GENETIC DIVERSITY AMONG FUNGICIDES RESISTANCE RICE BLAST PATHOGEN (*MAGNAPORTHE ORYZAE*) STRAINS ISOLATED FROM FARMERS' FIELDS IN NIGER STATE NIGERIA

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ABSTRACT

Rice blast caused by fungus *Magnaporthe oryzae* is one of the major fungal diseases affecting rice (*Oryz sativa*) cultivation. The resistance of this fungus to fungicide is imperative as the presence of fungicid resistant strains (biotypes) has been associated with control failure; therefore managing resistance is critic to disease control. This study was carried out to evaluate the genetic diversity among the strains of *M oryza* in relation to their fungicide resistance found in rice fields in Niger State. The blast infested leaves, stems an panicles of rice plants were collected in November, 2015 from five farmers' field located in Gbako, Katch and Lavun Local Government Areas in Niger state, Nigeria. Isolation of the pathogen was carried out o Potato Dextrose Agar (PDA). A total of ten *M. oryzae* strains designated as MOR001- 0010 were isolate from all the samples collected and subjected to two fungicides (Mancozeb and Benomyl). The strains wer analyzed using random amplified polymorphic DNA (RAPD) PCR. The results showed three distinct group among the *M. oryzae* isolates. MOR010 is genetically different from other isolates. MOR004 showe relatedness to MOR006 genome-wise than to MOR009 and MOR005. MOR004, MOR006, MOR005 an MOR009 were found to have common recent gene. MOR009 showed similarity with MOR005. MOR008 an MOR001 were genome wise related. Both group 2 (MOR004, MOR006, MOR005 and MOR009) and grou 3 (MOR001 and MOR008) have the same common late accessory different from accessory of group (MOR010). However MOR002, MOR003 and MOR007 that did not show any band do not amplify with al the primers screened.The results revealed that among the three groups, only group 2 was found to contain th two isolates (MOR004 and MOR005) that were completely resistant to both fungicides used in this study. This information could be useful in rice breeding programs aiming at developing a lineage exclusion method in breeding for durable fungicide-blast-resistant relationship in rice cultivars.

Keywords: blast, genetic diversity, rice, *Magnaporthe oryzae*, accessory, genome

INTRODUCTION

In major rice growing areas, yield loss due to disease is one of the most important biotic factor that hindered production goals. Among these diseases, rice blast caused by *Magnaporthe oryzae* remains a particular threat because of its unpredictable outbreaks as well as resistance to fungicidal effects. Rice blast is an infectious fungal disease which is distributed worldwide and prevailing in more than 85 countries of the world (Scardaci *et al.*, 1997; Jamal-U-deen *et al.*, 2012). It is the most important disease of rice worldwide that threatens food security. Rice blast is one of the most serious and widespread constraint of rice cultivation in West Africa (Akator *et al.*, 2013). In Nigeria, Rice blast pathogen (*Magnaporthe oryzae)* remains the most devastating agent causing serious damage to upland rice. *Magnaporthe oryzae* (syn *Pyricularia oryzae*) the rice blast pathogen, is a filamentous, haploid heterothallic fungus belonging to the phylum Ascomycota (Gilbert *et al.*, 2004).

The fungus *M. oryzae* attacks at all stages of the rice plant and symptoms appears on leaves, nodes, neck and panicle (Ghazanfar *et al.*, 2009; Seebold *et al.*, 2004). Heavy yield losses have been reported in many rice growing countries like India (75%), Phillipines(50%) and Nigeria(40%) as a result of the pathogen (Ghazanfar *et al.*, 2009).It is estimated that about 14-18% yield reduction was caused by these disease worldwide (Mew and Gonzales, 2002; Jamal-u-deen Hajano *et al.*, 2012). Blast occurs in upland and rainfed rice ecology because water deficiency predisposes the rice field to severe infection in all environments. Control of fungal disease through chemotherapy is receiving much attention as a result of its efficacy and non having any deleterious effect on the vegetation and other component of the ecosystem (Ibiam *et al.*, 2008). In the past, several fungicides have been employed in the control of fungal disease of rice and other crops.

however new and potentially virulent species are being discovered every day (Ibiam *et al.*, 2008) as a result of their resistance.

