Molecular Characterization of *Pyricularia oryzae* Causing Blast Disease in Rice (*Oryza sativa* L.) in different Zones of Karnataka.

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Abstract-

Rice blast is one of the deadliest disease and also one of the most widely distributed, occurring in every rice growing zones of Karnataka. Fifteen isolates of Pyricularia oryzae causing rice blast disease were evaluated for their genetic characterization using RAPD marker. The molecular polymorphism among isolates by means were analysed by means of RAPD-PCR and the genetic coefficient matrix derived from the scores of the RAPD profile showed that minimum and maximum per cent similarities among isolates were in the range of 90 to 45 per cent respectively. The cluster analysis by unweighted pair group method with arithmetic average (UPGMA) separated the isolates into two major groups. Through RAPD grouping of isolates suggested that genetic variability among P.oryzae isolates should be taken into account for the screening of blast resistant rice genotypes and utilize in the future breeding programme.

***Key words**: Pyricularia, RAPD, Cluster, Virulence*

Introduction:

Rice blast disease is caused by *Pyricularia oryzae* is an important fungal disease known to occur in entire rice producing areas of the globe (Ou, 1985). The disease can strike all aerial parts of the plant. Most infections occur on the leaves, causing diamond-shaped lesions with a grey or white centre to appear, or on the panicles, which turn white and die before being filled with grain (Scardaci *et al.,* 1997). *P. oryzae* is highly specific to rice, although certain strains that don't attack rice can harm weeds in the rice field. Once on a rice plant, the fungus rapidly produces thousands of spores, which are carried readily through the air, by wind or rain, onto neighbouring plants (Rick and Lee, 2000). Blast was first reported in Asia more than three centuries ago and is now present in over 85 countries. It is highly adaptable to environmental conditions and can be found in irrigated lowland, rain-fed upland, or deep water rice fields (Rao 1992). The disease results in yield loss as high as 70-80% (Ou,1985) when predisposition factors (high mean temperature values, degree of relative humidity higher than 85-89%, presence of dew, drought stress and excessive nitrogen fertilization) favour epidemic development (Piotti *et al.,* 2005). The analysis of genetic variation in plant pathogen populations is an important prerequisite for understanding coevolution in the plant pathosystem (Mc Donald *et al.,* 1989). Populations of rice blast pathogen throughout the world have been studied for their phenotypic and genetic variation

(Levy *et al.,* 1991; Kumar *et al.,* 1999). Although earlier studies focused on pathotypic variability (Ou, 1985) but recent studies utilized molecular markers to characterize population diversity. The use of molecular markers in population genetic studies has unravelled epidemiologic information to levels of accuracy not previously possible. Unlike traditional markers, molecular markers are direct manifestations of genetic content and can therefore serve as reliable indices of genetic or pathotypic variation which provide a framework to understand the taxonomy and population structure. They are not influenced by environmental factors and therefore are highly reproducible. Besides, these are cost effective and less cumbersome. Polymerase chain reaction (PCR)-based molecular markers are useful tools for detecting genetic variation within populations of phytopathogens
(Vakalounakis *et al.,* 1999; Kolmer *et al.,* 2000) Random amplified polymorphic DNA (RAPD) (Welsh *et al.,* 1990; Williams *et al.,* 1990) markers have been widely used for estimating genetic diversity in natural populations (Annamalai *et.al.,* 1995), mainly because the technique does not need previous molecular genetic information and increases marker density for evaluating genetic relationship. Many phytopathogenic fungi have been characterized using this technique (Malvickan and Grau, 2001; Vakalounakis and Fragkiadakis, 1999; Muller *et al.,* 2005; Jahani *et al.,* 2008). The present study is planned to investigate the diversity of *P.oryzae* from different Zones of Karnataka of by using morphological, pathological, and RAPD analysis.

