

Morphological and Molecular Variability among Rice Blast Pathogen (*Magnaporthe oryzae*) Isolates in Southern India

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Abstract Rice is a staple food crop for majority of human population worldwide. Significant grain yield losses are reported due to blast disease caused by *Magnaporthe oryzae* across all crop growing areas of the world. Though, presently available blast management strategies reduce disease significantly, blast epidemics are still common, thereby causing devastating yield losses. Our present study therefore aimed at determining variability among blast pathogenic isolates that ultimately enables formulation of area-wise blast management strategies, besides preventing indiscriminate usage of chemical fungicides. Leaf blast samples were collected from crop growing areas of Telangana and Andhra Pradesh during *kharif* 2014 and 2015 and *M. oryzae* (n=20) isolates were obtained using standard laboratory protocols. These isolates were further evaluated for their morphological and genetic characterization. In morphological studies, variability was determined based on colony diameter, color and shape of conidia under *in vitro* conditions. Genetic diversity was determined based on molecular methods using PCR with 3 MGM markers. Our results indicated that morphologically, the blast

pathogenic isolates differed and their colony diameter ranged from 71.6 to 87.6 mm. On oat meal agar, colony color of the isolates was usually grey and greyish-white with good growth. Genetic diversity studies on *M. oryzae* isolates using 3 MGM markers indicated less polymorphism at DNA level. The cluster analysis of MGM data grouped the isolates on the basis of their origin with few exceptions. A total of seven amplicons were obtained from 20 *M. oryzae* isolates. Cluster analysis of the blast isolates revealed the average pair-wise similarities in the range of 0.08 to 1.0, thus suggesting variations among the isolates. Our research results on variability of blast pathogen will be useful in devising location specific disease management strategies for rice blast.

Keywords Genetic diversity, *Magnaporthe oryzae*, Rice blast, Variability.

Introduction

Rice (*Oryza sativa* L.) is the world's most important crop and a primary source of food for more than half of the world's human population. More than 90% of the world's rice is grown and consumed in Asia [1]. Rice is widely cultivated in China followed by India, Indonesia, Bangladesh, Vietnam, Thailand, Myanmar. The crop occupies about 165.2 M ha worldwide, with an annual production of 740.9 Mt and a productivity of 4485.87 kg ha⁻¹ [2]. Rice is a rich source of protein, carbohydrate, dietary fiber, minerals and vitamins [3].

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In India, rice is grown to an extent of 43.94 M ha with a production of 159.2 Mt and a productivity of 3623.12 kg ha⁻¹. In Andhra Pradesh, the crop is grown to an extent of 3.62 M ha with a production of 11.4 Mt and productivity of 3173 kg ha⁻¹. In India, the productivity of rice is less than those in agriculturally advanced countries. This is attributed to the poor agronomic practices and partially due to biotic and abiotic stresses. However, major diseases are rice blast, brown spot, bacterial leaf blight, sheath blight, sheath rot, Bakanae, stem rot, tungro, false smut and post-harvest diseases [4].

Rice blast caused by a filamentous, ascomycete fungus *Magnaporthe oryzae* (syn: *Pyricularia oryzae* Cav.) is one of the most important diseases of rice worldwide and is one of the major hindrances for profitable rice production [5]. Several rice blast epidemics have occurred in different parts of the world, resulting in 50 to 90% of the grain yield losses. There was a severe epidemic of rice blast in 1978, when incidence of panicle blast was more than 40% in some cultivars. Blast was first recorded in India during 1913 and a devastating epidemic occurred in 1919 in the Tanjore delta of Tamil Nadu. Blast disease is known to attack nearly all the above ground parts of the plant and all the crop growth stages. Reports have shown that the fungus has the capacity to infect plant roots also [6].

Several management strategies have been proposed and evaluated to minimize the blast disease incidence. Although the use of resistant cultivars is known to be the most effective control strategy, it also carries certain issues relating to development of pathogenic races. For example, the major difficulty in controlling rice blast with genetic resistance is that there are many races of the pathogen and cultivars containing a single gene conferring resistance to a specific race of the pathogen. But they often become susceptible over time with the development of new races and can infect plants with that specific resistance gene. Thus the use of resistant cultivars is limited to a certain place and time. Knowledge of the genetic variation within and among pathogen populations is an important component of understanding the population biology of pathogenic fungi. Research

in these aspects also infers the impact of driving force influencing the evolution of pathogen populations. It is therefore precisely at this juncture, the studies on diversity of rice blast pathogen using morphological and molecular approaches assume significance. The long term goal of this study is to devise strategies for developing resistant cultivars and to evolve new molecules for rice blast management.

