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Assessment of genetic diversity of finger millet blast isolates in Tamil Nadu

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ABSTRACT

Blast disease caused by *Magnaporthe grisea* is one of the major production constraints in finger millet. Fourteen *M. grisea* isolates collected from blast infected leaves and panicle from different locations of Tamil Nadu were subjected to randomly amplified polymorphic DNA (RAPD) analysis using 16 different random primers for assessing diversity. A total of 83 DNA fragments in the range of 200 to 2000 bp were amplified of which, 62 bands (74.7%) were polymorphic. Cluster analysis with unweighted pair group method of arithmetic averages (UPGMA) identified two main clusters.

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INTRODUCTION

Finger millet (*Eleusine coracana* L.), one of the ancient crops in India, is grown extensively in semi arid regions of India and Africa as a rainfed crop. Finger millet ranks fourth in importance among the millets in the world after sorghum, pearl millet and foxtail millet (Upadhyaya *et al.*, 2007). It constitutes as a staple food for the population in the arid and semi arid tropics and subtropics of the world and is considered as important component of food security in these regions (Sreenivasaprasad *et al.*, 2007). The crop is well recognized for its nutritional importance because of its high content of calcium (0.38%), dietary fibre (18%) and phenolic compounds (0.3-3%) (Devi *et al.*, 2011). The grain is also rich in iron, methionine and tryptophan (Tatham *et al.*, 1996). Its nutritive value and storability make it ideal for the management of malnutrition among children and adults, diet for diabetics and it is a good diet for patients.

Finger millet is cultivated in India in an area of 1.19 million ha producing 1.98 million tonnes with a productivity of 1.661 t/ha (Directorate of Economics and Statistics, Govt. of India, 2013-'14). In Tamil Nadu finger millet is grown in 1.187 lakh ha with a productivity of 3053 kg/ha (Directorate of Economics and Statistics, Govt. of India, 2013-'14). Blast disease caused by *Magnaporthe grisea* is the serious constraint in finger millet production wherever the crop is grown since most genotypes are susceptible to this disease (Mgonja *et al.*, 2007). The yield loss caused by this pathogen has been estimated to be around 28 -36 per cent (Nagaraja *et al.*, 2007) and in epidemic or endemic situations it causes yield loss upto 80-90 per cent (Rao, 1990).

Genetic analysis of plant pathogen population is

fundamental for understanding epidemiology, plant-pathogen coevolution and resistance management (Milgroom and Fary, 1997). Despite the huge economic loss associated with blast disease, specific studies to evaluate intra specific genetic variability and spatial distribution of the pathogen are rather limited. Molecular markers are useful tools to analyze the genetic variation in populations of plant pathogenic fungi. Randomly amplified polymorphic DNA (RAPD) markers are discrete fragments of DNA defined in the genome by annealing single decamers with random sequences to prime either ends of DNA, that form fingerprint of a genome (Williams *et al.,* 1990 and Sobral and Honeycutt, 1993). In the present investigation RAPD fingerprinting method is used to detect genetic variations and similarities among the isolates of *M. grisea* collected from different parts of Tamil Nadu.

MATERIAL AND METHODS

Fungal isolates :

Finger millet showing typical symptoms of blast disease was collected from farmers' fields and experimental plots at different geographical locations of Tamil Nadu during 2013-2014 monsoon seasons. The pathogen was isolated from symptomatic tissues of leaves or panicle by tissue segment method, using prune juice agar medium (composition per lit.: 40 ml prune juice, 5 g lactose, 1 g yeast extract and 20 g agar; pH 6.5) (Waller, 2002). Infected portions of leaves/ panicle were surface sterilized with 0.1 per cent mercuric chloride solution for one minute and rinsed in three changes of sterile distilled water. These bits were blot dried on sterilized filter paper and placed (four pieces per plate) on solidified prune juice Agar medium in sterilized Petri dishes. The dishes were incubated at room temperature (26±2°C) and when the growth of the fungus was visible, hyphal tips were transferred aseptically to fresh media and incubated at room temperature for ten days (Tuite, 1969). The fungal cultures were then purified by single spore isolation technique and pure cultures were maintained for further studies (Kotasthane and Agrawal, 2010).