Material and Methods:

Genomic DNA isolation:

P. oryzae isolates were revived and grown on potato dextrose agar (PDA, Hi Media) plates at 25°C for 5 days. For DNA extraction, isolates were grown in 100 ml of potato dextrose broth for 4 days at 25°C in a rotary shaker at 100 rpm. Mycelial mat was filtered, dried and ground to a fine powder in liquid nitrogen. Powdered mycelia were vortexed in pre-warmed lysis buffer [100 mM Tris (pH 8.5), 250 mM NaCl, 0.5 mM EDTA and 0.5% SDS], incubated at 65°C for 30 min followed by the addition of 1.7 M potassium acetate solution. The contents were gently mixed and incubated on ice for 30 min. Samples were then extracted with chloroform and the total nucleic acid was precipitated with chilled isopropanol. The pellet after centrifugation and drying was dissolved in TE (10 mM Tris and 1 mM EDTA, pH 8.0). After RNAase treatment, the DNA was purified with phenol: chloroform (1: 1; v/v) and chloroform: isoamylalcohol (24: 1; v/v) and precipitated with chilled ethanol after adding 1/10th volume of 3 M sodium acetate. The DNA was dissolved in TE buffer (Hamer and Givan 1990) (Fig.1).

Molecular diversity analysis of pathogen (*Pyricularia oryzae* L.) using RAPD analysis:

PCR amplification was carried out according to the protocol of Williams *et al.,* 1990 with minor modifications. For RAPD analysis, initially fifteen random primers were selected after screening of 25 RAPD primers (Table 1). Genomic DNA was amplified in a 20 µl reaction volume containing 10 mM Tris–Cl, pH 8.3, 50 mM KCl, 2 mM MgCl 200 µM each of dNTP, 0.2 µM primer, 50 ng of genomic DNA and 0.5 U of Taq DNA polymerase (Merck India, Pvt. Ltd.) in BIO-RAD PTC-100 thermal cycler (USA). The PCR program with an initial denaturation, 94 ºC for 5 min., followed by 1.0 min. denaturation at 94 ºC, annealing

temperature at 35 ºC for 1 min, and 72 ºC temperatures for 2 min. elongation were repeated 40 times with the final elongation step at 72 ºC for 5 min. The amplified DNA fragments were resolved through electrophoresis in 1.2 % agarose gel prepared in TAE (Tris Acetic Acid) buffer. Finally the gel was stained with ethidium bromide (0.5 µg/ml) and visualized in a gel documentation system (Alpha Digi Doc System, USA). Most informative RAPD primers were scored based on polymorphism information content (PIC) values of individual primers.

PIC= 1- $\sum pi^2$; where: pi is the frequency of the i^{th} allele (Smith *et al.,* 1997)

Results and Discussion:

Rice blast is the most serious disease in all rice growing regions of the world. *Pyricularia oryzae* a filamentous ascomycete fungus i.e. causal agent of the rice blast disease. The fungus has an ability to overcome resistance within a short time after the release of a resistant cultivar and thus breeding for resistance has become a constant challenge. The analysis of genetic variation in plant pathogen populations is an important pre-requisite for understanding coevolution in the plant pathosystem (McDonald *et al.,* 1989).The population structure of *P. oryzae* rice isolates from the different zones of Karnataka were analysed using RAPD markers, which showed high genotypic variation in the pathogen population (Rathore *et al*.,2004).

Isolation of DNA from Blast disease casual organism *Pyricularia oryzae* isolates

The total DNA extracted from the fifteen different isolates of *Pyricularia oryzae* were checked through agarose gel electrophoresis method and all the fifteen isolates showed good quality and concentration of DNA. The DNA quality was considered to be good if the spectrophotometer value at 260/280nm should be more than 1.80.

RAPD (Random Amplified Polymorphic DNA) analysis of total DNA of *P. oryzae.*

RAPD analysis of DNA product of fifteen isolates of *P. oryzae* were digested with ten distinct RAPD primers *viz.*,OPB-2, OPB-4, OPB-5, OPA1, OPC-6, OPD -9, OPF-19, OPB-10, OPF-1 and OPF-2 (Table 1). Each RAPD primers were generated distinct types of banding patterns which were slightly different for the isolates studied, indicating that, there was a slight variability among the *P. oryzae* isolates. Estimated sizes of the RAPD profile fragments generated using ten primers are summarized below.

The profiles generated from the RAPD digestion of different isolates of *P. oryzae* isolates with OPB-4 primer showed that, DNA was cut into one to eight distinct fragments of 100 to 1500 bp length in different isolates of *P. oryzae*. The banding pattern was observed in different base pair lengths viz., 300bp, 400bp, 500bp up to 1500bp. The maximum bands were observed in isolate po-2 (8 distinct bands at different lengths) comparing with 1500bp ladder. The isolate po-8 generated seven bands at different lengths. The least bands (one) were observed in po-15 isolate (Fig.2).

