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Morphological and Molecular Characterization of *Pyricularia Oryzae* Causing Blast Disease in Rice (*Oryza Sativa*) from North India

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Abstract- Rice blast is not only one of the earliest known plant disease but also one of the most widely distributed, occurring in every region of the world where rice is grown. Ten isolates of *Pyricularia oryzae* causing rice blast disease were evaluated for their morphological, pathogenic, virulence and genetic characterization using RAPD marker. Isolates were classified into three groups based on pathogenicity *viz.* severely pathogenic, moderately pathogenic and mildly pathogenic and also into four another gropus i.e. PG-I, PG-II, PG-III and PG-IV based on cultural & morphological characteristics. The molecular polymorphism among isolates by means were analyzed 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 80 to 35 percent respectively. The cluster analysis by unweighted pair group method with arithmetic average (UPGMA) separated the isolates into two major groups at 0.53 of similarity coefficient. Through morphological characterization, pathogenicity and RAPD grouping of isolates suggested no correlation among test isolates but genetic variability among *P.oryzae* isolates should be taken into account for the screening of blast resistant rice genotypes.

Index Terms- Blast disease, Rice, RAPD, Pathogenecity,

I. Introduction

Blast disease is caused by *Magnaporthe oryzae* (Hebert) Barr., anamorph *Pyricularia oryzae* (Rossman *et al.,* 1990) is an important fungal disease of rice known to occur in most rice producing areas of the world (Ou, 1985). The disease can strike all aerial parts of the plant. Most infections occur on the leaves, causing diamond-shaped lesions with a gray or white center 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 neighboring 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 deepwater 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) favor 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 unraveled 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 geographical regions of north India by using morphological, pathological, and RAPD analysis.

II. MATERIAL AND METHOD

Collection and Isolation of blast pathogen:

Samples of typical blast symptoms on rice leaves were collected from different rice growing regions of noth Indiaduring 2010-11. The infected portion were cut into small pieces and surface sterilized by dipping in 0.1% $HgCl_2$ for 1 min. and rinsed three times with sterile distilled water and transferred onto the surface of water agar. The mycelium growing out of the plant tissue was subcultured to potato agar (PDA) and incubated at 25^0C for 7 to 10 days. The isolates were identified based on the morphological and cultural characteristics of pathogen. After confirming microscope examination, one monoconidial culture from each isolate was prepared and used in this study (Table 1).

Examination of cultural and morphological characteristics:

The isolates were cultured on PDA as well as on OMA (Oat meal agar) media at 25^0C for 7 days, after which mycelia disks were transferred to the center of a new PDA/OMA medium. The colony morphology and colony colour of each isolates on PDA/ OMA medium were examined daily from 5 -10 days. For sporulation the culture were maintained in 12 hr night and dark alternatively, then conidia were harvested from each isolates and mounted in water. The shapes of conidia were measured under image analyzer.

Pathogenicity test under in vitro and glasshouse condition:

Pure culture of each isolates are grown on OMA for 7-10 days at 25^0C under alternating 14 hour of fluorescent light and 10 hour dark cycle to induce sporulation (Barksdale and Asai, 1961). The conidial suspension was harvested, filtered and centrifuged at 5000 rpm. The mass of spore sedimentation was collected, resuspended with sterilized distilled water and spore density was adjusted to a concentration of 1x 10^6 spore/ml using heamocytometer. Freshly collected immature and untreated leaves were washed under running tap water for 60 seconds followed by surface sterilization and immersing the leaves in 70% ethanol for 3 minutes, 1% sodium hypochlorite solution for 3 minutes and then rinsing three times in sterilized distilled water for 2 minutes each time and drying with sterile tissue paper and then air drying. The surface rice leaves were pinpricked with sterile needle then placed in the petridish which is equipped with moist cotton. The drop of 6 µl of 10^6 spores/ml was placed on the pinpricked or wounded spots and incubated in moist chamber at 25^0C and 95% relative humidity (Barksdale and Akai, 1961). The sterile water was used instead of spore suspension served as control under in vitro condition. In another experiment, the conidial spore suspension @1 x 10^6 spore/ml was prepared and sprayed at 3-4 leaf stage on rice leaves under glass house condition. The inoculated plants were covered with polythene sheets for incubation and maintenance of temperature and relative humidity. The appearance of symptom was observed four days after inoculation (Padmanabhan, 1965 and 1965b).

Genomic DNA isolation:

P. oryzae isolates were revived and grown on potato dextrose agar (PDA, Hi Media) plates at 25°C for 5 days (Table 2). 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).

