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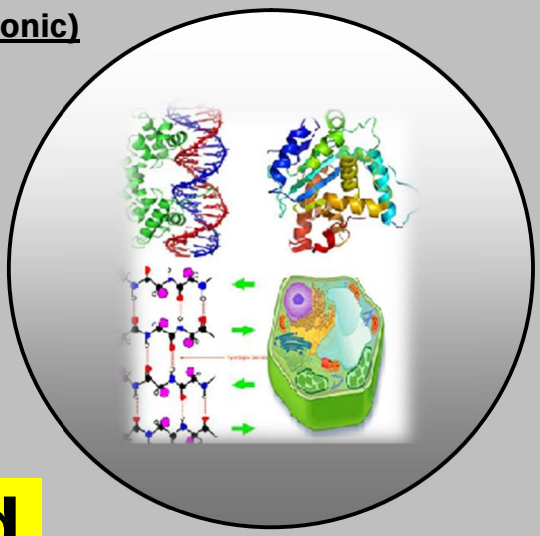

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RAPD Based Genetic Variation in *Rhizoctonia* Sp. in India

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ABSTRACT

Rhizoctonia solani is the causal pathogen of rice sheath blight disease. Variations among R. solani isolates are causing serious concern during screening sheath blight resistant varieties. Therefore, study on genetic diversity of the pathogen can provide information on its behavior in field. The objective of this study was to assess the genetic diversity of R. solani isolates collected from different locations of India. A total six isolates of R. solani were analyzed using ten random amplified polymorphic DNA primers. Polymorphism range shown by RAPD primers was 33.33 to 62.5 %, while the range of total loci scored was from 07 to 11. The results obtained confirmed the genetic diversity of rice sheath blight pathogens in India.
Key words: Rice, Rhizoctonia solani, RAPD, Polymorphism.

INTRODUCTION

Rhizoctonia solani Kuhn (Teleomorph: *Thanatephorus cucumeris*), is a widely distributed soil-borne plant parasitic-saprophytic fungus (Mirmajlessi et al. 2012). Sheath blight disease caused by *R. solani* Kuhn, is becoming a major constraint to rice production, especially in the intensified cultivation system (Jayaprakashvel and Mathivanan, 2012). Rice sheath blight is a particularly important component of the rice disease complex, occurring in most rice-producing areas (Dagupta and Vilgalys, 1997), including India.

Rice sheath blight has been the subject of several population studies measuring variation based on virulence (Neeraja et al. 2002), isozymes (Neeraja et al. 2002), RFLPs (Rosewich et al. 1999), or simple-sequence repeat PCR (Banniza and Rutherford, 2001).
Sheath blight disease caused by *R. solani* Kuhn was first recorded as minor disease of rice in West Bengal (Roy, 1978). Yield losses due to this disease is reported to range from 5.2 to 50%, depending on environmental conditions, crop stages at which the disease appears, cultivation practices and cultivars in India (Rajan, 1987). Sclerotial diseases of rice (*Oryza sativa*) caused by *Rhizoctonia* and *Sclerotium* species display similar symptoms on the leaf sheaths of the plants. Some of these diseases cause 20 to 35% yield losses and inferior quality of rice (Inagaki, 2001). Molecular techniques have become reliable and are highly suitable tools for identifying pathogen species and for assessing genetic variation within collections and populations (Sundravadana et al. 2011). With the advent of various molecular marker technologies, the studies of genetic diversity in plant pathogens have become feasible. Random amplified polymorphic DNA (RAPD) markers have been successfully applied to numerous filamentous fungi in different fields of experimental mycology (Pollastro et al. 2000). RAPD offers a promising, versatile and informative molecular tool to detect genetic variation within population of plant pathogens (Chiochetti et al. 1999). The main advantages of RAPD are the speed and the simplicity of the technique, and also the fact that closely related strains of a pathogen can be distinguished without prior knowledge of the nature of polymorphic regions by the use of RAPD.
Variability in molecular characters is used for determining resistant cultivars (Thirumalasamy et al., 2006) and for the evaluation of the germ plasm resistant line (Shekhar et al., 2006). Understanding the genetic structure of pathogenic fungi is critical for developing appropriate strategies for disease management; however, little is known about the genetic structure of *Rhizoctonia* spp. in natural population (Vilgalys and Cubeta, 1994). Thus, the present study was undertaken to assess the molecular variability using RAPD markers to distinguish the isolates collected from different agro-ecological zones of Northeast India.