Pathogenicity test :

Finger millet seedlings (cv. CO15) were raised under glasshouse conditions. To test pathogenicity of the isolates, the single spore isolates were inoculated on sterilized *Brachiaria mutica* stem bits supplemented with lactose (1%) in Erlenmeyer flasks using 5 mm mycelial bits and incubated at room temperature at 12 h light and 12 h dark (Madhavan, 2013). After fifteen days the stem bits fully covered with mycelia were placed in a sterile test tube containing 25 ml sterilized distilled water and shaken well to dislodge the spores. Tween 20 (0.02%) was added to the spore suspension (2-4 x 10^4 conidia per ml) and sprayed on 20 days old seedlings. Seedlings sprayed with water and tween 20 (0.02%) served as control. The pot containing seedlings were covered with polythene bag and kept in glasshouse for symptom development. The pathogen was reisolated from the lesions produced on the seedlings and the virulent isolates were maintained for further studies.

DNA isolation :

Total DNA was extracted from the isolates of *Magnaporthe* collected from finger millet using modified CTAB (cetyl trimethyl ammonium bromide) method (Murray and Thompson, 1980). Each isolate was grown in 100 ml Prune Juice broth in 250 ml Erlenmeyer flask at 25±2°C for ten days. The mycelial mat obtained was washed with sterile distilled water, dried and mashed with liquid nitrogen to fine powder. It was stored at -20°C. One gram of the mycelial powder was vortexed for two minutes with two ml pre-warmed CTAB extraction buffer [0.1 M Tris Cl (pH 8.0), 1.4 M NaCl, 0.5 M EDTA pH 8.0, CTAB (1%), SDS (0.5%), β-mercaptoethanol (1%) and polyvinyl pyrrolidone (PVP) (1%)] and incubated at 65°C for 1 hour with occasional shaking. The samples were allowed to cool down at room temperature. Thereafter the samples were extracted with 750 µl chloroform: isoamyl alcohol (24:1 v/v) and centrifuged at 10,000 rpm for 10 min (Rota 4 R-V/FA, Plasto Crafts). The upper aqueous phase was transferred to a fresh eppendorf tube and the extraction was repeated once. The upper aqueous phase was pipette off to a new eppendorf tube and mixed gently with an equal volume of ice cold isopropanol (2-propanol). The precipitated DNA was collected by centrifugation and the pellet was washed with 70 per cent ethanol. The pellet was resuspended in TE buffer (10 mM Tris HCl and 1 mM EDTA, pH 8.0) with 2 µl RNase (10 mg/µl) and incubated at room temperature for 30 min. Further purification was done with phenol: chloroform (1:1 v/v) and chloroform: isoamyl alcohol (24:1 v/v). Finally DNA was precipitated with 3M sodium acetate and chilled

ethanol. Thereafter it was centrifuged at 12000 rpm for 15 minutes, washed with 70 per cent ethanol and the DNA pellet was dried at room temperature. The pellet was dissolved in TE. DNA was checked by agarose gel (0.8%) electrophoresis and the concentration was determined by using a spectrophotometer (Genway Genova, Bibby Scientific Ltd., UK).

Primer screening :

A total of 20 random primers (OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, OPA-06, OPA-07, OPA-08, OPA-09, OPA-10, OPB-11, OPC-10, OPD-06, OPD-20, OPE-03, OPF-03, OPG-03, OPG-06, OPG-17 and OPK-07) were initially screened for 14 monoconidial isolates of *M. grisea* from finger millet to determine amplification and production of clear, sharp and repeatable amplicons and 16 random primers were selected. A control PCR with no genomic DNA was done for each primer to check contamination. The primers generating no or weak patterns were not selected.

RAPD analysis :

Random Amplified Polymorphic DNA markers were determined using 16 random decamer primers (Integrated DNA Technologies, Inc.). Polymerase chain reaction was carried out in a 20 µl reaction mixture containing 10 µl Genei Red Dye Master mix (Banaglore Genei Pvt. Ltd., Bangalore), 50 ng genomic DNA and 0.1µM primer. Single primer was used in each reaction. Amplification was carried out in Palm Cycler (Corbett) programmed at (i) initial denaturation (1 cycle) at 94°C for 3 min, (ii) 40 cycles of 94°C for 1 min for denaturation, 36°C for 1 min for annealing and 72°C for 1 min for extension and (iii) final extension of 10 min at 72°C. The amplified PCR products were resolved by using 1.4 per cent agarose gel stained with ethidium bromide (0.5 µg/ml), in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1mM EDTA, pH 8.0) at 80 V for 2 hours (Genei Maxi Sub System). The banding patterns were documented using UVItec, Cambridge gel documentation system. At least two replications of the assay were performed with template DNA to ensure consistency of banding pattern.