This RAPD banding pattern of different isolates digested with primer OPB-4 indicates that, there is a slight variability among the different isolates of *P. oryzae*. This variability due to the samples were collected from different location, soil type, variety and different environmental conditions. The dendrogram similarities also revealed that, the close

similarities between the isolates po-4 and po-3 (7.1%) and po-5, po-6, po-7 and po-8 (4.5%) (Fig. 5).

The next RAPD banding pattern of different isolates digested with primer OPB-5 indicates that, there is some variability among the different isolates of *P. oryzae*. This variability due to the samples were collected from different zones/location, soil type, variety and different environmental conditions (Table 2). The dendrogram similarities also revealed that, the close similarities between the isolates Po-4 and Po-3 (5.6%) and Po-13 and Po-14 (7.1 %), Po-1 and Po-2 (2.9 %). Other primer OPB-10 dendrogram indicates that Po6, Po4, Po2 (4.5%), Po12, Po9, Po10 (4.5%) (Fig. 2), Po3, Po1 (7.1%). Primer OPF-1 dendrogram indicates that Po2, Po1 and Po3 (5.6%) (Fig. 3), Po10, Po9, Po8, 7 (4.5 %), the primer OPF-2 Po13, Po11, Po6, Po4 (16.7%), Po14 (7.1%) (Fig 4). The primers OPC-6, OPA-1, OPB-4 shown variability across the *Pyricularia oryzae* isolates.

Analysis of the diversity of the plant pathogen has been revolutionized by molecular techniques and particularly PCR techniques have helped to understand taxonomy and population structure. According to Burdon and Silk (1997), plant pathogenic fungi most commonly rely on mutation and recombination as the main source of genetically based variations. Molecular marker has been used widely to characterize fungal plant pathogen populations, in particular for the assessment of genetic diversity, phylogenetic relationships and the characterization of pathotypes. The level of degree of genome coverage and the type of DNA sequence variation being assayed. In present study total 10 RAPD primers were used among them 8 were amplified that gives total of 253 band out of them 158 band were polymorphic. Similarly 32 primers were used for the assessment of genetic diversity of Indian isolates of rice blast pathogen (*Pyricularia oryzae*) by Sonia and Gopalakrishna (2005a) and gives total of 269 band among them 171 band were polymorphic. Sere *et al*., (2007) used ten primers out of 82 primers that showed polymorphism among individual isolates. The amplification reactions with the 10 primers generated 153 bands, 108 of them being polymorphic. The primers showed polymorphism and consistently produced 2 to 7 bands of 0.3kb to 2 kb although majority was below 1kb. However this value was lower than the study of Kumar *et al.*, 2010 ranges from 40 bp to 4.2 kb. But nearly similar result was found by Chada and Gopalakrishna (2005b) with REMAP marker and value ranges from 0.1 to 2 kb. Dendrogram constructed based on Jaccard's similarity coefficient using the marker data from *P. oryzae* isolates with UPGMA analysis separated into two major groups A and B at 0.53 of similarity coefficient and this coefficient was lower than the Rathour *et al.*, 2004 i.e. 0.84 who tested 48 *P.oryzae* isolates with total of 65 RAPD primers among them 5 were polymorphic. Similarities among ten blast fungus isolates based on RAPD profile were ranged from 35 to 80%. Our result was in agreement with previous studies in which similarity values ranged from 20 to 80% (Sharma *et al.*, 2002).

CONCLUSION

Concluded that *P. oryzae* from various zones of Karnataka consists of variable populations based on RAPD analysis. Molecular phylogenetic grouping obtained by RAPD analysis did not correlate with morphological characteristics and virulence pattern. The result of the present study demonstrates that there is a certain level of genetic diversity among isolates of *P.oryzae* from various zones of Karnataka. The genetic variability among the

isolates of *P. oryzae* should be taken in to account when *P. oryzae* are used for screening of rice genotypes for blast resistance. On the basis of the present study, it is concluded that the population of rice blast fungus collected from different zones of Karnataka is genetically heterogeneous and the interrelationships amongst the different isolates of blast disease can be easily, precisely and reliably explained by RAPD.