MATERIAL AND METHODS

Collection of plant samples and Isolation of fungal pathogens

Rice plant infected by *R. solani* and showing typical sheath blight symptoms were collected from paddy fields of different agro ecological regions of Northeast India in sterile polythene bags and kept airtight. It is then brought to the laboratory and stored at 4º C until isolation.
The diseased plant parts were washed in running tap water followed by sterile distilled water to remove impurities. The diseased sample was cut with a sterile blade 1-2 cm in length ensuring that fresh plant tissues were also included.

It was then immersed in 0.2% $HgCl_2$ solution for 2 minutes followed by 2-3 times washings with sterile distilled water. It was then immersed in 70% ethanol for 20-30 seconds and washed with sterile distilled water 2-3 times. After that the plant part was blotted dry with sterile filter paper. The thin sections were inoculated on potato dextrose agar medium (PDA, Hi Media, India), supplemented with 100 mg streptomycin sulphate/L (Gad, 2012). Petri plates were incubated at 25±2ºC for 2-4 days or until colony appears. The fungal colony was repeatedly sub-cultured on PDA medium, followed by microscopic examination of the colony characters till the pure culture were obtained. The colonies thus obtained were then cultured in PDA slants and stored at 4ºC until further use.

Identification of fungal cultures

Cultural, morphological and microscopic properties were under taken to identify the isolated fungal pathogens according to Gilman (1957), Barnett (1960), Booth (1977) and Singh (1982). Final identification of a microbial culture was done from Indian Type Culture Collection of Indian Agricultural Research Institute, (IARI), New Delhi. The identified fungi were maintained in potato dextrose agar medium (PDA, Himedia, Mumbai) and stored at 4º C for further studies.

Genomic DNA isolation

The total genomic DNA was isolated using the modified CTAB protocol (Roger and Bendish, 1988). Initially, 0.5 g of fungal mycelium mat was taken and grinded with the help of liquid nitrogen. DNA quality and concentration was checked by running 5 µl DNA sample on 0.8% agarose gel by electrophoresis.

RAPD Fingerprinting and PCR conditions

PCR conditions were optimized by varying concentrations of template DNA, Taq DNA Polymerase, dNTPs and MgCl2. Ten, 10 *mer* random primers (OPA and OPN series, synthesized by Sigma-Aldrich, Bangalore) were used for DNA finger printing.

The PCR reaction was performed in a 25µl PCR mix containing the following components: (Double distilled water: 14.1 µl, 10x PCR buffer: 1.5 µl, MgCl2: 2.0 µl, dNTP: 2.0 µl, Taq DNA: 0.9 µl, Target DNA: 2.0 µl, Random primer: 2.0 µl). RAPD-PCR reaction was then performed on Thermal Cycler (Applied Biosystems 9700).

PCR conditions were maintained as follows: (94ºC/5 min for 1 cycle); (94ºC/45 sec, 35ºC/60 sec, 72ºC/90 sec for 10 cycle); (94ºC/45 sec, 37ºC/90 sec, 72ºC/60 sec for 40 cycle); (72ºC/10 min for1 cycle); (4ºc/ Infinity for 1 cycle).

RAPD amplification reaction was analyzed in a 1.2 percent agarose gel electrophoresis in 1X TBE buffer as stated in MIF method.

RAPD profile was viewed under UV light and documented with a Gel Doc system (Syngene, UK), capture the image in computer. RAPD bands were tabulated.

Data analysis

Polymorphic bands were considered as binary characters and scored as '1' for presence and '0' for absence of each band in individual lanes. These scores were entered as a binary matrix for analysis by the software NTSYS-pc version 2.02 (Raholf, 1998).

RESULTS AND DISCUSSION

Isolation and identification of the causal organisms:

R.solani isolates were isolated from diseased rice plant samples and identified according to their morphological criteria and final identification was done from IARI, New Delhi is shown in (Table 1).

Table 1. Collection source of *R. solani* isolates.

