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Characterization of Finger Millet Blast Pathogen Population of *Magnaporthe grisea* by DNA Fingerprinting Analysis

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ABSTRACT: *In the present study,* M. grisea *samples were collected from different geographical regions of Tamil Nadu and AICSMIP centre. Collected* M. grisea *samples may be genetically heterogeneous and the interrelationship amongst the different isolates can be easily, precisely and reliably by RAPD and PCR. Twenty DNA primers, 10 nucleotides long were used (OPA01, OPA02, OPA09, OPB01, OPC20, OPE04, OPF01, OPF05, OPF06, OPF07, OPF08, OPF10, OPF11, OPF12, OPF14, OPG19, OPZ13, OPZ19, C3 and R3). RAPD markers used in this investigation increases the marker density to find out genetic relationships, blast samples from eleven different geographical regions in Tamil Nadu, AICSMIP centre. The dendrogram revealed that the geographic origin of strains does not play crucial role in lineage formation, as in each lineage (group), there were mixed populations of the eleven geographical regions. It is concluded that Indian ragi blast like rice blast fungus may be genetically heterogeneous and the interrelationships amongst the different isolates can be easily, precisely and reliably RAPD-PCR technology.*

Keywords: *Blast, PCR, RAPD,* M. grisea

INTRODUCTION

Finger millet (*Eleusine coracana* (L.) Gaertner. commonly known as ragi is one of the most consumed as a stable food in the rural community of Tamil Nadu, Andhra Pradesh, Karnataka and Maharashtra. Finger millet blast incited by *Magnaporthe grisea* (Hebert) Barr. anamorph of *Pyricularia grisea* (Cooke) Sacc; is a heterothallic, filamentous fungus, one of the major destructive disease causing excessive damage to this crop from seedling to ear head forming stages. The disease occurs during all growing seasons and on almost all finger millet varieties cultivated. Yield loss due to blast can be as high as 50% when the disease occurs in epidemic proportions.

Magnaporthe grisea (Hebert) Barr. (Anamorph: *Pyricularia grisea*) which is the most important constraint to finger production in most finger millet growing environments. *M. grisea* parasitizes over 50 grasses, including economically important crops like wheat, rice, barley and millet [16]. All aerial parts of the plant can be affected in moistened environment, leaf surfaces become speckled with oval lesions and plants are liable to lodging if stems are infected. A severe yield loss is recorded when the panicle is affected by blast disease. Surveys confirmed that blast remains among the most serious constraints to yield in South Asia [25,4]. Host plant resistance is the most promising method of blast disease control [1]. The analysis of genetic variation in plant pathogen populations is an important prerequisite for understanding co-evolution in the plant pathosystem [13]. Populations of rice blast pathogen throughout the world have been studied for their phenotypic and genetic variation [2,9-11,21]. Globally, random amplified polymorphic DNA (RAPD) markers are also reported to be useful in identification [6,8,22,24] and analysis of genetic divergence [12,20].

In the present study, *M. grisea* samples were collected from different geographical regions of Tamil Nadu and AICSMIP centre. The single spores of *M. grisea* were isolated and maintained in the laboratory using potato dextrose agar. The genomic DNA was isolated using standard cetyl trimethylammonium bromide (CTAB) protocol with minor modifications 20 random primers (10 mer) were used to generate

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genomic finger prints of twenty four *M. grisea* isolates of different geographical regions. All RAPD markers depicted high polymorphism (71 - 90%). The banding patterns generated by these markers were used to generate "squared Euclidean distances". The linkage distances were further used to construct a dendrogram using "biostastica" and "unweighted pair group averages". This study revealed that populations of *M. grisea* in hilly geographical region of Tamil Nadu and AICSMIP centre (India), may be genetically heterogeneous and the interrelationship amongst the different isolates can be easily, precisely and reliably explained by RAPD polymerase chain reaction (PCR) technology.

MATERIALS AND METHODS

Samples of infected leaves, necks and finger were collected from Krishnagiri, Dharmapuri, Coimbatore, Ranchi, Dholi, Vijayanagaram, Dindori, Berhampur, Sesai, Rewa and Bangaluru (Table 1). The collected samples were packed in paper bags and stored at 4°C till further use. Twenty four monoconidial isolates were obtained by directly transferring conidia (from one lesion per leaf/neck/finger of a plant) on petri plates having potato dextrose agar medium. For DNA extraction, the fungus was grown in potato dextrose broth medium. After seven days of incubation at room temperature, fungal mycelium was harvested by filtration through Whatman filter paper and washed with distilled water. The mycelial mats were transferred to sterilized butter papers (150 x 100 mm size) and freeze dried for 16 hours at -400^{0}C, in a lyophilizer. Total DNA was extracted following the procedure of [14] for plant DNA with modifications for mini-scale preparation as described by [19].The concentration of the DNA was measured by using U. V. spectrophotometer (Thermo Spectronic - Biomate 5) and adjusted to 40ng/ìL and quality was assessed by a mini-gel electrophoresis (1.0 % agarose gel).