Fungicide resistance is a stable, inheritable adjustment by fungus to a fungicide, resulting in reduced effectiveness of the fungicides. In Nigeria, yield loss due to rice blast caused by *Magnaporthe oryzae* remains a particular threat because of its unpredictable outbreaks as well as resistance to fungicidal effects. The presences of different strains of this pathogen leading to frequent resistant to fungicides, has called for a great concern on the biology of *M. oryzae*. However, the information available about the different strains of the pathogen in the study area is not adequate. Therefore, this study evaluated the genetic diversity among the strains of *M oryzae* found in rice fields in Niger State in relation to their fungicide resistance using random amplified polymorphic DNA (RAPD) PCR.

MATERIALS AND METHODS

Collection of Materials

Blast infested rice leaf, stem and panicle samples were collected in polythene bags from five farmer's field in Edozhigi, Chanchaga, Kataeregi, Busu and Agaie across Gbako, Katcha and Lavun Local Governments in Niger State, Nigeria. The samples were taken to the Department of Biological Science laboratory (FUT) Minna, Nigeria for analysis. Mancozeb (Dithane M45) and Benomyl (Benlate WP 50) were purchased from Agrochemical shops in Minna

Isolation of Pathogen

Infected plant parts were sterilized in mercuric chloride (0.01%) and 5 discs taken from the periphery of necrotic region were placed on Potato Dextrose Agar (PDA), to which streptomycin ($1mlL^{-1}$) has been added and incubated at $28\pm2^{o}C$ for 3 days. A Single conidium was picked up with a sterile needle under microscopic observation, transferred individually to PDA plates and incubated at ambient temperature (Gomathinayagam *et al.*, 2011). The monoculture was prepared and stored on PDA slants at $4^{o}C$. Subculture was made at regular intervals. The fungal isolates were identified using the fungal family of the world mycological monograph (Cannon and Kirk, 2007; Adebola and Amadi, 2008) under microscopic observation

Pathogenicity Test

The rice seed variety (FARO 52) that was used for pathogenicity test was collected from National Cereal Research Institute (NCRI) Badeggi, Niger State, Nigeria. Pathogenicity of *M. oryzae* was tested on healthy forty five days old FARO 52 rice plants grown in the greenhouse located in the Department of Biological sciences, Federal University of Technology, Minna (F.U.T.) garden. The rice plants were sprayed with mycelial suspension of the strains of pathogen (*M. oryzae*) (10ml/pot) by means of automizer. The plants were covered with individual polythene bags to provide adequate humidity. The inoculated plants were observed after 7 days for characteristic symptoms of blast (Subramanian *et al.*, 2013).

Evaluation of Genetic diversity of Fungicides Resistance and Sensitive *M. oryzae* strains using RAPD marker

DNA Extraction

The DNA of the *Magnaporthe oryzae* fungicides resistant and sensitive strains were extracted using protocol described by Wang *et al.* (2011)

The test fungal *Magnaporthe oryzae* was grown in potato dextrose broth on a shaker and 1ml of the culture suspension was transfer into a sterile crysogenic storage tube containing 200 ul of sterile glycerol and store at -70°C. The fungal tissues (~2.5 mg) from the isolates were inoculated into a sterile 1.5ml micro centrifuge tube and 400ul of API buffer was added (DNeasy Tissue kit). Freeze/thaw cycle was applied to lyse the fungal cells using crushed ice/ethanol and a boiling water bath; the cycle was repeated seven times and was also boiled in a water bath after the last cycle of freeze/thaw cycle. 1ml micropipette tip was used to grind any visible tissue in the tubes for 5secs. The tubes were centrifuge at 10,000 rpm for 10 minutes at 4°C and the supernatant was collected in 1.5 ml centrifuge tube. Fifty (50) µl of TE buffer was added to dissolve the DNA. The DNA was eluted in 50 µl buffer and was stored at −20°C for PCR amplification.

The DNA was quantified using Nanodrop Spectrophotometer and diluted to 10µl for Polymorphic Chain Reaction (PCR) analysis. The samples were screen with six (6) primers; ORY 1; 5'-CAATCGCCGT-3', ORY2; 5'-CAGGCCCTTC-3', ORY 3; 5'-AGTCAGCCAC-3', ORY4; 5'-GGTGACGCAG-3',ORY 5; 5'-TGGATTGGTC-3', ORY 6 5'-TACAACGAGG-3'RAPD markers (Laura *et al.*, 2015).