Serial No.	Isolate Code	Location
1	R1	Kaul, IARI 4110
2	R2	Tamil Nadu, IARI 2060
3	R3	Manipur, RRL03
4	R4	Meghalaya , RRL02
5	R5	Assam, RRL01
6	R6	Nagaland, RRL04

Molecular variability by using RAPD technique

Ten different RAPD primers (10 *mer*) have been used to detect the molecular variation among different isolates of *R.solani* collected from different locations of Northeast India. Each RAPD primer in this study generated clear and distinctive bands and revealed polymorphism among all fungal species (Figure 1, 2).

A total of 92 bands were obtained from the 10 primers among which OPA-03 amplified the highest number of bands (11 bands) (Table 2). The number of bands generated by each primer varied from 07 to 11. Polymorphism range shown by RAPD primers was 33.33% (OPN-10) to 62.5% (OPN-05). The range of total loci scored ranged from 07 (OPN-04) to 11 (OPA-03). The primer OPN-05 revealed maximum polymorphism percentage of 62.5%. Among the 92 fragment generated by 10 primers, only 48.9 fragment were polymorphic in nature. These results indicate molecular variation of phytopathogenic fungi, *R.solani* exits in India isolates. Based on the presence or absence of bands a UPGMA based dendrogram was constructed (Figure 3).

The results of our present study indicate significant level of variation among the six isolates of *R. solani*. The range of variation estimated is from 33.33 to 62.5 %, among the six isolates. It is noteworthy that such wide variability has been found only among the few isolates used in our present study.

RAPD-PCR has been successfully used for studying variability among populations from different as well as the same geographical regions (Sundravadana et al. 2011). The main difference between the Texas and Indian populations was that clones were not shared among Indian populations, while some shared clones were found among the Texas populations. The regular out crossing and high gene flow indicated for R. solani AG-1 IA has significant implications for effective control and resistance breeding strategies in India (McDonald and Linde, 2002).

Table 2. Primers sequences and details of loci detected with 10 RAPD primers.

S/N	Operon Code	Nucleotide Sequence 5' to 3'	Total loci	Polymorphism	
				No of loci	%
1	OPA-02	TGCCGAGCTG	10	4	40
2	OPA-03	AGTCAGCCAC	11	6	54.54
3	OPA-10	GTGATCGCAG	9	5	55.55
4	OPA-13	CAGCACCCAC	10	5	50
5	OPA-18	AGGTGACCGT	9	5	55.55
6	OPN-04	GACCGACCCA	7	3	42.86
7	OPN-05	ACTGAACGCC	8	5	62.5
8	OPN-07	CAGCCCAGAG	10	5	50
9	OPN-08	ACCTCAGCTC	10	4	40
10	OPN-10	ACAACTGGGG	9	3	33.33
Total			92	45	48.9

On analyzing 24 isolates of *R. solani* from different locations and crops using 11 RAPD primers, Sharma et al. (2005), reported a similarity index of 0.16 to 0.53 and the markers revealed host-specific band profiles.

These results are in consonance with that of Sharma et al. (2005), indicating high genetic variability in the pathogen population in different epidemiological regions of West Bengal. High genetic variability present in *R. solani* population has significant implications for effective control and resistance breeding strategies against sheath blight of rice disease in India.

RAPD markers used in this investigation increases the marker density to find out genetic relationships. In the present study, rice sheath blight samples from different states of Northeast India were collected. The analysis of RAPD polymorphism in isolates of *R. solani* from different states across India revealed the occurrence of different levels of polymorphism, indicating a diverse genetic base. Individual strains were grouped based on their genetic relatedness, which was estimated from pair wise comparisons between their fingerprints.

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). It is in the light of this background the present study on the characterization of rice fungal pathogens were under taken. The dendrogram study revealed that the geographic origin of strains does not play crucial role in lineage formation. Similar results have been shown that most isolates are different from each other, indicating that both local and geographical polymorphisms exist.

It is anticipated that this study will lead to a better understanding of the diversity of sheath blight pathogens, and to its potential application in rice breeding programs aiming at development of durable sheath blight-resistant rice cultivars.