Genomic DNA isolation

The total genomic DNA was isolated using the standard CTAB protocol [17] and making minor modifications. Initially, 0.5 g of fungal mycelium mat was taken and grinded with the help of liquid nitrogen. The grinded powder was transferred into two micro centrifuge tubes of 2.0 ml capacity each. Then, 500 ìl extraction buffer (0.1 M Tris buffer) was added. After vortexing the tube, 50 µl 10% sodium dodecyl sulphate (SDS) was added and after incubation at 37°C for 1 h, 60 µl of CTAB/NaCl solution (10% CTAB in 0.7 M Nacl) was added. Again, incubation was done for 30 min at 65°C. After this, equal amount of (~610 µl) chloroform (24): isoamyl alcohol (01) was added and centrifuged at 10,000 rpm for 15 min, the supernatant was transferred into new centrifuge tube and 2.0 ìl of RNase was added and further incubated for 30 min. The micro centrifuge tube was transferred at -20°C for overnight after adding 2/3 volume of ice cold isopropanol. The tube was spinned at 10,000 rpm for 15 min and pellet was washed twice with 70% ethyl alcohol. Finally, pellet was dissolved in 100 µl TE buffer.

RAPD-PCR analysis

RAPD-PCR analysis was performed to all the isolates. Twenty DNA primers, 10 nucleotides long (Table 2) were used for the experiment (OPA01, OPA02, OPA09, OPB01, OPC20, OPE04, OPF01, OPF05, OPF06, OPF07, OPF08, OPF10, OPF11, OPF12, OPF14, OPG19, OPZ13, OPZ19, C3 and R3). The concentrations of each DNA sample were 10 ng/µl. Amplifications were performed in a 20 µl reaction mixture consisting of genomic DNA (2 µl) and PCR mixture (18 µl). PCR mixtures for 12 portions were made by mixing these chemicals in sequence: DDW (147.6 µl), PCR kit buffer 10X (24 µl), dNTP *mixture* (18 µl; 2.5 µmol/µl) and primer (12 µl; 10 pmol/µl), DDW (12 µl) dan *gene Taq NT* (2.4 µl; 5U/µl). A single primer was used in each reaction. Amplification was performed in a Progene Thermal Cycler PCR Machine for. The first program (1 cycle) was divided into three segments (i) 94^{o}C for 3 min; (ii) 36^{o}C for 1 min; (iii) 72^{o}C for 2 min. The second program (28 cycles) was divided into three segments: 94^{o}C for 1 min, 36^{o}C for 1 min and 72^{o}C for 2 min. The third program (1 cycle) was also divided into three segments: 94^{o}C for 1 min, 36^{o}C for 1 min and 72^{o}C for 5 min. The amplification products were resolved by electrophoresis in a 0.8% agarose gel (250 ml; containing 12.5 µl (10 mg/ml) ethidium bromide) using TBE buffer 0.5X at 400 Volt for 15 min, then at 20 Volt for 19 h. A 30 cycles divided into three programes SmartLadder (720 ng/5µl) Nippongene was included as a molecular size marker. Gels were visualized and photographed by iluminator UV which was connected with FAS III Toyobo.

Agarose gel electrophoresis

Horizontal submerged gel electrophoresis unit was used for fractionating RAPD primers on agarose gel. After amplification, 10 µl of each amplified product was electrophoresed in a 1.5% agarose gel prepared in 1 X TAE buffer. 6 X DNA loading dye was mixed in the ratio of 5: 1 v/v to amplified product. Ethidium

Table 1
***M.grisea* isolates collected from different finger millet growing areas of Tamil Nadu and AICSMIP centers**