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Materials and Methods

Isolation of mono-conidial isolates of *Magnaporthe oryzae*

Rice leaves showing typical symptoms of the blast disease were selected and washed with sterile distilled water. Small piece of diseased tissue along with some healthy tissue were cut with the help of sterile scalpel and surface sterilized with 1% sodium hypochlorite for one minute, rinsed thrice in sterile water and dried with sterilized filter paper. The surface sterilized and dried leaf pieces were later transferred aseptically on to the sterilized petri dishes containing OMA medium and plates were incubated at 28°C for 4 days. The pathogen was further sub cultured on oat meal agar medium and was purified by using single spore isolation method. The culture was maintained on oat meal agar medium slants and preserved at 4°C for further studies [5]. The isolates were designated as MG1 to MG20.

Genomic DNA isolation

Seven day old pre-inoculated *M. oryzae* agar block was transferred into sterile 2% Yeast Extract Glucose (YEG) broth and incubated at 28°C for 7 days for mass production of the fungal mycelium. The obtained mycelium (~250 mg) was lyophilized using liquid nitrogen and used for DNA extraction. The DNA was extracted by following CTAB method [7]. A working DNA solution was made by diluting DNA stock to approximately 10–20 ngµl⁻¹.

DNA fingerprinting of M. oryzae isolates

Polymerase Chain Reaction (PCR) was performed in a final volume of 25 µl consisting of 0.25 mM of each dNTP's, 1 unit of Taq DNA polymerase 15–20 ng of DNA template, 0.3 µM of primer (Integrated DNA Technologies, Iowa, USA), 1 × PCR buffer (containing 1.5 mM MgCl₂) and sterile distilled water. The reactions were carried in a Thermal Cycler PT-100 (MJ Research, MA). Thermal profile was 94°C for 5 min, followed by 36 cycles of 94°C for 45 sec, 50–60°C for 1 min with final extension of 72°C for 7 min. The PCR products were electrophoresed at 100 v for 3 h on 3.5% agarose gels in 0.5 × TBE buffer for MGM primers and on 1% agarose gel for Pot 2 primer. Gels were stained with ethidium bromide and visualized using UV Trans-illuminator. Twelve MGM Primers and a Pot 2 primer were used for PCR amplification of 20 *M. oryzae* isolates.

Variability studies

Morphological characteristics

The morphological characters of all monoconidial isolates of *M. oryzae* were recorded by growing them on OMA medium for 15 days at 28°C. Morphological characteristics of *M. oryzae* isolates collected from different locations were studied for radial growth (mm), size of conidia and sporulation. Spores of *M. oryzae* of different isolates collected from the infected host tissue were mounted in lacto phenol cotton blue on a clean slide. Spores were measured under high power objective lens (40X) using a precalibrated ocular micrometer. The average size of spore was then determined and shape of the spores were recorded. Photomicrographs were taken to show the typical spore morphology of the pathogen.

Molecular variability in *Magnaporthe oryzae* using *Magnaporthe grisea* Microsatellite (MGM) markers and Pot 2 primer

Molecular variability among the isolates of *M. oryzae* collected from different locations was studied using the *Magnaporthe grisea* Microsatellite (MGM) mark-

ers and Pot2 primer. A set of 11 markers were selected based on the *M. oryzae* linkage map reported by Mohan et al. [7].

Molecular data analysis

The profiles generated by different primers were compiled to determine the genetic relatedness among the different *M. oryzae* isolates. The presence or absence of each band in all isolates was scored manually by binary data matrix with 1 indicating the presence of the band and 0 indicating the absence of the band. Data were generated separately for each primer. A similarity matrix was generated from the binary data using Jaccards similarity coefficient in the SIMQUAL program of the NTSYS-pc package. Cluster analysis was performed with the unweighted pair group arithmetic mean method (UPGMA) in the SHAN program of the NTSYS-pc package [7].

Results and Discussion

Morphological and colony characteristics of *Magnaporthe oryzae*

Morphological and colony characteristics of the fungus are the important basic factors for identification of a fungus and its variability. The morphological characteristics such as color of the mycelium, texture and radial growth of the mycelium of the isolate, size and shape of the conidia and sporulation (number of spores observed per microscopic field) were studied among the isolates of *Magnaporthe oryzae*. The isolates were morphologically characterized by measuring the size (length and width) of conidia at a magnification of 40X.