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Table 1: Primers used in the Study

Primers	Sequences
OPB-2	5 TGA TCC CTG G-3
OPB-4	5 GGA CTG GAG T-3
OPB-5	5 TGC GCC CTT C-3
OPA1	5 CAG GCC CTT C-3
OPC6	5 GAA CGG ACT C-3
OPD 9	5 CTC TGG AGA C-3
OPF19	5 CCT CTA GAC C-3
OPB-10	5 CTG CTG GGA C-3
OPF-1	5 ACG GAT CCT G-3
OPF-2	5 GAG GAT CCC T-3

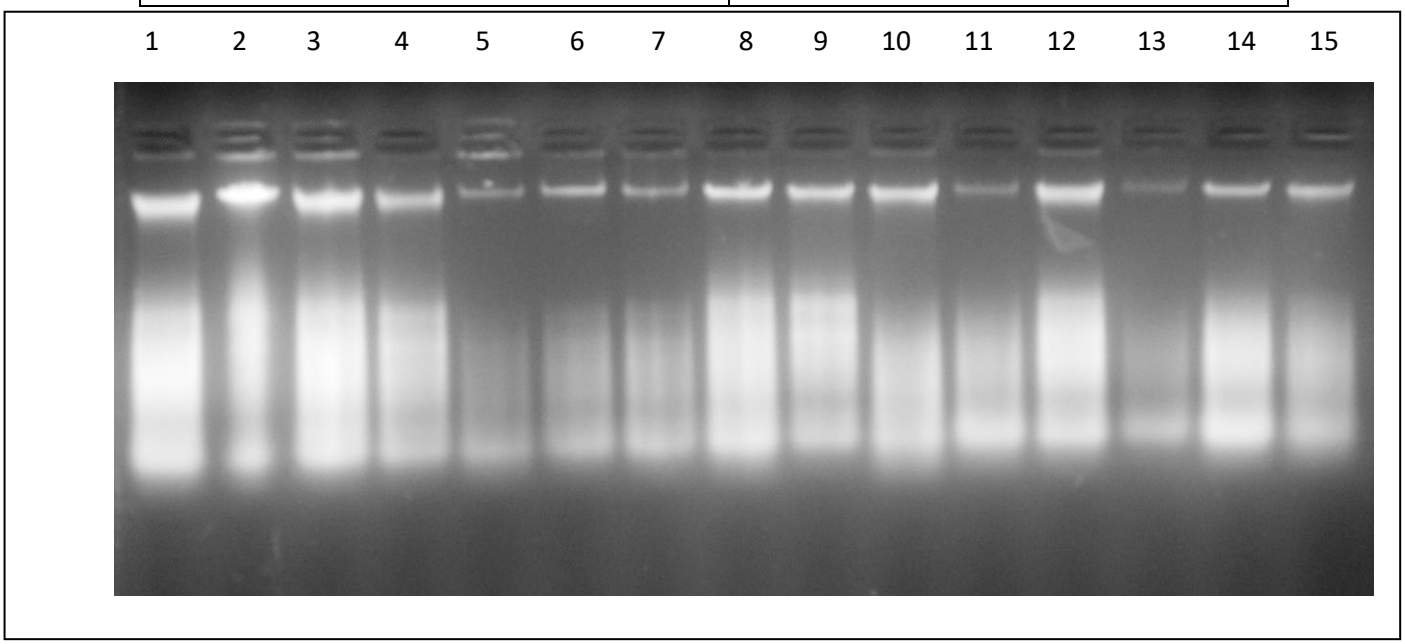


Fig. 1 DNA of Po1-Po15

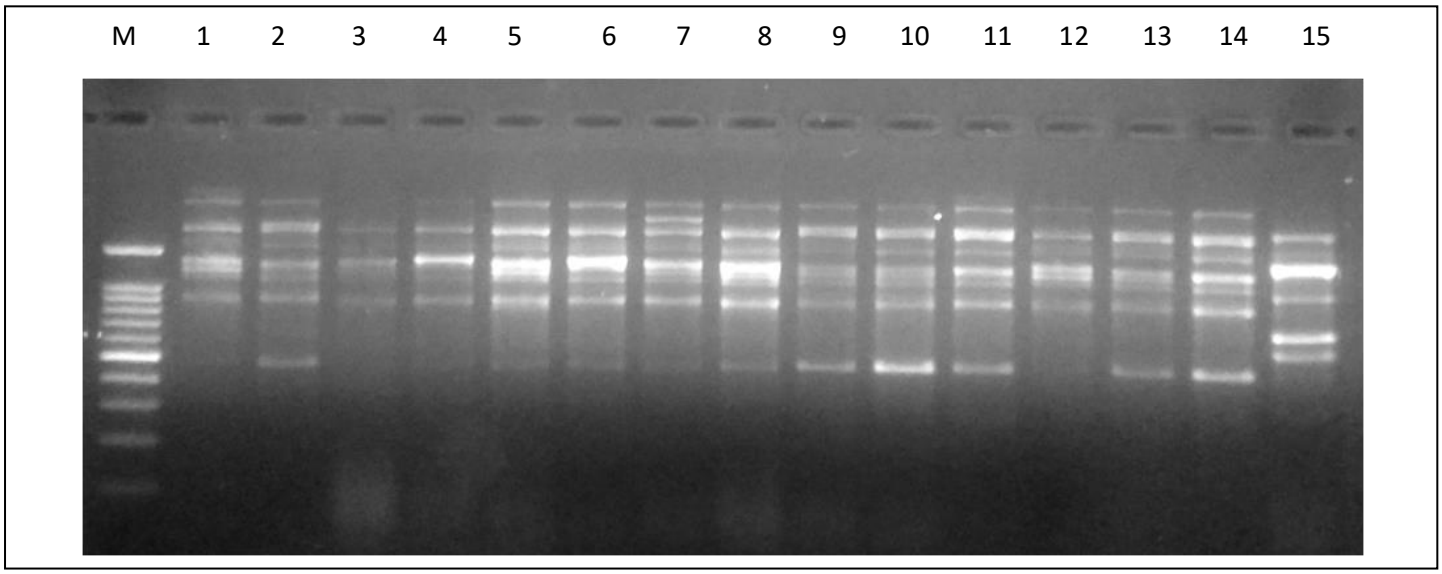