Polymerase Chain Reaction (PCR Amplification)

PCR reaction mixture contained mastermix (containing PCR buffer 1X, $MgCl_2$ 2.5Mm, dNTPs 0.2Mm, Taq DNA polymerase1U), primer 0.6uM and15ng of DNA template. The PCR reaction was carried out using Applied Biosystem PCR machine. The PCR machine was programmed to include initial denaturation at 94°C for 3min followed by denaturation at 94°C for 1min, annealing at 40°C for 2min and extension at 72°C for 2min and these steps were repeated for 45 cycles with a final extension at 72°C for 5 min and hold at 4°C. The PCR products were resolved using 1.2% agarose gel in 0.5 X TBE buffer containing ethidium bromides (5 µg/ml of gel). The gel was visualized on a UV transilluminator. The RAPD products bands were scored as presence (1) and absence (0) to generate a binary matrix for analysis (Bayraktar *et al.*, 2007). The binary band data matrix, from molecular analysis, was used to construct dendrogram to depict molecular phylogenetic relationship among the strains

RESULTS

Isolated Pathogens

The results from five sample collection sites (Edozhigi, Badeggi, Kataeregi, Bussu and Agaie farmer's field) and the number of *M. oryzae* pathogens strains are presented in Table 1. A total of ten *M. oryzae* strains were isolated from all the samples collected. The colonies were identified based on colour, morphology and shape on PDA

Table1. 1; Number of *M. oryzae* strains isolated

S/N	Strains code*	Locality/collection site
1	MOR 001	Edozhigi
2	MOR 002	Edozhigi
3	MOR 003	Chanchaga
4	MOR 004	Chanchaga
5	MOR 005	Chanchaga
6	MOR 006	Kataeregi
7	MOR 007	Busu
8	MOR 008	Busu
9	MOR 009	Busu
10	MOR 010	Agaie

Key; MOR- *Magnoporthe oryzae* strains from rice

The microscopic features of the isolated fungi showed that in all the ten isolates, the shape of the conidia was typically pyriform with rounded base, narrowed apex and 2-3 septate (Plate I). The mycelia were highly branched, septate, superficial, bearing conidia at the tip and bunch at the side of the conidiophores (Plate II). The conidiophores of the isolates were slender, straight grayish white to grayish black, smooth bearing clusters of conidia which are typically of pyriform and 2-3 septate.

Pathogenicity Test

The result of the symptoms (the appearance of small spot and later enlarged as either roundish or slightly elongated necrotic sporulating spots on leaves which later developed into narrow or slightly elliptical lesions of more than 3 mm long with a brown margin) observed upon inoculation of *M. oryzae* on the rice phylosphere in the green house and their re-isolation confirmed *M. oryzae* as the causal organism of rice blast. It is therefore pathogenic on rice plant.

