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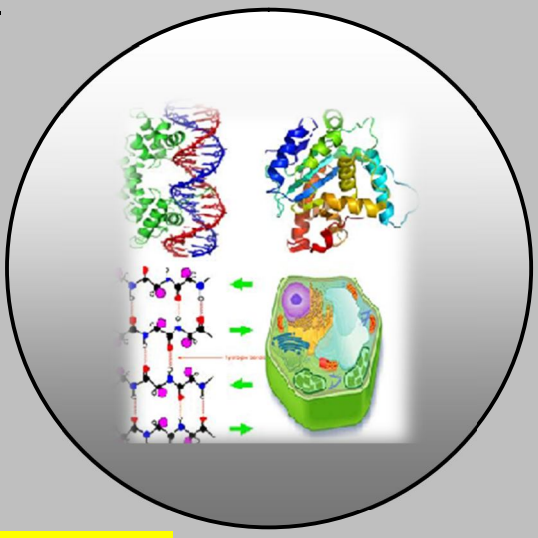

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REVIEW ARTICLE

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Molecular Biodiversity in Phytopathogenic Fungi, Pyricularia Spp.

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ABSTRACT

Pyricularia grisea is the causal pathogen of rice blast disease. Variations among P. grisea races are causing serious concern during screening blast resistant varieties. Therefore, study on genetic diversity of the pathogen can provide information on its behavior in field and can also bring new strategies to breeders. This study was done using random amplified polymorphic DNA markers to find out genetic diversity in P. grisea isolates collected from farmer's fields in Northeast India. 28S rRNA gene sequences of the isolates were compared with other related taxa retrieved from Gene Bank database. The results obtained confirmed the genetic diversity of rice blast fungus in Northeast India. From phylogenetic evidence gathered in this study it is concluded that all isolates represent a novel species of the genus Pyricularia, for which the name Pyricularia grisea is proposed. Our results confirmed that RAPD offers an inexpensive and speedy means of generating markers for analyzing the population structure of the blast fungus.
Key words: Pyricularia grisea, Rice, RAPD markers, Polymorphism, Gene Bank.

INTRODUCTION

Pyricularia grisea (Cooke) Sacc. [teleomorph: *Magnaporthe grisea* (Herbert) Barr] (Rossman et al. 1990 and Couch and Kohn, 2002) is the causal pathogen of rice blast disease and is the most important disease of rice and cause severe losses in most rice growing areas.

The fungus produces spots or lesions on leaves, nodes, panicles, and collar of the flag leaves. Leaf lesions range from somewhat diamond to elongate shape with tapered, pointed ends. The centre of the spot is usually gray with brown or reddish-brown margin. Blast constitutes one of the main constraints to intensification for increasing rice production. The use of resistant varieties is the most economical and effective way of controlling rice blast. Unfortunately, the causal fungus is able to overcome this resistance within two to three years after these plants are cultivated widely (Babujee and Gnanamanickham, 2000). The breakdown in resistance has been attributed to the high variability of the pathogen and there are numerous reports that this diversity may be due to continuous generation of novel pathogenic variation. The analysis of genetic variation in plant pathogen populations is an important pre-requisite for understanding co-evolution in the plant patho system (McDonald et al. 1989). Traditional markers used to estimate genetic variation within *M. grisea* populations include pathogenicity, mating type, auxotrophic mutants, melanin deficiency or drug resistance, and isozyme polymorphism (Leung and Taga, 1988). Information on fungal population diversity is crucial for developing strategies to increase the durability of resistance (Xia et al., 2000).
There is a significant shift in the types of approaches used to identify and characterize plant pathogens with the advent of molecular biology. Advances in molecular biology and genetic modification enable genes and traits to be tracked precisely and via marker based studies. DNA based technologies such as the polymerase chain reaction (PCR) has been the bases for molecular detection in modern plant pathology. Accurate detection, identification and early detection of pathogens is the cornerstone of disease management in many crops. Many plant pathogens are difficult to identify using morphological criteria, which can be time consuming and challenging and requires extensive knowledge in taxonomy. Molecular detection techniques can generate accurate results rapidly enough to be useful for disease management decisions (Levesque 2001). Molecular markers based upon sequence variation have proved extremely effective tools for distinguishing between closely related genotypes, with the advent of PCR technology, methods have been developed that use amplified DNA sequences as molecular markers. These methods require very little template DNA, which can usually be obtained using a simple mini separation protocol. The production of marker bands is very fast and far less labour intensive than when using restriction fragment length polymorphism (RFLP) technology, (Munthali et al. 1992; Williams et al. 1990; Welsh and McClelland 1990; Caetano-Anolles et al. 1991). The utility of proteins, isozymes and nucleic acids (DNA, RNA) as potential markers to define variation in the blast pathogen was explored by many researches (Hunst et al. 1986). Different marker systems such as RFLP, Random Amplified Polymorphic DNA (RAPD), Microsatellite (Simple Sequence Repeats), and Inter Simple Sequence Repeat (ISSR) markers proved to be more informative in understanding population structure of *M. grisea* and other pathogenic fungi (Tenzer et al. 1999 and Sharma et al. 2002).

