Genetic analysis of rice blast fungus of southern Karnataka using DNA markers and reaction of popular rice genotypes

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Twenty-seven monosporic blast cultures were isolated from infected leaves of susceptible rice cultivars from three major rice growing areas of Karnataka. They were fingerprinted using thirty Random Amplified Polymorphic DNA (RAPD) primers. Genetic variation analysis and grouping of these isolates were done using cluster analysis (NTSYS). Three distinct lineages were observed. Only one isolate, PPT-4, was distinct and formed a separate group. Nineteen isolates of Ponnampet had representative isolates in all the three distinct lineages identified. Isolates from the other two locations clustered into one group indicating no significant genetic differences existing among the lineages operating in the two locations. With a view to identifying resistant sources for the most distinct lineages, seventeen rice genotypes including some improved varieties, land races and pyramided rice genotypes with major blast resistance genes, were inoculated with PPT4 isolate. Of the genotypes tested, IRAT177, Moroberekan, Apura, Doddi (local land race) and pyramided lines, containing known resistance genes *Pi*1, *Pi*1+ *Pi*4 and *Pi*2 + *Pi*1 showed high degree of leaf blast resistance. Rest of the genotypes showed susceptible reaction.

RICE blast, caused by the filamentous fungus *Magnaporthe grisea* (Hebert) Barr, is one of the oldest reported diseases of rice, which has received wide attention[1]. The disease appears severely in upland and rain-fed lowland environments that are prone to drought[1]. An estimated quantity of 157 million tonnes of rice was lost between 1975 and 1990 worldwide due to blast alone[2]. Many pathogenic races have been identified in *M. grisea* and the pathogenic variability has been cited as the principal cause for the breakdown of resistance in rice varieties[3]. In disease-conducive environments, the lifespan of many disease-resistant cultivars has been known to be ephemeral[4,5]. Wide pathogenic variation has been reported from single spore isolates, from single lesions and monoconidial sub-cultures of *M. grisea*[6]. Earlier studies on the variability of this fungus relied mainly on the phenotypic characters and virulence tests using a set of host differentials. Most of these phenotypic traits are highly variable as this pathogen is genetically unstable[1]. Such kinds of study are labour-intensive and time-consuming, require a large greenhouse space and often lead to ambiguous results. Furthermore, they are influenced by environmental conditions, inoculation techniques and human errors in scoring.

With the advent of molecular biology, isozyme analysis[7], ribosomal DNA, internal transcribed spacer sequence analysis, repetitive DNA polymorphism analysis[8], restriction analysis of mitochondrial DNA[9] and RAPD analysis[10,11] have been successfully used to study the genetic variation of different pathogens. Characterization has been accomplished using RAPDs and pathogen-specific MGR586, an RFLP probe[12–14].

Keeping all these intricacies in view, we made an attempt to study genetic variability of *M. grisea* isolates of southern Karnataka, India, using RAPD markers. Using different genetic groups of blast fungus present in the locations, we made an attempt to identify resistant sources among popular rice genotypes grown in the region and their possible exploitation in breeding for leaf blast resistance, as some of these genotypes contain known major genes for blast resistance.

Severely blasted leaves of HR12, CO39 (universally known susceptible varieties) and Intan rice varieties, showing large spindle-shaped lesions with dark-brown margin and greyish centre were collected from Ponnampet, Mandya and Bangalore in Karnataka during the wet season 1996–97 (Table 1). Well-developed susceptible lesions were identified, excised and washed in running water for 2 h. The leaf bits were surface-sterilized with mercuric chloride. They were then washed serially with sterile double-distilled water and allowed for sporulation on sterilized glass slides by incubating in a moist chamber at 28°C for 48 h. Well-sporulated lesions were placed in double-distilled water in test tubes and vortexed for 1 min. About 1 ml of spore suspension was added to sterilized plates and 4% lukewarm agar was added. Single spores were located and picked up microscopically. Each spore was eventually transferred to potato dextrose agar (PDA) slants. The slants were incubated at 28°C for 2 days and stored at 4°C. Twenty-seven monosporic cultures representing all the three regions isolated from diverse genetic background were used for DNA analysis in the study (Table 1).