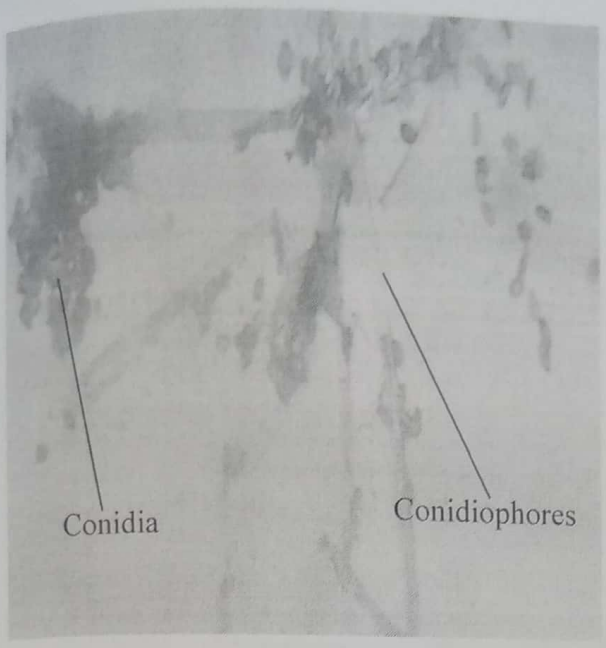

Plate I: Conidia attachec to conidiophores

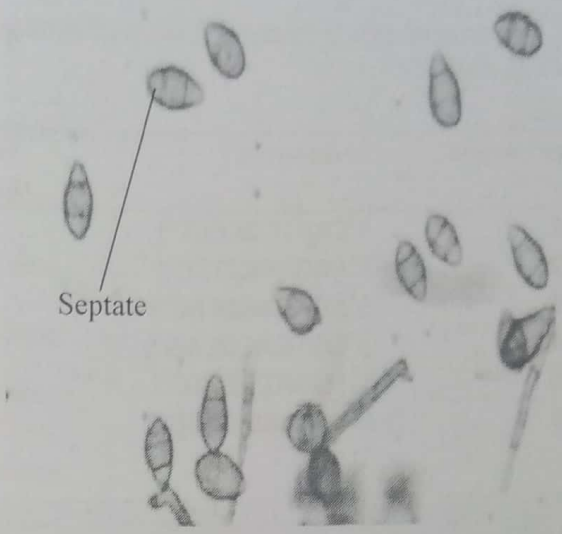

Plate II: Conidia showing septate

Figure 1 showed that isolates "MOR004" and "MOR005" were resistance to both fungicides. Isolates "MOR001", "MOR002", "MOR004", "MOR005" and "MOR008" were resistance to Mancozeb while isolates "MOR004", "MOR005" and "MOR010" were resistance to Benomyl, however MOR003, MOR006, MOR007 and MOR009 are susceptible to both fungicides

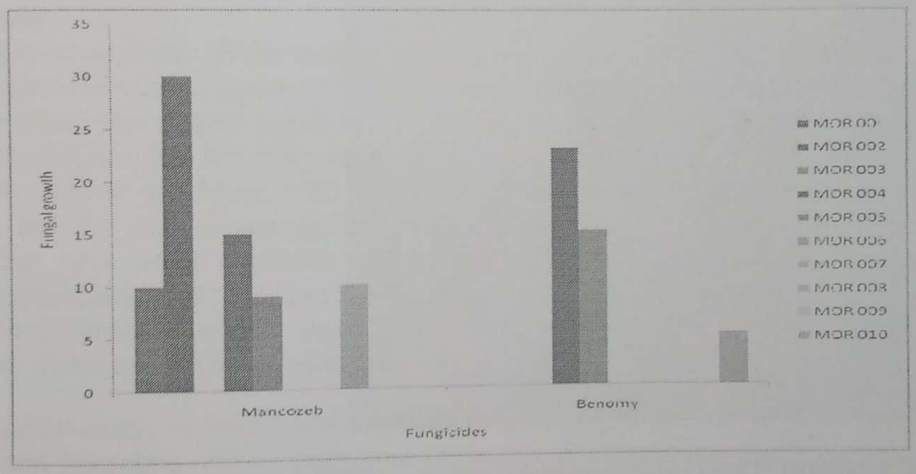

Fig 1; *M. oryzae* growth on medium amended with fungicides

Genetic diversity of Isolated Pathogen using PCR-RAPD marker

The PCR amplifications were performed with 6 RAPD primers to access the level of polymorphism in the ten (10) isolates of *M. oryzae*. Polymorphism of all the RAPD primers was 100% (Table 2). The band score ranged from 1 (ORY 5) to 3 (ORY 1, 2, 3, and 4) and 9 (ORY 6). One primer (ORY 6) out of the 6 primers tested showed highest polymorphic band among the individual isolates (Table2). The amplification reactions with the primer (ORY 6) generated 9 polymorphic bands with size ranging between 200 and 1000 base pairs. Each individual was identified based on the distinct amplified DNA band pattern obtained (Plate III). For instance, isolate MOR006 gave unique bands when its DNA was amplified with all the primer used, suggested that these bands could be used to characterize and identify it.

In the present study, six (6) RAPD markers were used to construct phylogenetic relationships among the *M. oryzae* isolates. Three (3) major groups and 3 sub groups at 50% similarity coefficients were obtained, while

all the isolates were distinct at 70% similarity coefficients (Fig II). Group 2 was the largest group representing 57.14% of the isolates analyzed; group 1 and group 3 belong to 28.57 and 14.28% respectively.