Positions of explicitly scorable bands were scored manually for their presence (1) or absence (0) across the isolates and assembled to a binary character matrix ('1' for the presence and '0' for the absence of a band at a particular position). Data on clearly resolved bands generated by the primers were used to estimate genetic similarities among the isolates based on Jaccard's similarity co-efficient. The matrix was analyzed using Unweighted Pair Group Method with Arithmetic averages (UPGMA) following the Sequential Agglomerative Hierarchical Nested (SAHN) cluster analysis module to derive the dendrogram. All these computations of independent markers were done using NTSYS-pc (Exeter software, Setauket, NY, USA) (Rohlf, 2000).

RESULTS AND DISCUSSION

A total of 15 isolates (Pg1-Pg15) were recovered from finger millet blast affected samples from different regions of Tamil Nadu (Table 1). The cultures were purified by single spore isolation technique and were confirmed as *M. grisea* by comparing the morphology with authentic cultures maintained at Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. Pathogenicity of the isolates was tested by artificial inoculation using cv. CO15 under glass house conditions. All the isolates except Pg15 collected from Bathalapalli, Krishnagiri District produced blast lesions on the leaves 4-6 days after inoculation. These isolates were reisolated from the lesions produced on seedlings and used for further studies.

RAPDs are especially useful in differentiating clonal lineages for fungi that reproduce asexually (McDonald, 1997). In the present study RAPD is used to assess the variability associated with 14 different monoconidial isolates of *M. grisea* at the intraspecific level using 16 random primers selected from the 20 random primers screened intially. The 16 primers (OPA-01, OPA-02, OPA-03, OPA-04, OPA-06, OPA-07, OPA-08, OPA-09, OPA-10, OPB-11, OPC-10, OPD-06, OPE-03, OPF-03, OPG-03 and OPG-06) led to amplification of 83 fragments of which 62 bands were found to be polymorphic (74.7%) and 21 bands were found to be monomorphic (25.3%). The size of the amplification products varied from ~200 bp to ~2000 bp. The largest molecular weight band was amplified by primer OPA-07 (~2000bp) while the smallest molecular weight band (~200bp) was amplified by OPC-10. The numbers of amplicons generated by each primer varied from three to seven (Table 2). The primers OPA-02, OPA-09 (Plate 1a) and OPB-11 (Plate 1b) produced greater number of polymorphic bands (6-7) compared to OPA-03 and OPA-06 which produced only 2 polymorphic bands. The level

of polymorphism ranged from 50 per cent (OPC-10 and OPE-03 where 3 out of 6 bands were polymorphic) to 100 per cent (OPB-11 where all the 7 bands were polymorphic). RAPD-PCR product by OPC-10 at ~1100bp was found to be specific for Pg1 isolate while a band at ~1500bp by OPA-10 was unique for the isolate Pg11. Amplification of *M. grisea* with arbitrary primers has proved to be successful for characterization of genetic diversity as already established by Madhavan *et al.* (2013).

Cluster analysis by UPGMA grouped the 14 isolates into two main groups (Fig. 1). Group 1 comprising single isolate Pg1 while group 2 comprising all the other isolates. Even the isolates collected from same district grouped in different cluster substantiating genetic diversity among the isolates. Thus RAPD technique has been used to detect genetic variation among isolates within a species (Boyd and Carris, 1997). All the strains were 54 per cent similar to each other. Dendrogram depicted that, genetically the most similar isolates were Pg4 and Pg7

Table 1 : *Magnaporthe* isolates collected from finger millet

Sr. No.	District	Locality	Plant part	Isolate number
1.	Coimbatore	Department of Millets, TNAU, Coimbatore	Leaf	Pg1
2.		Thondamuthur	Leaf	Pg2
3.		Thondamuthur	Neck	Pg3
4.	Villupuram	Tirukoilur	Leaf	Pg4
5.	Thiruvannamalai	Thiruvannamalai	Neck	Pg5
6.	Dharmapuri	Annasagaram	Leaf	Pg6
7.	Theni	Andipatti	Neck	Pg7
8.	Namakkal	Kolli hills	Leaf	Pg8
9.	Erode	Burgur	Neck	Pg9
10.		Anthiyur	Leaf	Pg10
11.	Salem	Mettur	Leaf	Pg11
12.		Kannamoochi	Leaf	Pg12
13.	Krishnagiri	Denkanikottai	Neck	Pg13
14.		Krishnagiri	Leaf	Pg14
15.		Bathalapalli	Leaf	Pg15