All the monosporic blast cultures were revived and each culture was inoculated with 100 ml of potato dextrose broth (PDB) in Erlenmeyer's flask and incubated at 28°C for 7 days on an orbital shaker at 100 rpm. Later, the mycelial mat was separated and freeze-dried. About 0.5 g of lyophilized mycelia was ground to a fine powder in the pre-cooled pestle and mortar using liquid

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Table 1. Monosporic cultures of *Magnaporthe grisea* collected across three locations of southern Karnataka, India

Isolate	Place of collection	Host
B2	Bangalore	HR12
B4	Bangalore	HR12
MDH2	Mandya	Mandya Vijaya
MDH3	Mandya	HR12
MDH4	Mandya	HR12
MDH5	Mandya	Mandya Vijaya
MDH7	Mandya	Mandya Vijaya
MDH10	Mandya	HR12
PPT1	Ponnampet	HR12
PPT3	Ponnampet	HR12
PPT4	Ponnampet	HR12
PPT12	Ponnampet	HR12
PPT13	Ponnampet	Mandya Vijaya
PPT14	Ponnampet	Mandya Vijaya
PPT15	Ponnampet	Mandya Vijaya
PPT17	Ponnampet	Mandya Vijaya
PPT18	Ponnampet	Mandya Vijaya
PPT19	Ponnampet	Mandya Vijaya
PPT21	Ponnampet	Intan
PPT22	Ponnampet	Intan
PPT23	Ponnampet	Intan
PPT24	Ponnampet	Intan
PPT25	Ponnampet	Intan
PPT26	Ponnampet	Intan
PPT27	Ponnampet	Intan
PPT28	Ponnampet	Intan

PPT, Ponnampet; MDH, Mandya; B, Bangalore.

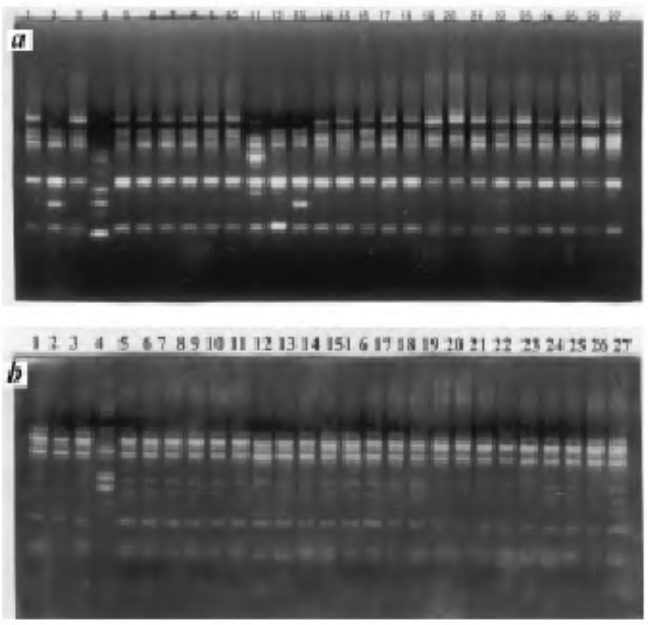

Figure 1. RAPD banding pattern of twenty-seven selected blast isolates of *Magnaporthe grisea* generated by random primers. ***a***, OPB-02; and ***b***, OPB-03.

nitrogen and transferred to 1.5 ml sterilized Eppendorf tubes. Then 500 µl of extraction buffer (50 mM Tris-HCl, 150 mM NaCl and 100 mM EDTA) was added and vortexed until evenly suspended, and incubated at 37°C for 1 h after adding 50 µl of 10% SDS. Later, 75 µl of 5 M NaCl and 60 µl of CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) were added and mixed thoroughly. The tubes were incubated at 65°C for 15 min and equal volume of chloroform : isoamyl alcohol (24 : 1) was added to extract DNA. The Eppendorf tubes were vigorously shaken and centrifuged at 10,000 rpm for 12 min. Aqueous viscous supernatant was transferred to fresh Eppendorf tubes, two-thirds volume of ice-cold isopropanol was added and incubated at –20° for 8 h. The tubes were centrifuged at 10,000 rpm for 15 min and the supernatant was discarded. Pellet was washed with 70% ethanol, air-dried, dissolved in 100 µl of 1 × TE buffer and used in the PCR reaction.

The 25 µl PCR reaction mixture comprised of 15 pmol of each primer (Operon Technologies Inc., USA); 200 µm each of the dATP, dCTP, dGTP and dTTP and 0.5 units of Taq DNA polymerase in 1X PCR buffer (Bangalore Geni Pvt Ltd, India). Template DNA consisted of 30 ng of genomic DNA extracted from each fungal isolate. PCR reaction was carried out using PTC 200, DNA Engine (MJ Research Inc., USA). The PCR profile was programmed at 94°C for 1 min for denaturation followed by primer annealing at 36°C for 1 min and primer extension at 72°C for 2 min with a total of 35 cycles. The initial denaturation of DNA was for 2 min at 94°C. The PCR products were analysed on 1.2% agarose gel containing ethidium bromide (0.5 µg/ml) and electrophoresed at 80 volts for 2 h. The gel was viewed on a UV-transilluminator and the bands were scored (Figure 1 *a* and *b*).