Genetic analysis of virulence in the rice blast fungus, *M. grisea* was reported by (Leung et al. 1988). In a DNA fingerprinting study carried with a dispersed repeated sequence, pathotype diversity in the rice blast fungus *M. grisea*, was achieved by many workers (Levy et al. 1991and Wilde et al. 1992).

DNA finger prints and pathotypes on international differentials for 151 isolates of rice blast fungus *P. grisea* in Columbia. Using the *P. grisea* repetitive DNA sequence MGR586 identified 115 haplotypes which partitioned into 6 discretely distinct genetic lineages determined by (Levy et al. 1993).

Genetic diversity in *Pyricularia* isolates from various host revealed by polymorphisms of nuclear ribosomal DNA was reported by (Kusaba et al. 1999). The isolates of *P. oryzae* from *O. sativa* and *P. grisea* from 26 other gramineous species were studied in the investigation. The fungal isolates were subjected to RFLP and sequence analyses of rDNA to clarify their genetic relationships.

Based on rDNA-RFLP patterns produced by digestion with 2 restriction enzymes, 71 isolates from 27 plants species were grouped into 13 rDNA types. To estimate their genetic relatedness, a UPGMA dendrogram was constructed based on the rDNA-RFLP profiles. The ITS 2 regions of 13 representative isolates were amplified by PCR and directly sequenced to construct another dendrogram.

A study on regional population diversity of *P. grisea* in Arkansas and the influence of host selection was conducted by (Xia et al. 2000). MGR 586 DNA finger printing was conducted to characterize population diversity of the rice blast pathogen. RFLPs and virulence phenotyping were used to examine genetic diversity of *P. grisea* in Arkansas. A total of 470 monoconidial isolates were recovered from eight rice cultivars in 18 commercial fields in nine countries in Arkansas. Variation in virulence in the rice blast fungus *M. grisea* in Sao Paulo state was reported by (Urashima et al. 2002).

Twenty nine isolates of *M. grisea* collected from different rice growing regions in Thailand and analyzed their genetic diversity (Pimpisitthavorn et al. 2003). Genetic diversity was assessed by RAPD analysis, virulence patterns were determined by spray-inoculating more than 50 rice cultivars carrying different resistant genes, including their rice cultivars, with each isolates. Cluster analysis of molecular band profiles grouped the isolates in five groups sharing 80 percent of a higher percentage of band similarity. The isolates showed a diversity of virulence patterns, and only two of the isolates tested showed an identical pattern. The authors concluded that there was a high degree of pathotype diversity among their isolates of the rice blast pathogen.

Virulence and rep-PCR analysis of *P. grisea* isolates from two Brazilian upland rice cultivars were investigated by (Prabhu et al. 2007). Forty-six isolates of *P. grisea* were collected from cv. Primavera 31 and from cv. Maravilla and studied in the experiment. The rep-PCR fingerprint profiles of the isolates exhibited differences and similarities in banding pattern varying from 4 to 15 fragments of 300 bp to 3.5 kb in length.

The banding pattern of twenty sample isolates from cv. Primavera (27 to 46) was distinctly different from six isolates (47 to 52) retrieved from cv. Maravilha.

DNA SEQUENCE BASED PHYLOGENETIC IDENTIFICATION

Molecular markers may be used for diagnostic or phylogeny. The main difference in diagnostic markers is related to the amount of sample needed, RAPD-PCR based methods requires very limited amount of target DNA samples. But in order to perform a diagnostic test using probes and RFLP analysis it require a higher amount of target DNA samples or it needs to be coupled to PCR, RAPD and RFLP analysis may produce molecular marker, to distinguish inter and intra specific level (Woo *et al*., 1998).