Molecular diversity analysis 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 $MgCl_2$ 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- Σpi2;

where pi is the frequency of the ith allele (Smith *et al.,* 1997)

III. Results

Examination of cultural and morphological characteristics:

Ten isolates of *P. oryzae* assigned to six morphological groups (PG-I to PG-VI) based on the differences in morphological characteristics (colony color, colony morphology and conidia shape). Various isolates produced ring like, circular, irregular colonies with rough and smooth margins on OMA media having buff color, grayish black to black color. The colony diameters of different groups were ranged from 67.40 to 82.50 mm. The colonies of group PG-I produced ring like smooth margin with buff to grayish color, PG-II produced circular smooth margin with buff color, PG-III produced circular rough margin with grayish black to black color, PG-IV produced Ring like cottony colony with buff color, PG-V produced irregular rough margin colony with buff color, PG- VI produced circular smooth colony with raised mycelium having buff color. The conidia shape of the different groups was pyriform (pear-shaped) with rounded base, and narrowed towards the tip which is pointed or blunt (Table 1).

Pathogenicity test:

The existence of strains of *P. oryzae* differing in pathogenicity was first noticed by Sasaki (1922). The variable pathogenicity was observed upon inoculation of *P. oryzae* on the rice leaves. All the isolates were pathogenic and produced blast symptoms. On leaves symptoms first appear as small spot and later they enlarge up roundish, slightly elongated necrotic sporulating spots to narrow or slightly elliptical lesions more than 3 mm long with a brown margin. Based on the blast lesions and affected area of leaves isolates were designated into three groups *i.e.* PGV-I, PG-II, PG-III. The first group, PGV-I was designed as mildly virulent strain consisting of two isolates Blast B4, Blast 2. Five isolates, Blast H-1, Blast 4, Blast 6(2), Blast H-8 and Blast H-3 were assigned to group PGV-II designated as moderately virulent strain with the remaining isolates assigned to group PGV-III Blast 6(2)B, Blast H-13, Blast H-5 severely virulent isolates. .

Random amplified polymorphic DNA (RAPD) analysis:

A total of 10 isolates of *Pyricularia oryzae* were tested or their genetic variability by RAPD analysis using twenty five random primers. Of these, eighteen primers produce easily scorable and consistent banding patterns, which were used for RAPD analysis of test isolates (Table 2). The generated fingerprints were evaluated for overall clearness of the banding pattern. The primers showed polymorphism and consistently produced 2 to 7 bands of 0.3kb to 2 kb although majority was below 1kb. All eighteen primers were polymorphic and having PIC values ranging from 0.54 to 0.90 (Fig 1)

The RAPD scores were used to create a data matrix to analyze genetic relationships using the NTSYS-pc software program version 2.02 described by Rholf, (1993). 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. In group A three isolates Blast B4, Blast 2, Blast H1 separated whereas in group B seven isolates Blast 4, Blast 6(2), Blast 6(2)B, Blast H13, Blast H5, Blast H8 & Blast H3.Major group A is again divided into two subgroups i.e. subgroup IA and IIA. Subgroup IA is clustered with two isolates Blast B4 and Blast 2. Blast H1 is clustered alone in subgroup IIA (Fig 2)

Major group B was again divided into four subgroups i.e. subgroup IB, IIB, IIIB, IV B. Blast 4 and Blast 6(2) B separated in the same group and Blast 6(2) separated alone in subgroup IB. In subgroup IIB Blast H13 and Blast H5 separated while Blast H8 and Blast H3 separated in subgroup IIIB and IV B respectively.

Maximum similarity among ten blast fungus isolates based on RAPD profile were found in between Blast H5 and Blast H13 (80% of similarity) followed by in between Blast 6(2) B and Blast B4 (75% of similarity) whereas minimum similarity were found in between Blast H3 and Blast H1 (35% of similarity) followed by in between Blast H3 and Blast 6(2) (43% of similarity).

IV. 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 northwestern Himalayan region of India was analysed using RAPD markers, which showed high genotypic variation in the pathogen population (Rathore *et al.,* 2004). Studies on morphological character of different isolates of *P. oryza* revealed variation with respect to colony color, morphology and conidia shape. Colony colour varies from buff colour to black colour and producing smooth and rough margin colony. However, no variation with respect to conidial shape was noticed. Where conidia was pyriform, almost hyaline to pale olive, 2-septate, 3-celled. These characters are in agreement as described by Shirai (1896). Rice hosts of *P. oryzae* show a continuous array of symptoms in reaction to the infection of various isolates of the fungus-from very minute brown specks (resistant), to roundish lesions a few millimeters in diameter with small, grey necrotic centers and brown margins (intermediate), to large elliptical lesions, with large, grey necrotic centers and brown or grey margins (susceptible).The lesion morphology commonly observed with the rice infection are the typical eye shaped with grayish center and brown margin. Tremendous variation in virulence has been documented in field population of the blast fungus (Bonman *et al.,* 1986; Correa-Victoria *et al.,* 1993; Lee and Chao, 1990; Ou, 1980, 1985; Zeigler *et al.,* 1995) and to some degree among asexual derivatives of single spore isolates (Latterell and Rossi, 1986; Valent *et al.,* 1991). In present study Blast 6(2)B, Blast H-13, Blast H-5 posses higher pathogenicity collected from north India (Siddhathnagar and Delhi) respectively whereas two local isolate Blast B4 and Blast 2 were mildly virulent collected from Kumarganj, Faizabad and Siddharthanagar respectively. Differences in pathogenicity between individual isolates have been used for a long time to assess variation in natural pathogen populations (Burdon, 1993).