Table 2 : Polymorphism exhibited by RAPD primers

Primer	Nucleotide sequence (5'-3')	No. of amplified bands	No. of polymorphic bands	Polymorphism %
OPA-01	CAGGCCCTTC	4	3	75.0
OPA-02	TGCCGAGCTG	7	6	85.7
OPA-03	AGTCAGCCAC	3	2	66.7
OPA-04	AATCGGGCTG	4	3	75.0
OPA-06	GGTCCCTGAC	3	2	66.7
OPA-07	GAAACGGGTG	7	4	57.1
OPA-08	GTGACGTAGG	4	3	75.0
OPA-09	GGGTAACGCC	7	6	85.7
OPA-10	GTGATCGCAG	5	4	80.0
OPB-11	GTAGACCCGT	7	7	100.0
OPC-10	TGTCTGGGTG	6	3	50.0
OPD-06	ACCTGAACGG	5	4	80.0
OPE-03	CCAGATGCAC	6	3	50.0
OPF-03	CCTGATCACC	4	3	75.0
OPG-03	GAGCCCTCCA	6	5	83.3
OPG-06	GTGCCTAACC	5	4	80.0
Total/ Mean		83	62	74.075

Table 3 : Similarity matrix for 14 different isolates of *M. grisea* from finger millet

	Pg1	Pg2	Pg3	Pg4	Pg5	Pg6	Pg7	Pg8	Pg9	Pg10	Pg11	Pg12	Pg13	Pg14
Pg1	1.0000													
Pg2	0.5588	1.0000												
Pg3	0.5571	0.7833	1.0000											
Pg4	0.5890	0.7813	0.8571	1.0000										
Pg5	0.4868	0.6818	0.6522	0.7286	1.0000									
Pg6	0.5200	0.7500	0.6667	0.7183	0.8030	1.0000								
Pg7	0.6087	0.8167	0.8065	0.8889	0.6812	0.7727	1.0000							
Pg8	0.5075	0.7069	0.6721	0.6769	0.5821	0.6462	0.7627	1.0000						
Pg9	0.5352	0.8136	0.7460	0.7727	0.6765	0.7692	0.8361	0.7586	1.0000					
Pg10	0.5606	0.7719	0.7931	0.7619	0.6615	0.7302	0.7966	0.7143	0.8571	1.0000				
Pg11	0.4921	0.7037	0.6102	0.5938	0.5714	0.6129	0.6441	0.6111	0.6379	0.6792	1.0000			
Pg12	0.5522	0.7895	0.7213	0.7231	0.6269	0.7460	0.7833	0.7018	0.8103	0.7679	0.6981	1.0000		
Pg13	0.5522	0.7586	0.6935	0.6716	0.6769	0.7188	0.7541	0.7636	0.7797	0.8000	0.6364	0.7857	1.0000	
Pg14	0.5441	0.7167	0.6825	0.6618	0.5714	0.6567	0.6875	0.6897	0.7667	0.7544	0.5965	0.7119	0.8036	1.0000