S No.	*Isolate code*	*Infected Parts*	*Place*
1	TNLB1	Leaf	Krishnagiri
2	TNLB2	Leaf	Krishnagiri
3	TNLB3	Leaf	Dharmapuri
4	TNLB4	Leaf	Coimbatore
5	TNNB1	Neck	Krishnagiri
6	TNNB2	Neck	Krishnagiri
7	TNNB3	Neck	Dharmapuri
8	TNNB4	Neck	Coimbatore
9	RALB1	Leaf	Ranchi
10	RANB1	Neck	Ranchi
11	DHLB1	Leaf	Dholi
12	DHNB1	Neck	Dholi
13	DHFB1	Finger	Dholi
14	VINB1	Neck	Vijayanagaram
15	DINB1	Neck	Dindori
16	BENB1	Neck	Berhampur
17	BENB2	Neck	Berhampur
18	SENB1	Neck	Sesai
19	SENB2	Neck	Sesai
20	SEFB1	Finger	Sesai
21	RELB1	Leaf	Rewa
22	RELB2	Leaf	Rewa
23	RENB1	Neck	Rewa
24	BANLB1	Leaf	Bangaluru

Table 2
Amplification performance of oligonucleotide on the isolates from *Magnaporthe grisea*

S.no.	*Primer Code*	*Nucleotide Sequence*
1	OPA-01	5′CAGGCCCTTC3′
2	OPA 02	5′ TGCCGAGCTG 3′
3	OPA-09	5′AGTCAGCCAC3′
4	OPB-01	5′GTTTCGCTCC3′
5	OPC-20	5′ACTTCGCCAC3′
6	OPE-04	5′GTGACATGCC3′
7	OPF-01	5′ACGGATCCTG3′
8	OPF-05	5′CCGAATTCCC3′
9	OPF-06	5′GGGAATTCGG3′
10	OPF07	5′CCGATATCCC3′
11	OPF-08	5′GGGATATCGG3′
12	OPF -10	5′GGAAGCTTGG3′
13	OPF -11	5′ACGGTACCAG3′
14	OPF -12	5′GGCTGCAGAA3′
15	OPF-14	5′TGCTGCAGGT3′
16	OPG-19	5′GTCAGGGCAA3′
17	OPZ-13	5′ GACTAAGCCC 3′
18	OPZ-19	5′GTGCGAGCAA3′
19	C3	5′CGGCTTGGGT3′
20	R3	5′TGCCGAGCTG3′

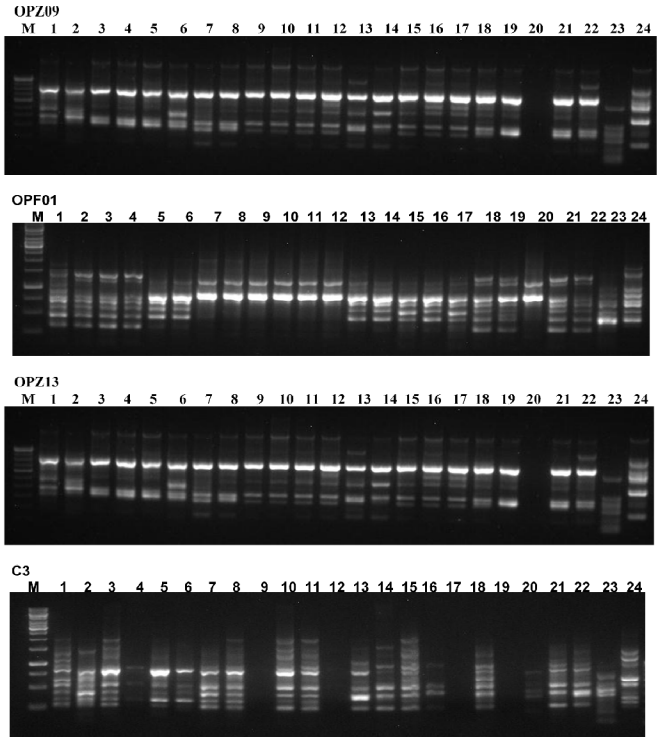
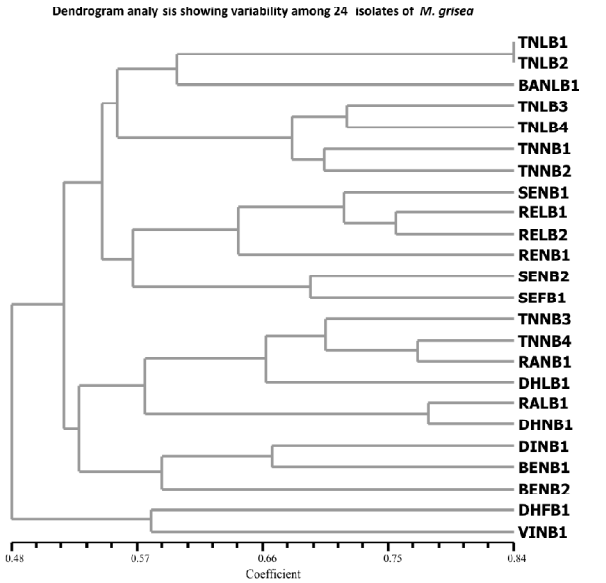