Table 2; Oligonucleotide primers that showed genetic discrimination among the *M. oryzae* isolates using RAPD-PCR analysis

Primer names	Sequences	Number of Bands	Total Bands	% Polymorphism
ORY 1	5'**CAATCGCCCT**'3	3	3	100
ORY 2	5'**CAGGCCCTTC**'3	3	3	100
ORY 3	5'**AGTCAGCCAC**'3	3	3	100
ORY 4	5'**GGTGACGCAG**'3	3	3	100
ORY 5	5'**TGGATTGGTC**'3	1	1	100
ORY 6	5'**TACAACGAGG**'3	9	9	100

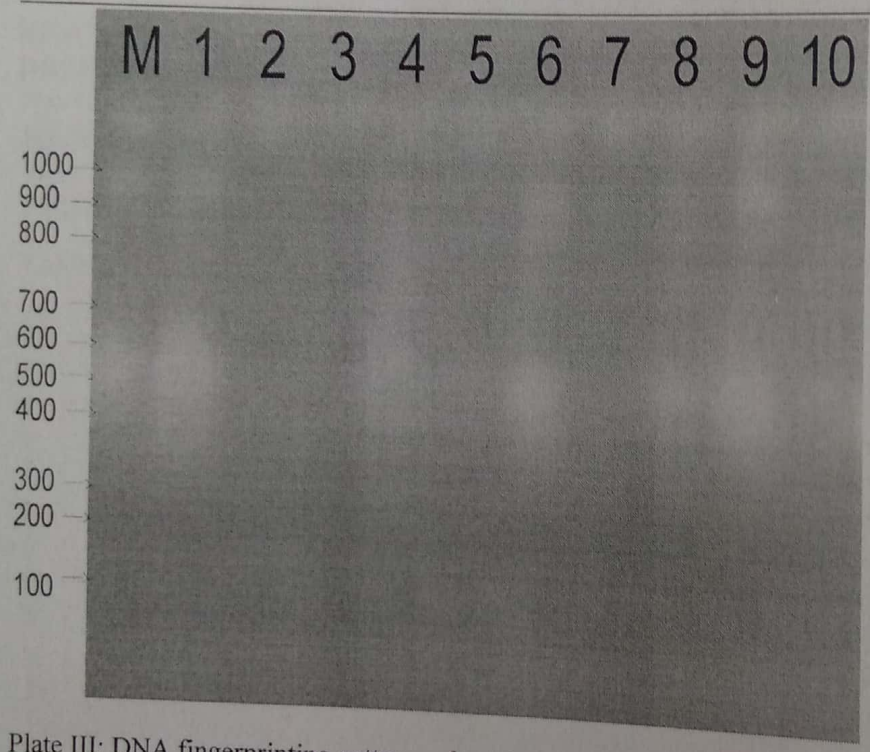

Plate III: DNA fingerprinting patterns of ten (10) *M. oryzae* isol ates using ORY 6 primer

DNA Fingerprint using RAPD Amplification

The results showed three distinct groups among the *M. oryzae* isolats. Group1 contains just one isolate which is MOR010. Group 2 has 2 sub-groups containing two isolates each; MOR009 and MOR005 in one class, and MOR006 and MOR004 in another one class. Group 3 also contains 2 sub-groups with one isolate each namely MOR008 and MOR001.

The result showed that MOR010 is genetically different from other isolates. MOR004 showed relatedness to MOR006 genome-wise than to MOR009 and MOR005 though, they (MOR004, MOR006, MOR005 and MOR009) have common recent gene. MOR009 shows similarity with MOR005. MOR008 and MOR001 are genome wise related. Both group 2 (MOR004, MOR006, MOR005 and MOR009) and group 3 (MOR001 and MOR008) have the same common late accessory different from accessory of group 1 (MOR010). However MOR002, MOR003 and MOR007 that did not show any band do not amplify with the primers screened.

The results revealed that among the three groups, only group 2 was found to contain the two isolates (MOR004 and MOR005) that are completely resistant to both fungicides used in this study.