Fig2: PCR amplicons with OPB 10 primers

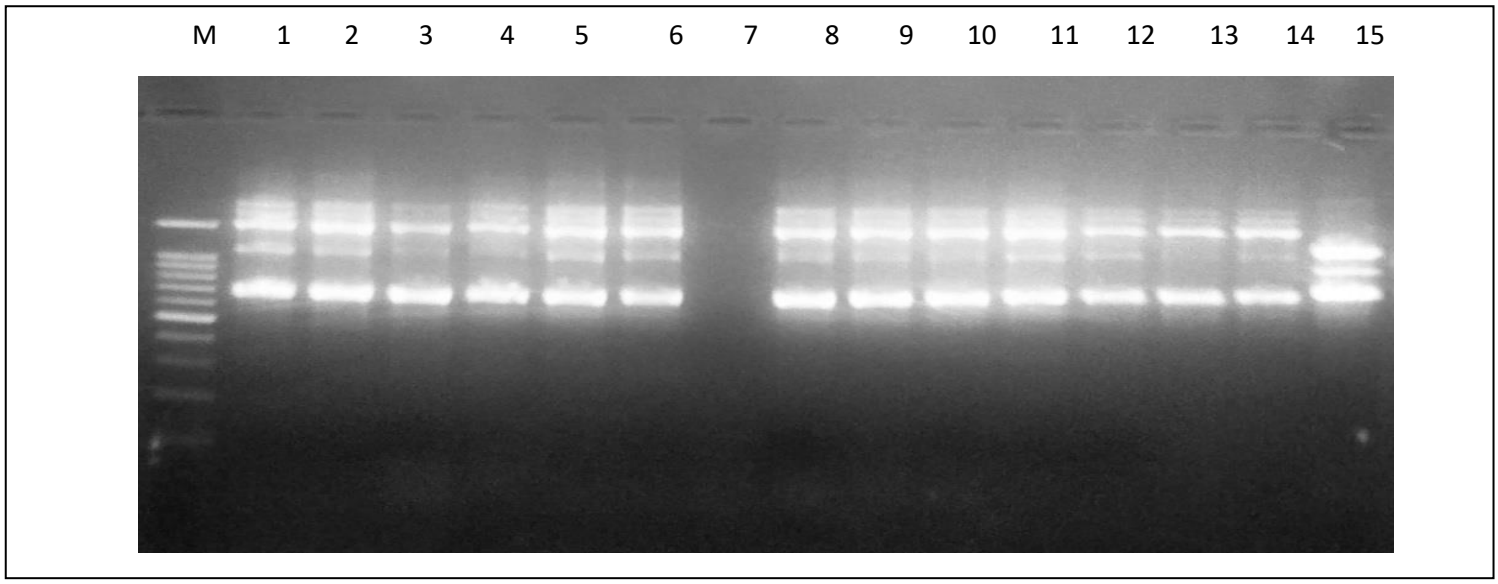


Fig.3: PCR amplicons with OPF 1 primers

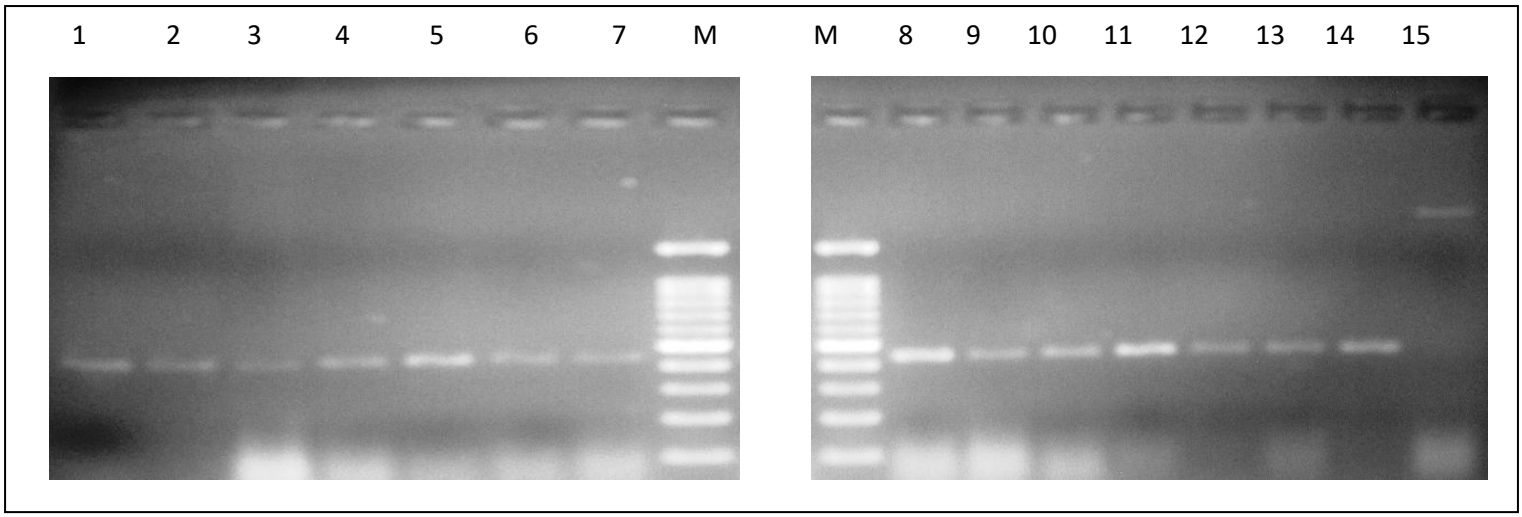
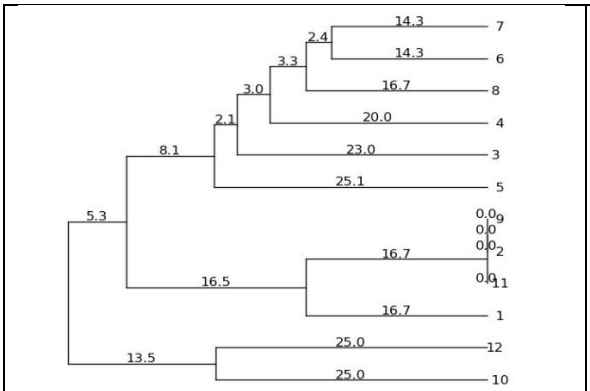
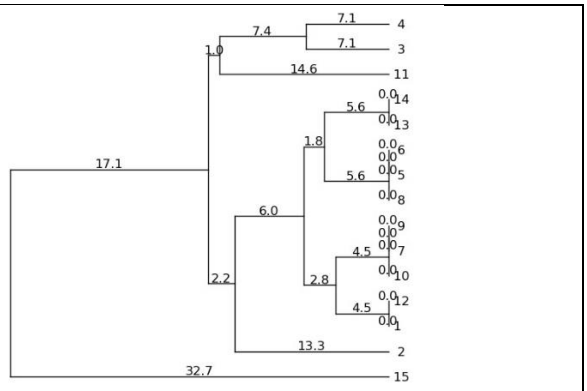