Figure 1: DNA finger printing patterns of 24 M.grisea isolates using RAPD primer. M: 1kb molecular size marker



bromide was used in gel to stain DNA bands. Electrophoresis was performed at 80 V for 4 h in 1 X TAE buffer. 250 ng of 100 bp DNA ladder was also loaded in the same gel to estimate the molecular weight of the amplified product.

Data analysis

DNA banding pattern generated by RAPD primers were scored as "1" for presence of an amplified band

and "0" for its absence. All gels were scored twice manually and independently. Presence or absence of unique and shared polymorphic bands was used to generate "Suared Euclidean Distances". The linkage distances were then used to construct a dendrogram using "Biostastica" and "Unweighted pair group averages".

RESULT AND DISCUSSION

The PCR amplification performed with 20 RAPD primers was to access the level of polymorphism in 24 isolates of *M. grisea*. 24 isolates were grouped in two major groups, that is, "A" having 13 isolates and "B" having 9 isolates. 2 isolates (DHFB1 and VINB1) of the remaining 2 were grouped was placed alone in the dendrogram. Group "A" at 25% linkage distance was further sub grouped into A1 and A2 consisting of 7 and 6 isolates, respectively. Sub group A1 included TNLB1, TNLB2, BANLB1, TNLB3, TNLB4, TNNB1and TNNB2, while sub group A2 comprised of SENB1, RELB1, RELB2, RENB1, SENB2 and SEFB1. Group B was sub grouped into B1 and B2 at 35% linkage distance. B1 sub group comprised of 6 isolates that is TNNB3, TNNB4, RANB1, DHLB1, RALB1 and DHNB1and the remaining 3 isolates, that is, DINB1, BENB1 and BENB2 were grouped in B2 sub group.

The analysis of RAPD polymorphism in isolates of *M. grisea* from different regions across India revealed the occurrence of high level of polymorphism, indicating a wide and diverse genetic base. A repeat sequence termed MGR586 was identified in the genome of rice infecting strains of *M. grisea* [21]. This sequence has been widely used for DNA fingerprinting of *M. grisea* to investigate the epidemiology of the rice blast disease [9, 18,3,23]. Another retrotransposon, *fosbury* has also been used for genetic differentiation studies and the results indicate that isolates from Bangladesh lack both MGR586 and *fosbury*. MGR586 probe also failed to detect karyotypic changes [26]. Thus there is a need to develop different DNA fingerprinting techniques to identify various forms of *M. grisea* diversity. RAPD markers used in this investigation increases the marker density to find out genetic relationships in the present study, blast samples from eleven different geographical regions in Tamil Nadu and AICSMIP centre, India were collected. The dendrogram study revealed that the geographic origin of strains does not play crucial role in lineage formation, as in each lineage (group), there were mixed populations of the eleven geographical regions. Similar results have been shown by [15] in their study on isolates of *M. grisea* from different nurseries of Hunan province in China. The phylogenetic grouping based on our RAPD data did not appear to be harmonious with geographical locations. The topology of the dendrogram suggests that most isolates are about 25 - 40% different from each other, indicating that both local and geographical polymorphisms exist. Genetic mechanisms that could explain such diversity include simple mutations, meiotic recombination and mitotic (para sexual) recombination [27,28]. Further, the sexual cycle does not seem to be a source of variation for the rice blast pathogen in India [9].If sexual reproduction occurs in blast pathogen, it is probably infrequent and might not be significant in terms of rice blast epidemiology. The significant amount of diversity among Indian isolates of *M. grisea* can be explained mainly by evolution resulting from natural and stress-induced transposition [5]. Other mechanisms like horizontal gene transfer between rice blast fungus and its host [7], may also be of importance because varieties deployed within a region are based on crop seasons along with other biotic and geographic factors. On the basis of the present study, it is concluded that the Indian population of ragi blast like rice blast fungus may be genetically heterogeneous and the interrelationships amongst the different isolates can be easily, precisely and reliably explained by RAPD-PCR technology.

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