in assessing character

homologies.

Table-3 *Molecular genetic similarity coefficient matrix of Pyricularia grisea isolates from ragi and rice*

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
B1	1											
B2	0.56	1										
B3	0 .44	0.54	1									
B4	0.51	0.45	0.66	1								
B5	0.50	0.43	0.65	0.77	1							
B6	0.52	0.50	0.58	0.73	0.72	1						
B7	0.31	0.38	0.34	0.39	0.43	0.55	1					
B8	0.35	0.39	0.35	0.35	0.42	0.50	0.90	1				
B9	0.32	0.30	0.27	0.34	0.41	0.40	0.62	0.62	1			
B10	0.40	0.41	0.34	0.33	0.40	0.43	0.69	0.73	0.72	1		
B11	0.35	0.39	0.35	0.35	0.42	0.47	0.77	0.78	0.62	0.81	1	
B12	0	0	0	0	0	0	0	0	0	0	0	1

Table-4 *Per cent nucleotide sequences identity matrix between 12 isolates of M.griseafrom India and ITS M. grisea*

Seq	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	Mg1	Mg2	Mg3	Mg4	Mg5	Mg6	Mg7	Mg8	Mg9	Mg10
B1	1	0.2																				
		0	0.99	0.17	0.17	0.17	0.16	0.16	0.17	0.17	0.17	0.99	0.27	0.21	0.26	0.23	0.28	0.23	0.21	0.22	0.24	0.22
B2		1	0.28	0.27	0.27	0.27	0.27	0.27	0.28	0.27	0.27	0.20	0.27	0.28	0.28	0.25	0.25	0.24	0.23	0.21	0.24	0.33
B3			1	0.17	0.17	0.17	0.16	0.16	0.17	0.17	0.17	1	0.27	0.21	0.26	0.24	0.28	0.23	0.21	0.22	0.24	0.26
B4				1	0.99	1	0.96	0.97	0.93	1	0.99	0.17	0.20	0.22	0.20	0.24	0.19	0.26	0.25	0.28	0.26	0.24
B5					1	0.99	0.96	0.97	0.99	0.99	1	0.17	0.20	0.22	0.20	0.24	0.19	0.27	0.26	0.28	0.26	0.24
B6						1	0.99	0.97	0.99	1	0.99	0.17	0.20	0.22	0.20	0.24	0.19	0.26	0.25	0.28	0.26	0.24
B7							1	0.99	0.96	0.96	0.96	0.16	0.21	0.21	0.21	0.25	0.19	0.27	0.26	0.29	0.27	0.24
B8								1	0.99	0.97	0.97	0.16	0.21	0.21	0.21	0.24	0.19	0.27	0.26	0.29	0.27	0.24
B9									1	0.99	0.99	0.17	0.20	0.22	0.20	0.24	0.19	0.27	0.26	0.28	0.26	0.24
B10										1	0.99	0.17	0.20	0.22	0.20	0.24	0.19	0.26	0.25	0.28	0.26	0.24
B11											1	0.17	0.20	0.22	0.20	0.24	0.19	0.27	0.26	0.28	0.26	0.24
B12												1	0.27	0.21	0.26	0.24	0.28	0.23	0.21	0.22	0.24	0.22
Mg1													1	0.25	0.99	0.22	0.30	0.26	0.25	0.31	0.26	0.27
Mg2														1	0.25	0.23	0.22	0.22	0.24	0.24	0.21	0.22
Mg3															1	0.22	0.30	0.26	0.25	0.32	0.26	0.27
Mg4																1	0.25	0.23	0.3	0.21	0.23	0.22
Mg5																	1	0.24	0.25	0.22	0.23	0.22
Mg6																		1	0.29	0.31	0.93	0.29
Mg7																			1	0.28	0.28	0.23
Mg8																				1	0.30	0.25
Mg9																					1	0.28
Mg10																						1

Conclusion

Thus, the results brought out a new information that based on morphological and molecular variability, it is concluded that the Indian isolates of *P. grisea* of ragi and rice blast fungus is genetically heterogeneous and the inter-relationships amongst the different isolates can be easily, precisely and reliably explained by RAPD and ITS-PCR. This information will help in further identification of patho-types/ races prevalent in *P. grisea* for their effective control and eradication.

Conflict of Interest: None declared

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