Color and texture of the colony

Twenty isolates of *M. oryzae* that were isolated from leaf blast samples collected from different rice growing areas of Telangana and Andhra Pradesh were categorized based on colony color such as grey and greyish white, based on the texture of the colony as smooth and rough (Table 1). Among the 20 isolates, maximum number of eleven isolates have shown greyish white colonies with rough texture. This was followed by greyish white colonies with smooth texture

Table 1. Morphological characterization of various *Magnaporthe oryzae* (rice blast) isolates obtained from certain districts of Telangana and Andhra Pradesh during *kharif* 2014. Radial growth of rice blast isolates was recorded at 15 days after incubation on OMA at 28°C. Sporulation index (on 0–4 scale) was determined after 15 days after incubation in petri dishes containing OMA incubated at 28°C where in Excellent (4) = > 30 ; Good (3) = 20–30 ; Fair (2) = 10–20 ; Poor (1) = <10 and Nil (0) = 0 of the number of spores per microscopic field. Figures in the parentheses are angular transformed values.

Isolates	Radial growth of <i>M. oryzae</i> isolate (mm)		Conidia size (µm) (40x)		Color of the mycelium	Texture of the mycelium	Sporulation index
			Range	Average			
MG1	75.00	(59.97)	9-10 × 3-4	9.54 × 3.5	Greyish white	Smooth	3
MG2	78.33	(62.23)	8-11 × 3-4	9 × 3.2	Greyish white	Smooth	3
MG3	73.67	(59.10)	8-10 × 3-4	8.6 × 3.5	Greyish white	Rough	3
MG4	80.00	(63.41)	8-12 × 3-4	9.64 × 3.5	Greyish white	Smooth	4
MG5	75.67	(60.42)	8-10 × 3-4	8.96 × 3.4	Greyish white	Rough	3
MG6	74.67	(59.75)	9-10 × 3-4	9.4 × 3.4	Greyish white	Smooth	3
MG7	72.33	(58.24)	10-12 × 3-4	10.8 × 3.8	Greyish white	Rough	3
MG8	76.67	(61.09)	8-12 × 3-4	9.7 × 3.6	Greyish white	Smooth	4
MG9	74.67	(59.75)	8-12 × 3-4	10.2 × 3.5	Greyish white	Rough	2
MG10	80.67	(63.89)	8-10 × 3-4	8.8 × 3.3	Greyish white	Smooth	2
MG11	87.67	(69.41)	8-10 × 3-4	9 × 3.5	Greyish white	Smooth	4
MG12	80.33	(63.65)	8-12 × 3	11.8 × 3	Greyish white	Rough	3
MG13	81.33	(64.37)	9-10 × 3-4	9.5 × 3.6	Greyish white	Rough	3
MG14	76.67	(61.09)	8-10 × 3	9.3 × 3	Greyish white	Rough	3
MG15	75.00	(59.97)	8-10 × 3	8.9 × 3	Greyish white	Smooth	2
MG16	74.67	(59.75)	9-10 × 3	9.4 × 3	Greyish white	Smooth	4
MG17	78.67	(62.46)	9-12 × 3-4	10.3 × 3.6	Greyish white	Rough	2
MG18	71.67	(57.81)	8-10 × 3-4	9.1 × 3.6	Greyish white	Rough	3
MG19	75.67	(60.42)	8-9 × 3	8.6 × 3	Greyish white	Rough	3
MG20	79.67	(63.17)	8-10 × 3-4	9.2 × 3.8	Greyish white	Rough	1
Mean	77.15						
SE (m)	0.30						
CD	0.84						

category wherein six *M. oryzae* isolates (MG1, MG2, MG4, MG6, MG10 and MG15) were present. The remaining three isolates such as MG8, MG11 and MG16 were found to exhibit grey colony color with smooth texture. Absolutely none of the isolates of *M. oryzae* showed grey colonies with rough texture.

Radial growth, conidial size, shape and sporulation of M. oryzae isolates

Studies on the morphological characteristics such as radial growth, conidial size and conidial production of various *M. oryzae* isolates indicated that the radial growth as recorded at 15 days after incubation varied from 71.67 (MG18) to 87.67 mm (MG11) with a mean of 77.15 mm (Table 2). Highest radial growth at 15 days after incubation was exhibited by MG11 (87.67 mm) isolate and was significantly superior over others.