Fig.4: PCR amplicons with OPD 9 primers



Primer OPA1



Primer OPB4

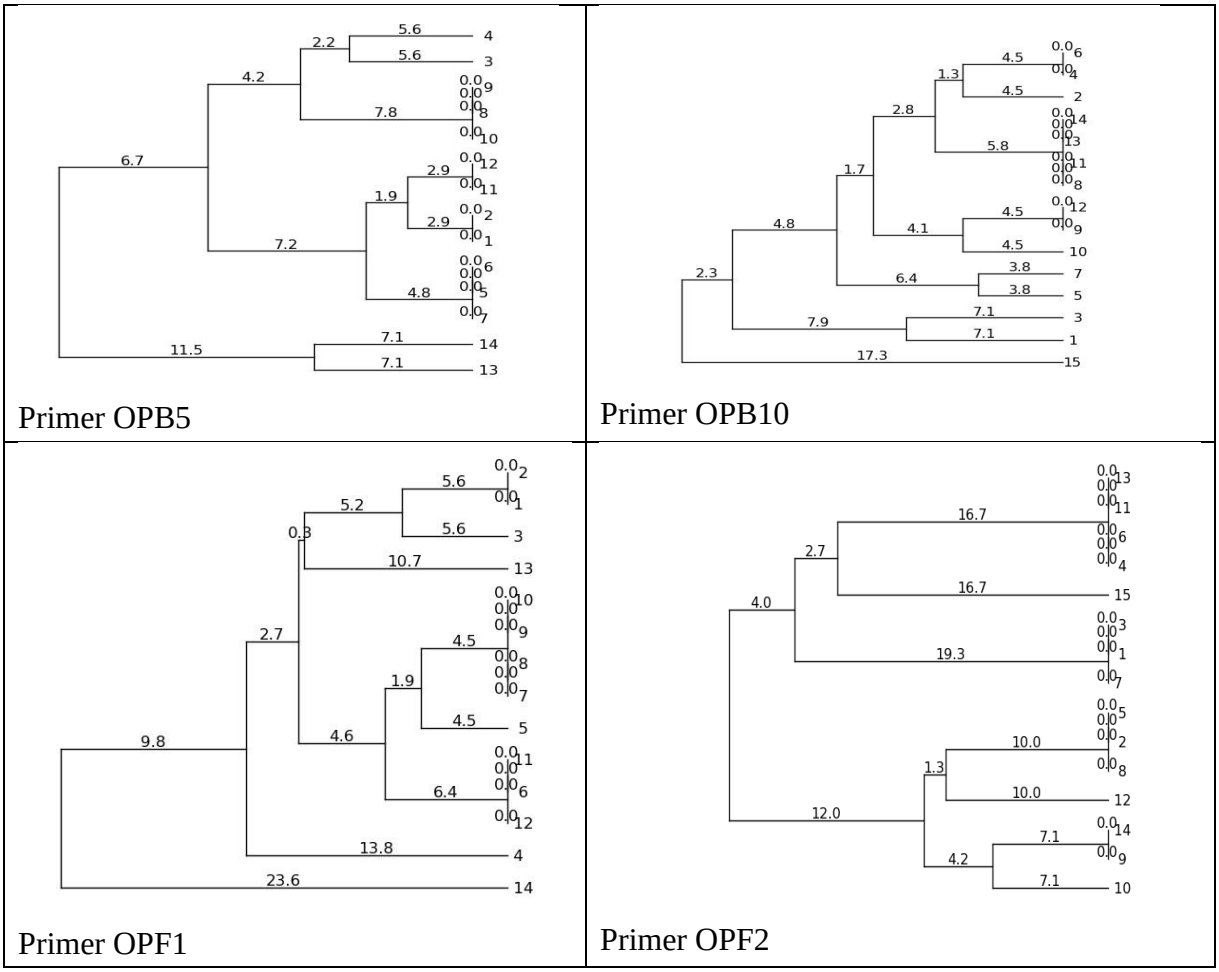


Fig.5: Dendrograms showing the RAPD analysis of similarities between *P.oryzae* isolates.

Table 2: Banding profile of primers for different isolates of *Pyricularia oryzae*

Banding profile of primer OPB05 for different isolates of *pyicularia oryzae*															
OPB 05	Po1	Po2	Po3	Po4	Po5	Po6	Po7	Po8	Po9	Po10	Po11	Po12	Po13	Po14	Po15
> 1500bp +3	1	1	0	0	1	1	1	1	1	0	1	1	1	0	0
> 1500bp +2	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
1500 bp +1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1000bp	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
700bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
600bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
500bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
400bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Banding profile of primer OPF1 for different isolates of pyicularia oryzae															

OPF1	Po1	Po2	Po3	Po4	Po5	Po6	Po7	Po8	Po9	Po10	Po11	Po12	Po13	Po14	Po15
> 1500bp+2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
> 1500bp+1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1500 bp	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
1000bp	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
700bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Banding profile of primer OPD 9 for different isolates of pyricularia oryzae oryzae															
OPD9	Po1	Po2	Po3	Po4	Po5	Po6	Po7	Po8	Po9	Po10	Po11	Po12	Po13	Po14	Po15
> 1500bp+1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
> 1500bp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
450bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Banding profile of primer OPF 2 for different isolates of pyricularia oryzae															
OPF2	Po1	Po2	Po3	Po4	Po5	Po6	Po7	Po8	Po9	Po10	Po11	Po12	Po13	Po14	Po15
>1500bp	1	1	0	0	1	0	0	1	1	1	1	1	1	1	1
1500 bp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1000bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
800bp	0	0	0	1	0	1	0	1	1	1	1	0	1	1	1