The polymorphic DNA bands generated by each isolate were scored at each marker level (Table 2). The presence of band was scored as 1 and its absence as 0. The genetic distance between the isolates was estimated using NTSYS software program to identify the number of clusters generated using qualitative similarity measures through SHAN clustering (Figure 2).

Seventeen selected rice genotypes were used to study the leaf blast reaction of the single-spore isolates. About fifty seeds of each line were sown densely in raised nursery beds in rows surrounded by two rows of susceptible spreaders (HR12 and CO39) in controlled condition. All the necessary operations for good growth of seedlings in the nursery were followed.

A monosporic culture, PPT-4 (an isolate of Ponnampet location), which showed distinct banding pattern and formed a separate lineage in the cluster, was used for inoculation. It was revived on PDA slants and kept in the incubator at 28°C for 8 days. Ten to twelve days prior to inoculation, 10 ml of distilled water was put in previously revived test-tube isolate and the spores were dislodged using a sterilized inoculation loop. The

Table 2. Details about DNA bands generated by thirty RAPD primers across twenty-seven isolates of *Magnaporthe grisea*

Total number of marker levels	176
Number of markers with monomorphic bands	12
Number of marker levels with polymorphic bands	164
Maximum number of bands generated by a primer	13
Minimum number of bands generated by a primer	1
Average number of bands generated	7
Per cent polymorphism	93.18

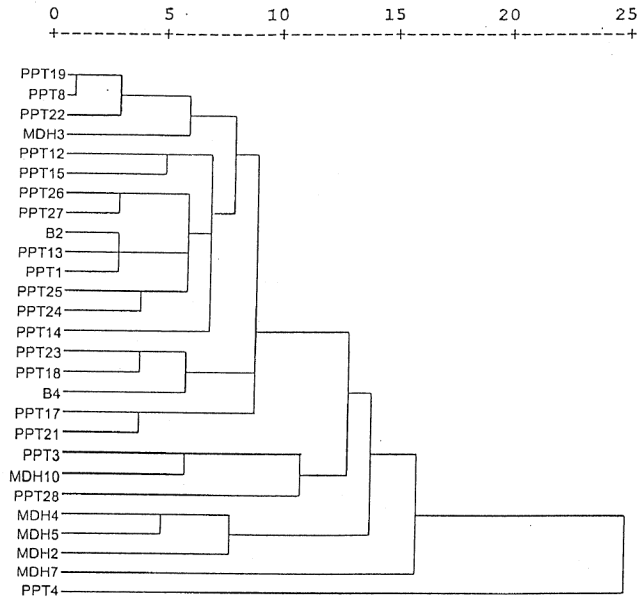

Figure 2. Dendrogram depicting the genetic relation of 27 blast isolates of *Magnaporthe grisea* based on DNA banding pattern generated by 30 RAPD primers.

dislodged spores and mycelial suspension were then poured onto cold oatmeal agar (OMA) plates. The plates were then incubated for 4–5 days at 28°C until mycelial growth was observed. The mycelial surface in the plates was disturbed with sterilized glass slides and exposed to fluorescent light continuously for 5–7 days to induce sporulation. About 10 ml of sterilized water mixed with a few drops of Tween-80 was poured onto each culture plate. Using glass slides, the spore surface was scraped and filtered through two-layered cheesecloth. The spore concentration was adjusted to 5×10^4 conidia/ml (ref. 15) for inoculation.

Fourteen-day-old rice seedlings were uniformly inoculated with spore suspension using an atomizer. The entire nursery bed was under a polythene cover for 48 h. Subsequently, the seedlings were misted 4–5 times a day and the required temperature and humidity for disease manifestation was maintained throughout the observation period.

The rice genotypes in the infected nursery were scored on seventh and fourteenth day after inoculation (DAI) for lesion type (LT), lesion number (LN) and diseased leaf area visual [DLA(V)], by selecting three seedlings per genotype randomly (Table 3). The observations were averaged genotype-wise and grouped[15].