Molecular tools have been successfully used to identify pathogenic strains and in some cases even races of the pathogen. Unfortunately, pathogenicity tests still rely largely on bioassays.

Nucleotide sequences from certain genes reflect phylogeny at various taxonomic levels. Identification of eukaryotic organisms is basically done on sequence information from PCR amplification of internal transcribed spacer (ITS) region from the conserved ribosomal RNA genes (O'Donnell *et al*., 2000).

Kusaba *et al.* (2006) studied genetic characterization and host specificity of *Pyricularia* isolates from annual ryegrass in Japan. Eighty-nine isolates from blast disease of annual ryegrass (*Lolium multiflorum*) in Japan were characterized by DNA analyses and pathogenicity assay. On the basis of sequence variation in the ITS-2 region, isolates from annual ryegrass could not be distinguished from those from t*riticum* and *Elusine*. All of the annual ryegrass isolates were closely related to those from *Elusine* but still distinct from those triticum. The results suggested that blast disease in annual ryegrass is caused by blast fungal isolates that were relative to Elusive isolates but specialized to annual ryegrass in their pathogenicity.

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

RAPD technique based on PCR has been one of the most commonly used molecular techniques to develop DNA marker. RAPD (Williams et al*.* 1990) or the arbitrarily primed PCR (AP-PCR, Welsh and McClelland 1990) use single primers of arbitrary sequence to generate DNA fragments. In RAPD, single primers of arbitrary sequence (generally 10 nucleotides) are used to generate DNA fragments. RAPD analysis has been observed to have a high level of variability among many isolates and used for identification of markers in fungi (Chiocchetti et al. 1999; Pasquali et al. 2003; Wilson et al. 2004; Balmas et al. 2005 and Bayrakar et al. 2008). As compared to other molecular techniques such as RFLP, AFLP (Amplified Fragment Length Polymorphism), DAF (DNA Amplified Fingerprinting)and ISSRs (Inter Simple Sequence Repeats), RAPD is simple and relatively faster (Wilson et al*.* 2004).

Molecular characterization of genetic variability in *Pyricularia grisea* isolates collected from different epidemiological regions of Himachal Pradesh was done by using RAPD markers. Forty 10 mer primers of arbitary nucleotide sequences were used for PCR amplification UPGMA analysis of total data set of RAPD markers obtained with two primers OPA10 and OPJ06 classified 62 *P. grisea* isolates into 8 DNA finger print groups. RAPD analysis of 24 isolates from 19 pathotype groups with primer OPA 10 however, have not shown any relationship between DNA finger print groups and pathotypes of *P. grisea* (Sharma et al. 2000).

Twenty-seven blast cultures were isolated from susceptible rice cultivars from three major rice growing areas of India; Karnataka *viz.,* Bangalore, Mandya and Ponnampet which were finger printed using 30 RAPD primers. One isolate, PPT-4 was distinct and formed separate group using cluster analysis (NTSYS).

Isolates of other 2 locations clustered into one group indicating no significant differences existing among the lineages (Shrinivasachary et al. 2002).

Populations of rice blast pathogen throughout the world have been studied for their phenotypic and genetic variation (Levy et al., 1991, 1993). Globally, RAPD markers are also reported to be useful in identification (Wang et al., 2010) and analysis of genetic divergence (Malode et al., 2010).

Genetic diversity of *M. grisea* from some blast nurseries of Human province in China was analysed by (Ngueko et al. 2004). Diseased samples were collected from three nurseries. Thirteen selected RAPD primers were used to test the forty-four isolates collected from those nurseries. The isolates were classified into 6 different genetic groups.

The genetic diversity of *M. grisea* has been widely studied in China (Shen et al. 2002) and other countries (Soubabre et al. 2001). RAPD was done to study genetic variation among all fungal cultures. RAPD analysis in our study revealed that there was variation among all the fungal cultures tested. Similar results were reported earlier by (Neto et al. 1991; Ngueko et al. 2004 and Wang et al. 2005). *M. grisea* population structure studies done in America (Levy et al. 1991, 1993), Europe (Roumen et al. 1997) and Asia (Chen et al. 1995 and Han et al. 1993) revealed simple population structures and suggested that *M. grisea* populations are generally composed of only a few clonal lineages.