This was followed by MG13 with 81.33 mm of radial growth, MG10 (80.67 mm), MG12 (80.33 mm), MG4

Table 2. Distribution of rice blast isolates into different clusters based on MGM marker analysis.

Cluster number	Sub cluster	Number of isolates	Isolate clustered
I.	Ia	4	MG1, MG2, MG3, MG5
	Ib	7	MG7, MG11, MG14, MG15, MG16, MG18, MG19
II.	IIa	5	MG4, MG9, MG10, MG17, MG20
	IIb	2	MG6, MG8
III.		2	MG12, MG13

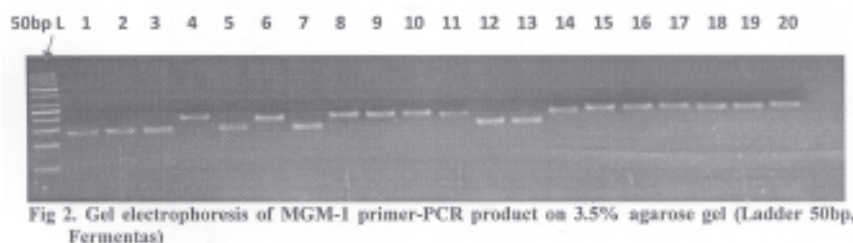


Fig 2. Gel electrophoresis of MGM-1 primer-PCR product on 3.5% agarose gel (Ladder 50bp, Fermentas)

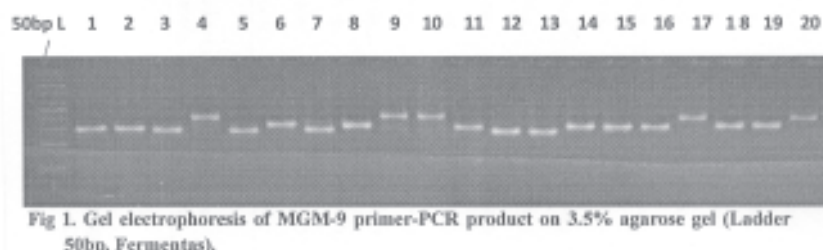


Fig 1. Gel electrophoresis of MGM-9 primer-PCR product on 3.5% agarose gel (Ladder 50bp, Fermentas).

Fig. 1. Gel electrophoresis of MGM-9 primer-PCR product on 3.5% agarose gel (Ladder 50bp, Fermentas). **Fig. 2.** Gel electrophoresis of MGM-1 primer-PCR product on 3.5% agarose gel (Ladder 50bp, Fermentas).

(80.00 mm) and MG20 (79.67 mm), with non-significant differences among them. For other isolates, lowest radial growth ranged from 71.67 mm (MG18, with least radial growth rate) to 78.33 mm (MG2).

The details of the conidial sizes of different *M. oryzae* isolates are given in Table 1. The mean conidial size ranged from 8.6 (MG19) to 11.8 μ m (MG12) in length and 3 (MG12, MG14, MG15, MG16 and MG19) to 3.8 μ m (MG7 and MG20) in width. Conidial size was apparently highest for MG7 isolate (10.8 \times 3.8 μ m). The conidia was 2 septate, 3 celled and shape of the conidia of all the isolates was pyriform.

Further, the sporulation of isolates as measured at 15 days after incubation indicated that 11 isolates (MG1, MG2, MG3, MG5, MG6, MG7, MG12, MG13, MG14, MG18 and MG19) exhibited good sporulation, with sporulation index of 3. Only four *M. oryzae* isolates viz., MG4, MG8, MG11 and MG16 exhibited excellent sporulation index (rating of 4). Further, the four isolates, MG9, MG10, MG15 and MG17 have exhibited fair sporulation index with a scale of 2. Only one out of the 20 isolates, MG20 exhibited poor sporulation index with a scale of 1 (Table 1).

The size and shape of the spores are important criteria for classification and identification of *Magnaporthe* species. The results of the present investigation indicate existence of variation in terms of mycelial color, texture, radial growth, size and sporulation among the isolates.

Polymorphic markers among the M. oryzae isolates

The breakdown of resistance in many released varieties was attributed to the high variability of the pathogen. Therefore, knowledge on population diversity would be useful for developing strategies to increase the durability of resistance. The aim of current study is to assess the diversity of the blast pathogen using *Magnaporthe grisea* Microsatellite Markers (MGM) and Pot2 primers.