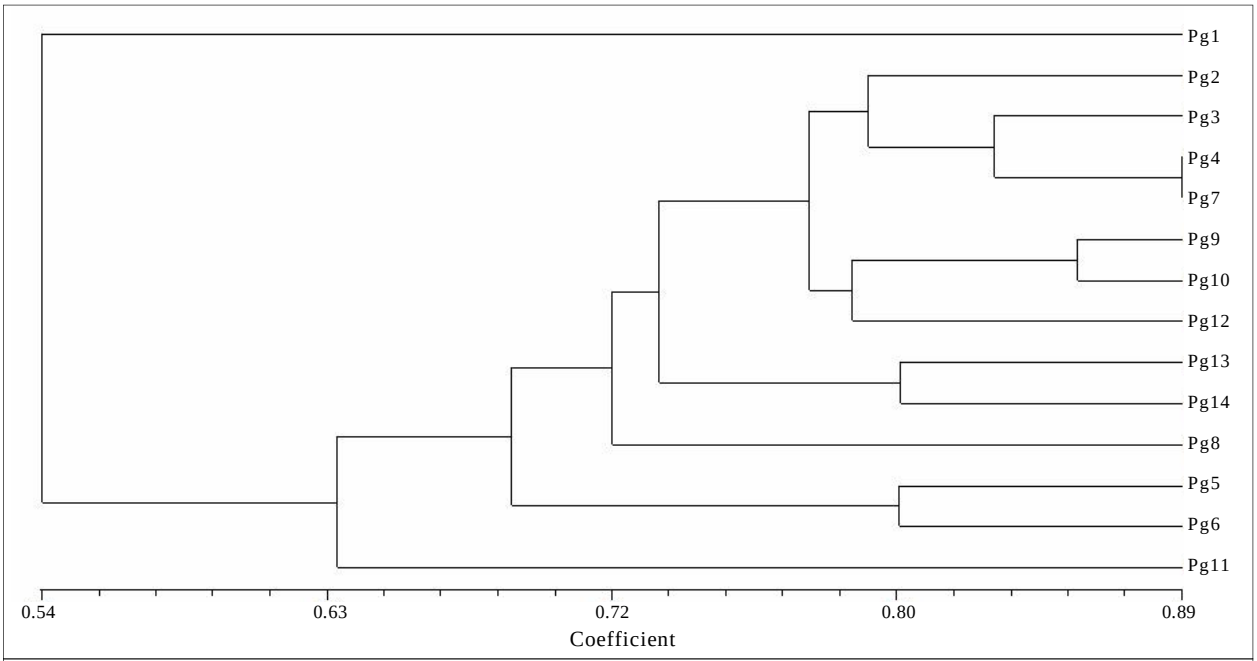

Fig. 1 : Dendrogram based on UPGMA clustering of RAPD data of the isolates of *M. grisea* from finger millet

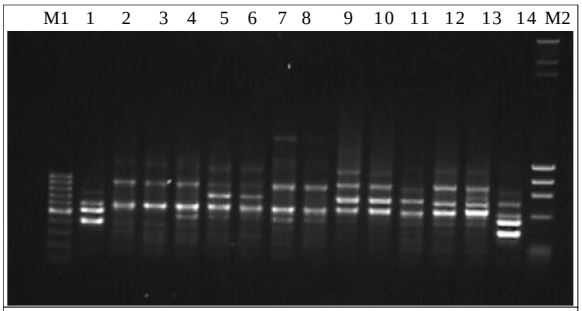

Plate 1A : RAPD of *M. grisea* isolates using primer OPA09

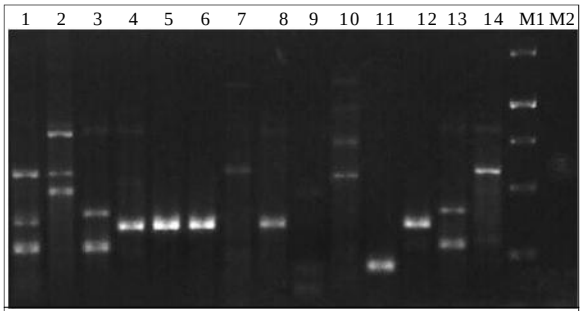

Plate 1B : RAPD of *M. grisea* isolates using primer OPB11

M1-100bp Marker, 1-Pg1, 2-Pg2, 3-Pg3, 4-Pg4, 5-Pg5, 6-Pg6, 7-Pg7, 8-Pg8, 9-Pg9, 10-Pg10, 11-Pg11, 12-Pg12, 13-Pg13, 14-Pg14, M2-1 kb Marker

(89%) followed by Pg9 and Pg10 (86%). The similarity co-efficient was in the range of 0.49 and 0.89 (Table 3) confirming the high genetic diversity among the isolates.

DNA finger printing techniques such as restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) provide a powerful approach to distinguish species and strains of filamentous fungi (Meyer *et al*., 1991). Welsh and McClelland (1990) and Williams *et al*. (1990) described another simple method for assessing genotypic variability based on amplification of nucleotides with primers of arbitrary sequences termed as arbitrary primed polymerase chain reaction (AP-PCR) or random amplification of polymorphic DNA (RAPD) markers.

M. grisea includes pathogens infecting rice and other grass species including finger millet. However individual isolates have a limited host range and they exhibit host cultivar specificity. The pathogen show high degree of variability in the field and new races frequently appear with the ability to attack previously resistant cultivars. To understand the mechanism of resistance breakdown in blast resistant cultivars, knowledge of genetic diversity in the pathogen population in the geographical region is important (Valent and Chumley, 1991 and Levy *et al*., 1993). Various potential mechanisms, including sexual recombination, heterokaryosis, parasexual recombination, and aneuploidy, have been proposed to explain frequent race changes in *M. grisea* (Zeigler *et al.*, 1997 and Kang and Lee, 2000). Such changes may cause new virulence shifts in the pathogen population. RAPD markers detect length polymorphisms arising from base sequence changes, insertions, deletions and substitutions either at or between the priming sites and provide useful information (Cook *et al.*, 1996). Utilizing these tools significant genetic diversity in *M. grisea* isolates from different graminaceous crops and graminaceous weeds has been described previously (Viji *et al*., 2000 and Madhavan *et al*., 2013). Finger millet mainly contains quantitative resistance to blast. Some of the lines identified are suitable for utilization in breeding programmes (Sreenivasaprasad *et al*., 2007). This information along with the knowledge of genetic diversity, pathogenic diversity and epidemiology of blast will be useful for the development of successful finger millet production systems.

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