The dendrogram study revealed that the geographic origin of strains does not play crucial role in lineage formation. Similar results have been shown by (Ngueko et al. 2004) in their study on isolates of *M. grisea* from different nurseries of Human province in China. The phylogenetic grouping based on RAPD data did not appear to be harmonious with geographical locations. Phylogenetic analysis suggests that most isolates are different from each other, indicating that both local and geographical polymorphisms exist. The significant amount of diversity among Indian isolates of *M. grisea* can be explained mainly by evolution resulting from natural and stress-induced transposition (Ikeda et al. 2001).

The results obtained in the present study are useful when assessing the suitability of commercial cultivars for planting in areas where there is a risk of *M. grisea* infection.
Indian isolates of rice blast pathogen were analyzed for genetic diversity by (Chadha et al. 2005) using molecular markers. The authors made an attempt to assess the genetic variability among *M. grisea* isolates and to establish possible genetic relationship among the Indian isolates of rice blast fungus. Initially they screened with 128 RAPD primers using three isolates Maruteru, Almora and Karjat CV4. The RAPD patterns among the 20 isolates exhibited an overall polymorphism of about 64 percent. The number of amplification products obtained was specific to each primer and ranged from three to fourteen. Overall, the RAPD patterns showed a high level of polymorphism. Out of a total of 269 bands, 171 polymorphic and around 64 low frequency bands (present only in 25% of less of the isolates) were obtained. RAPD has analysis advantages as a means of characterizing genetic variability. These include speed, low cost, less amount of DNA and lack of radioactivity. Prior sequence information about the target DNA is not required. An advantage of RAPD analysis is that it can be applied to any strain or species of a fungal or bacterial group without previous knowledge of that isolate. It is applicable to large number of isolates and enables analysis of variation at more than one locus (Bentley et al. 1998). RAPD analysis is a fast, PCR-based typing method for study of genomic polymorphism. RAPD has been found to be efficient in demonstrating the DNA polymorphisms as well as in accessing diversity in nature (Bereswill et al. 1994 and Dautle et al. 2002). Therefore, this technique has used extensively in the molecular characterization of several organisms, microorganisms like bacteria, yeasts, fungi (Bayrakar et al. 2008; Bentley et al. 1998; Assigbetse et al. 1994; Manulis et al. 1994; Migheli et al. 1998; Chiocchetti et al. 2001 and Jimenez-Gasco et al. 2004). The main disadvantage of RAPD technology is low reproducibility and reliability. It is unusually sensitive to change in reaction conditions and even some of the binding patterns can be DNA polymerase dependent (Li et al. 2010). The high homoplasy in data generated with RAPD primers, the lack of co-dominance, and its reproducibility also pose limitations in the use of the technique. However, development of more specific, sensitive and reproducible markers like RAPD based on sequence characterized arbitrary region (SCAR) can increase application of the molecular techniques. These markers are specific as they increase the reproducibility of RAPD markers and have been used for specific amplification of target DNA-PCR methods based on RAPD and SCAR primers analysis have a good sensitivity and are largely used in routine laboratory research. The use of SCAR markers to identify pathogens offers advantage of low cost and good specificity. Furthermore, these markers are an efficient means of screening large fungal populations and they are economical and relatively easy to analyse (Bayrakar et al. 2008; Chowdhury et al. 2001 and Lievens et al. 2009).

The use of RAPD in phylogenetic analysis was evaluated by (O'Donnell et al. 2000) employed two 10 *mer* and one 15 *mer* provided a better resolution than those inferred from the individual and combined data generated with the 10 *mer*. RAPD analysis revealed the underlying phylogenetic structure as accurately as DNA sequence data. The author concluded that it would be better to use longer primers (15 to 20 *mer*) for phylogenetic studies to reduce the homoplasy associated with shorter RAPD primers. They also suggested that trees inferred from RAPD analysis should be treated cautiously, especially when 10 mer are used.

RAPD may also fail to differentiate between pathogenic and non-pathogenic isolates reported by (Woo et al. 1998).

Therefore, it is necessary to screen a large number of primers in order to reach to an effective RAPD system, since all primers cannot resolve isolates.

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