Twenty *M. oryzae* isolates from various regions in Telangana and Andhra Pradesh were selected for the present study to gain an insight into the genetic diversity of the fungal population. A set of 10 *Magnaporthe grisea* Microsatellite (MGM) markers and a repeat element specific primer Pot2 were used in the present study. Of these only 3 (27%) MGM

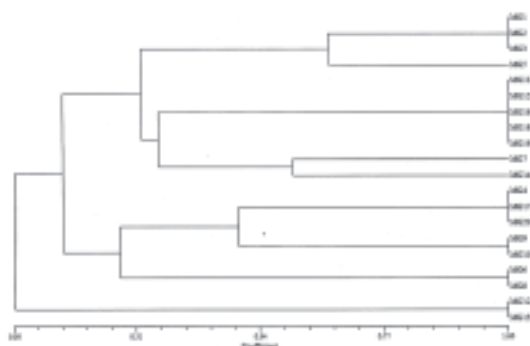


Fig. 3. Dendrogram constructed from MGM primers based fingerprint data from 20 rice infecting *Magnaporthe oryzae* isolates from Telangana and Andhra Pradesh. (MG1-Wyra, MG2-Medak, MG3-Atmakur, MG-4 Bapatla, MG5-Khammam, MG6-Nalgonda, MG7-Adilabad, MG8-Mahabubnagar, MG 9-Nandyal, MG10-Mahanandi, MG11-IIRR, MG-12-Nellore, MG-13-Marteru, MG15-Palem, MG16-Madhira, MG17-Aleru, MG18-Warangal, MG19-Piduguralla and MG20-Ragolu).

markers showed polymorphism among *M. oryzae* isolates. The genomic DNAs of only four *M. oryzae* isolates were amplified with Pot2 primer, eliminated from the analysis. Three MGM markers viz., MGM-1, MGM-9 and MGM -21 showed consistent banding pattern.

Allelic richness and diversity in M. oryzae

The MGM markers gave a low number of distinct scorable fragments per primer. All the bands that could be reliably read within the size range of 100–500 bp were treated as individual loci. A total of 7 amplicons were obtained from 20 *M. oryzae* isolates with 3 MGM markers. The number of loci amplified by each primer pair ranged from 2 to 4. The maximum number of fragments (4) was amplified by the marker MGM-9 (Fig. 1), whereas minimum number of fragments (2) was amplified by MGM-1 (Fig. 2). The PIC value varied from 0.46 to 0.6. The isolates showed polymorphism with two markers (MGM-1 and MGM-9) and monomorphism with one marker (MGM-21).

The polymorphic MGM markers in the present study showed 2 to 4 alleles. Variation in allele number in the present study could be due to the population size. In contrast to the present study, Mohan et al. (7)

reported that 6–8 alleles with an average of 7.8 alleles per locus. The difference in the number of alleles detected in *M. oryzae* isolates was significant and could be related to the sampling strategy used to recover isolates in these areas.

The PIC value for *M. oryzae* populations were 0.46 for MGM-1 and 0.60 for MGM-9. Nevertheless, the reported PIC values for these MGM primer pairs may be useful for selecting comparatively more informative markers in future for assessment of molecular diversity of *M. oryzae* isolates from Telangana, Andhra Pradesh or elsewhere.

Genetic variability among the isolates

In the present study, the DNA polymorphism did not reflect the geographical distribution of isolates. The MGM primers scores were used to create a data matrix to analyse genetic relationships using the NTSYS-pc software program version 2.02. Dendrogram constructed based on Jaccards similarity coefficient using the marker data from *M. oryzae* isolates with UPGMA analysis separated into clusters. Cluster analysis of the blast isolates revealed the average pair wise similarities in the range of 0.08 to 1.0 suggesting large variations among the isolates of the present study. All the isolates were grouped into three major groups (Table 2). Cluster-I consists of 11 isolates, which were further divided into two sub clusters (IA and IB). The sub cluster IA consists of four isolates in that, two isolates (MG1 and MG5) were collected from Khammam, one isolate (MG2) from Medak and one isolate (MG3) from Atmakur. All the isolates of this sub cluster shares 65% similarity. Whereas, sub cluster IB is comprised of seven isolates (MG7, MG11, MG14, MG15, MG16, MG18 and MG19) collected from Adilabad, IIRR (Rajendranagar), Nizamabad, Palem, Madhira, Warangal and Piduguralla. All the isolates of this sub cluster shares 35% similarity (Fig. 3).