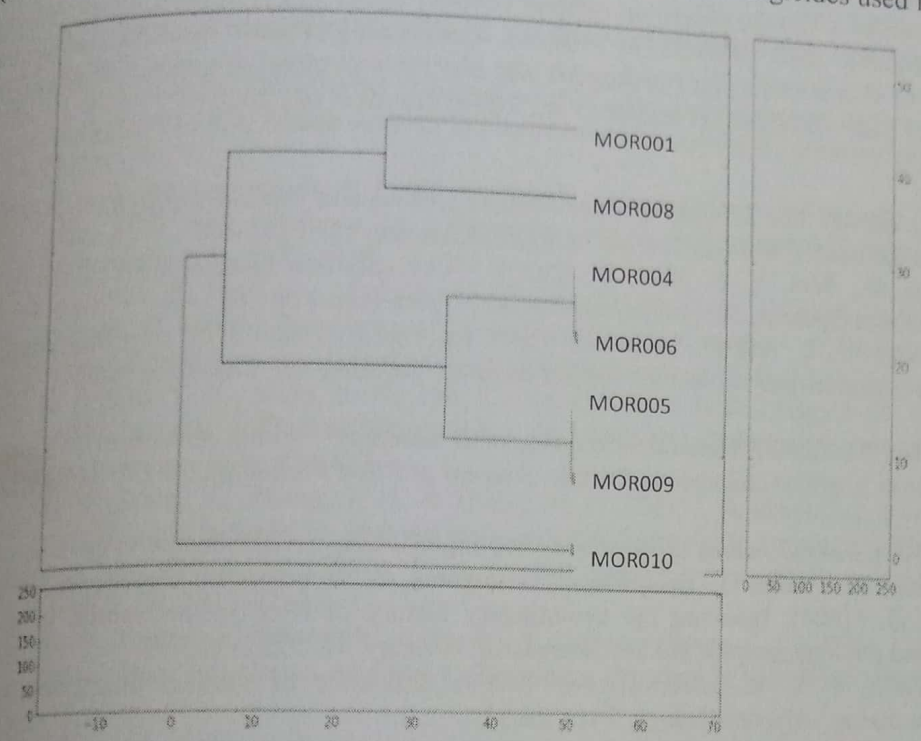

Fig 2: Dendrogram showing the relationship between the *M. oryzae* isolates derived from cluster analysis of the RAPD profile

Discussion

The result revealed that all the isolates that were resistance to both fungicide and either of the fungicide was related genomic wise as isolates "MOR004 and MOR005" were grouped together because of their related genome which was a likely possibility for their resistance to both fungicides. The high distinction pattern of each isolate obtained in this study suggests possible and frequent occurrence of mutants in *M. oryzae* (Levy *et al.*, 1991; Klister and Miao, 1992). The limited number of morphological and cultural characters of *M. oryzae*, and the lack of standardization of cultural conditions and virulence tests among the different researchers have led to confusion and uncertainty in the characterization of this pathogen (Babujee and Gnanamanickham, 2000). Distinct phenotypes usually consist of isolates that are genetically less related and such identification of isolates using cultural and morphological techniques often lacks consistency and precision (Babujee and Gnanamanickham, 2000).

In the current study, we have found that identification of genetic diversity in *M. oryzae* depends on resistance to different fungicides. For instance, four (4) isolate genotyped as group 2 were originated from 3 different sample collection sites but two of the isolates (MOR004 and MOR005) that was resistance to both fungicides used were isolated from the same site (Chanchaga), probably because there has been continuous application of this two fungicides in that collection site and the pathogen has develop resistance over time. The result is supported by the report of Sere *et al.* (2007), Singh and Kumar (2010) which reported separately that the high distinction pattern of each isolate obtained in their study suggests possible and frequent occurrence of mutants in *M. oryzae* in different host cells. The results obtained confirmed the genetic diversity and virulence complexity of rice blast fungus among samples. Globally, random amplified polymorphic DNA (RAPD) markers have been reported to be useful in identification (Singh and Katoch, 2008; Khan *et al.*, 2010; Kumar *et al.*, 2010; Wang *et al.*, 2010) and analysis of genetic divergence (Sere *et al.*, 2007; Malode *et al.*, 2010)

RAPD markers indicated possible relationship between, host origin, mutation and genetic variation among *M. oryzae* isolates and this demonstrated the finger prints and diagnostic potential of RAPD. Obviously, for these DNA band patterns to have practical meaning in the areas of plant pathology, population biology and

molecular epidemiology, specific DNA bands must be related to host origins, mutation, virulence and fungicide resistance genes (Welsh and McClelland, 1990). This information could be useful in rice breeding programs aiming at development of a lineage exclusion method (Zeigler *et al.*, 1994) in breeding for durable fungicide-blast-resistant relationship rice cultivars to different rice ecologies and localities. Molecular markers are used extensively to characterize plant pathogens and elucidate population genetic structure and the evolutionary relationship of plant pathogens (Fargette *et al.*, 2004; Traoré *et al.*, 2005).

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