700bp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Banding profile of primer OPB4 for different isolates of *pyricularia oryzae*															
OPB4	Po1	Po2	Po3	Po4	Po5	Po6	Po7	Po8	Po9	Po10	Po11	Po12	Po13	Po14	Po15
> 1500bp+3	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
> 1500bp+2	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0
> 1500bp+1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
1500 bp	1	1	1	o	1	o	1	1	o	1	0	1	0	0	1
900bp	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
600bp	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
500bp	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0
400bp	0	1	0	1	1	1	1	1	1	1	1	1	0	0	0
300bp	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0
Banding profile of primer OPA1 for different isolates of pyricularia oryzae															
OPA1	Po1	Po2	Po3	Po4	Po5	Po6	Po7	Po8	Po9	Po10	Po11	Po12	Po13	Po14	Po15
> 1500bp+3	0	0	0	0	1	0	1	1	0	1	0	0	0	0	0
> 1500bp+2	0	0	0	0	1	0	1	1	0	1	0	0	1	0	0
> 1500bp+1	1	0	0	0	3	0	3	3	0	3	1	1	2	0	0
1500 bp	1	1	1	0	1	0	1	1	0	1	0	1	0	0	0
800bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Banding profile of primer OPC6 for different isolates of pyricularia oryzae															
OPC6	Po1	Po2	Po3	Po4	Po5	Po6	Po7	Po8	Po9	Po10	Po11	Po12	Po13	Po14	Po15
1500 bp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1000bp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
900bp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
800bp	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1
Banding profile of primer OPB10 for different isolates of pyricularia oryzae															
OPB 10	Po1	Po2	Po3	Po4	Po5	Po6	Po7	Po8	Po9	Po10	Po11	Po12	Po13	Po14	Po15
> 1500bp+3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
> 1500bp+2	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0
> 1500bp +1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1500 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
900bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
700bp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
600bp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
500bp	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0