Many pathogenic races are being reported in *M. grisea* incessantly and this could be the main cause for breakdown of resistance in several popular rice varieties[1]. The diversity and variability of the pathogen originate from the clonal mode of reproduction, coupled with mutation, migration, drift/selection, heteroploidy and parasexuality of the fungus[16]. In the present study, involving twenty-seven single-spore isolates collected across three locations, we observed three distinct lineages at 70% similarity index. All the nineteen isolates of Ponnampet (an internationally known hot spot for rice blast disease evaluation) largely showed much similarity with respect to banding pattern, indicating that most of the isolates more or less belong to same lineage. Average similarity of the first lineage, which comprised of twenty-five single-spore isolates was found to be 44%; however, PPT-4 and MDH 7 formed two separate lineages. Similarly, the two isolates from Bangalore (B2 and B4) did not show significant variation. The number of lineages and their composition prevailing in an area largely depend on the rice cultures grown in that region. Ponnampet has all the three lineages, including the ones observed in Mandya and Bangalore. This could be ascribed to the fact that this region is a potential rice-growing area where rice varieties with diverse genetic background are being grown, and this puts enormous amount of selection pressure on the

Table 3. Reaction of prominent rice genotypes for PPT-4 single-spore isolate of *Magnaporthe grisea*

	7 DAI		14 DAI		21 DAI	
Genotype	MLT	% DLA	MLT	% DLA	MLT	% DLA
Apura	0	0.00	0	0.00	0	0.00
Azucena	1	4.67	2	12.67	*	55.00
BKB	2	0.50	5	6.67	*	30.00
CO39	3	80.00	5	100.00	*	100.00
Doddi	Ph	0.10	1	0.70	2	10.00
HR12	3	80.00	5	100.00	Ph	5.00
Irat177	0	0.00	0	0.00	0	0.00
IR64	1	3.755	2	5.67	*	15.00
Irylon short	Ph	0.34	1	2.33	*	60.00
Jaya	2	6.00	5	21.66	*	35.00
Mandya Vijaya	3	0.75	4	10.00	*	12.00
Pokkali	3	3.667	5	20.00	*	35.00
*Pi*1	Ph	0.05	Ph	5.00	Ph	40.00
*Pi*4	3	6.33	5	21.66	*	25.00
*Pi*2X1	Ph	0.18	Ph	2.66	Ph	45.00
*Pi*1X4	Ph	1.85	Ph	7.00	Ph	40.00
Moroberekan	0	0.00	0	0.00	0	0.00

MLT, Maximum lesion type; DLA, Diseased leaf area; *, Lesions coalesced; Ph, Pinhead-sized lesions.

pathogen. Also, all the rice varieties/lines, identified/bred, for leaf and neck blast resistance in India and abroad, are routinely tested in this region annually. The continuous evaluation of highly susceptible and resistant rice lines brought from various countries may act as an additional source for the new pathotypes. Lavanya and Gnanamanickam[17] have summarized the work carried out on the characterization of rice blast fungus in India. From our investigation, it is evident that there are three distinct lineages of *M. grisea* operating in this region.

There has been considerable achievement in the development of blast-resistant varieties, particularly using vertical-resistant genes. Nevertheless, durable resistance alone can adequately protect the irrigated rice crops in the tropics. Exploitation of durable resistance has been proposed for less blast-conducive environments[18–21]. In our study involving the reaction of representative single-spore culture PPT-4 to rice varieties, Moroberekan, isolines of CO39, viz. *Pi*1, *Pi*2, *Pi*4, *Pi*2 + *Pi*1, *Pi*1 + *Pi*4 along with IRAT177, Apura and Doddi showed resistant reaction. Of these, *Pi*1, *Pi*2, *Pi*4, *Pi*2 + *Pi*1, and *Pi*1 + *Pi*4 are known to contain major genes conferring resistance to blast disease. The isolines have specific resistance genes from LAC 23, 5173 and Pai-Kan-Tao for *Pi*1, *Pi*2 and *Pi*4 genes, respectively[15,22]. Artificial inoculation indicated the availability of resistance sources among the genotypes tested for the prevailing races of this region. Moroberekan, a West African *Japonica* variety, has been reported to possess more than two major genes along with several QTLs conferring resistance to blast disease[23]. IR64 showed moderate level of resistance to the isolate PPT-4 and which corroborated the earlier findings indicating the presence of major genes and QTLs conferring blast resistance[24]. On the contrary, CO39 and HR12 varieties used as susceptible checks proved their susceptible nature for this group of isolates also. The results obtained in this study help in incorporating both major genes and QTLs into rice varieties based on their reaction to blast disease.

This study indicates that RAPD is a simple and convenient tool to measure the extent of genetic variability of *M. grisea* at the molecular level and identify the lineages. The preliminary results of our study are useful in identifying different lineages and population structure of blast fungus of this region. Further, it is possible to identify resistant genotypes to a particular group of pathogen and host differentials.

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ACKNOWLEDGEMENTS. We thank The Rockefeller Foundation, NY, USA, for financial support for this research (RF98007#671). Help rendered by the members of MAS Laboratory, Department of Genetics and Plant Breeding, GKVK, UAS, India is acknowledged.

Received 6 October 2001; revised accepted 17 January 2002