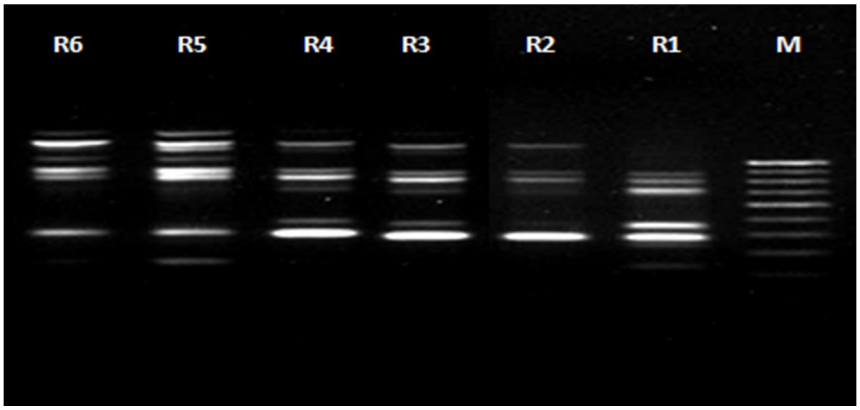

Fig 1. DNA fingerprinting patterns of six *R. solani* isolates using OPA-10 RAPD primer; M: 100 bp DNA marker.

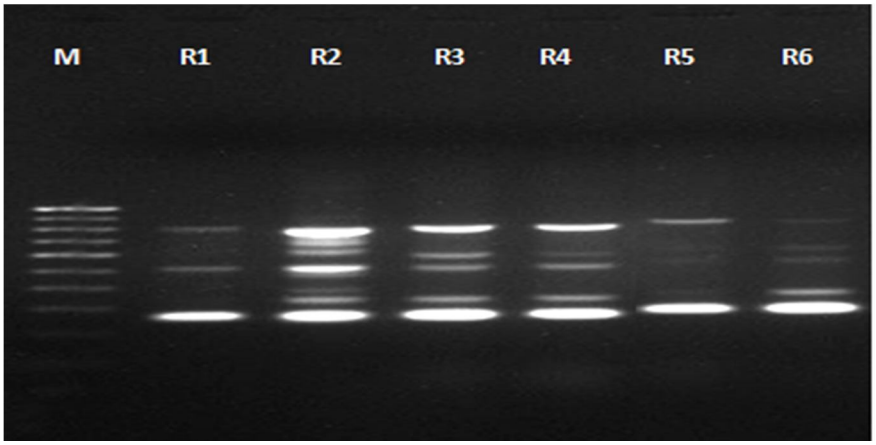

Fig 2. DNA fingerprinting patterns of six *R. solani* isolates using OPN-04RAPD primer; M: 100 bp DNA marker.

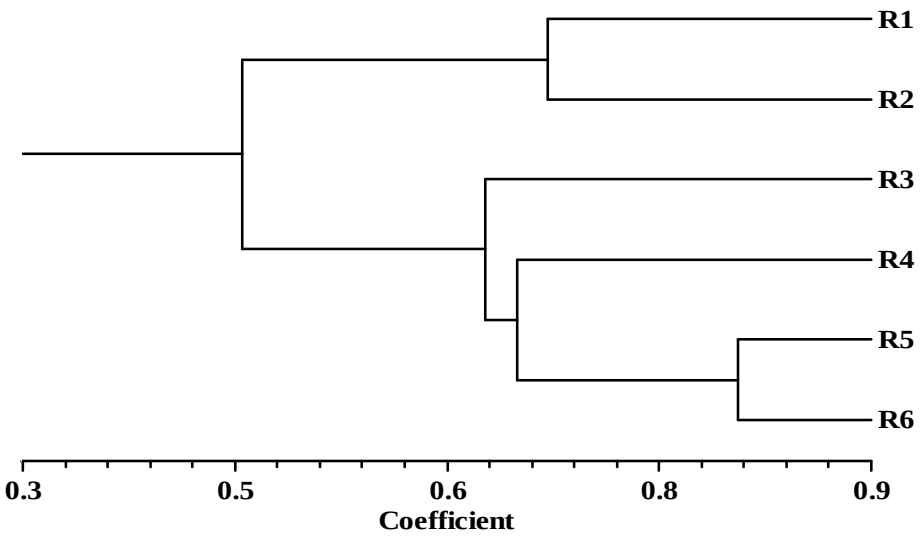

Figure 3. UPGMA dendrogram of six genotypes of *R. solani* based on ten random RAPD primers

CONCLUSIONS

The results obtained revealed that *R.solani* isolates collected from India during 2011 rice growing season may be genetically heterogeneous and the interrelationships amongst them can be easily, precisely and reliably explained by RAPD-PCR technology.

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