Cluster-II consists of 7 isolates, which were further divided into two sub clusters (II A and II B). The sub cluster II A consists of five isolates in which one each from Bapatla / Guntur (MG4), Nandyal (MG 9), Mahanandi (MG10), Aleru / Nalgonda (MG 17), Ragolu / Srikakulam (MG20). All the isolates of this

sub cluster share 52% similarity. Whereas, sub cluster II B is comprised of 2 isolates, MG6 from Nalgonda and MG8 from Mahbubnagar. The two isolates of this sub cluster shares 28% similarity. Cluster-III consists of 2 isolates, MG12 from Nellore and MG14 from Maruteru sharing 100% similarity (Fig. 3).

High degree of variation was observed within the isolates from different locations. Several clusters of the rice blast isolates were observed in the dendrogram depicting a genetic variation among the isolates.

Variability studies among plant pathogens is an important aspect for determining the virulence and disease severity along the transect of specific crop growing areas globally. In our studies, blast pathogenic isolates showed variability both in terms of morphology as well as through genetic studies. Several researchers have determined variability among *M. oryzae* isolates at different rice growing areas. Indeed these studies enable to determine and predict the extent of damage by blast pathogenic isolates based on the presence of virulent isolates.

These studies along with determining the virulence / avirulence genes associated with specific isolates at specific crop locations would further determine the selection pressure through which new blast pathogenic genotypes would evolve in course of time. In our studies, though the 20 isolates exhibited wide variability morphologically, comparatively, not that much of variation was noticed through molecular based detection methods. Earlier attempts by several researchers also witnessed both morphological and genetic variations among *M. oryzae* isolates. For example, Srivastava et al. [8] reported that the colony color of the *Magnaporthe oryzae* varies from buff color to black color with smooth and rough colony margin. However, no variation was observed with respect to conidial shape. The conidia are pyriform, almost hyaline to pale olive, 2-septate and 3-celled. Meena, [9] reported that the colony color of rice blast (*Pyricularia grisea*) isolates was usually buff with good growth on oat meal agar, greyish black with medium growth on host seed extract + 2% sucrose agar. Further, raised mycelial growth with smooth

colony margin was observed on potato dextrose agar, whereas raised mycelium with concentric ring pattern was noticed on Richards agar medium.

Genetic variability in rice blast pathogenic isolates was also determined by several researchers. For example, cluster analysis were made by Mohan et al. [7] who observed great extent of variation among the isolates collected from different endemic areas. Isolates collected from coastal Andhra Pradesh (Maruteru and Nellore) shares the high similarity of 64% with Assam isolates. Chadha and Krishna [10] reported high similarities between the isolates collected from various endemic areas of India like Uttaranchal, Himachal Pradesh than those of Madhya Pradesh and Karnataka using RAPD markers contributing to the possibility of seedborne transmission of the pathogen. Therefore it is expected that, the migration of the pathogen to a newer location operates.

Investigations on variability often form a basis to formulate area-wise blast management strategies. Overall, our studies in the long run would enable to emphasize the need to evolve blast management practices that are viable in the long run in a sustainable way and of course when these studies are accompanied with determining the virulence genes. Research on studying variability in blast pathogens through mapping of avirulence / virulence genes is also available. However, much attention is required to investigate upon the prevalence of these specific genes among different isolates and their correlation to manifest upon the symptoms on rice cultivar at field level. The research objectives henceforth, keeping the long term goals of sustainable management of blast should involve studying parameters of virulence, variability simultaneously on resistant, tolerant cultivars of rice and of course on popularly grown and ruling rice cultivars. This will enable advocating cost-effective control strategies to blast to the farmers in general and in South India specifically as in this case.

Conclusion

Rice blast isolates were studied to understand its diversity and characterization. We conclude that *M. oryzae* isolates from various regions of South India

consists of variable populations based on morphological and MGM marker analysis. Molecular phylogenetic grouping obtained by MGM marker analysis did not correlate with morphological characteristics. Genetic diversity of *M. oryzae* isolates from different locations using three MGM markers showed less polymorphism at DNA level. Information on variability of pathogen will be useful in devising location specific disease management strategies.

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