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 25 RAPD primers were used among them 18 were amplified that gives total of 193 band out of them 106 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 (2005 a) 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).

V. Conclusion

We conclude that *P. oryzae* from various regions of North India consists of variable populations based on cultural morphology, virulence pattern and RAPD analysis. Molecular phylogenetic grouping obtained by RAPD analysis did not correlate with morphological characteristics and virulence pattern. In the present study RAPD data failed to reveal relationship between clustering in the dendrogram and in pathogenicity but two isolates from Delhi having high pathogenicity were clustered in same group shows close genetic identity. However other isolates were genetically varied with respect to geographical distribution. Pathogen diversity plays a major role in disease dynamics and consequently, in the success of disease management strategies, including the development of cultivars resistant to diseases. The result of the present study demonstrates that there is a certain level of genetic diversity among isolates of *P.oryza* from various regions of north India. Pathogencity tests revealed that these isolates expressed different level of virulence. 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 regions of north India is genetically heterogeneous and the interrelationships amongst the different isolates can be easily, precisely and reliably explained by RAPD.

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Table 1: Cultural and morphological characterization of different isolates of *P. oryzae* casuing rice blast disease

Isolate	Location	Colony morphology	Colony colour	Conidia shape	Pathogenecity	Morphology group	Pathogenicity group
Blast B4	Kumarganj, Faizabad (U.P. India)	Ring like, smooth margin	Buff colour	Pyriform, small	Mild	PG-I	PGV-I
Blast 2	Dhensa-A, Siddharthnagar	Circular smooth margin	Buff colour	Pyriform, medium	Mild	PG-II	PGV-2
Blast H-1	IARI, New Delhi	Circular, rough margin	Grayish Black	Pyriform, medium	Moderate	PG-III	PGV-II
Blast 4	Kumarganj, Faizabad	Ring like, cottony, smooth margin	Buff colour	Pyriform, medium	Moderate	PG-IV	PGV-II
Blast 6(2)	Parsiya, Siddharthnagar	Circular smooth margin, raised myecilium	Buff colour	Pyriform, small	Moderate	PG-VI	PGV-II
Blast 6(2)B	Chhatra (Shoharatgarh), Siddharthnagar	Irregular rough margin	Buff colour	Pyriform, medium	High	PG-V	PGV-III
Blast H-8	IARI, New Delhi	Circular, rough margin	Black colour	Pyriform, large	Moderate	PG-III	PGV-II
Blast H-3	IARI, New Delhi	Ring like, smooth margin	Grayish black colour	Pyriform, large	Moderate	PG-I	PGV-II
Blast H-13	IARI, New Delhi	Circular, rough margin	Black colour	Pyriform, medium	High	PG-III	PGV-III
Blast H-5	IARI, New Delhi	Circular, rough margin	Black colour	Pyriform, large	High	PG-III	PGV-III

Table 2: RAPD primers used in the present study; their sequence, annealing temperature, % polymorphism and Polymorphic Information content (PIC) value

S.No.	**Primer**	**Primer sequence (5'-3')**	**Total no. of band amplified**	**Polymorphic band**	**Polymorphism (%)**	**PIC**
1	OPA 13	CAGCACCCAC	6	4	66.7	.90
2	OPA 16	AGCCAGCGAC	8	5	6 2.5	.82
3	OPA 17	GACCGCTTGT	9	6	66.7	.83
4	OPA 19	CAAACGTCGG	6	5	83.3	.80
5	OPB 08	GTCCACACGC	10	7	70	.85
6	OPB 11	GTAGACCCGT	11	6	54.5	.72
7	OPB 13	TTCCCGCGCT	8	6	75	.86
8	OPB 18	CCACAGCACT	7	5	71.4	.82
9	OPB 10	CTGCTGGGAC	10	6	60	.73
10	OPN 01	CTCACGTTGG	8	6	75	.87
11	OPN 07	CAGCCCAGAG	12	9	75	.85
12	OPK 11	AATGCCCCAG	10	6	60	.70
13	OPK 17	CCCAGCTCTG	7	4	57.2	.67
14	OPO10	TCACAGCGCC	11	9	81.8	.79
15	OPO 12	TCACAGCGCC	8	7	87.5	.82
16	OPO 11	GACAGGAGGT	-	-	-	-
17	OPC 07	GTCCCGACGA	-	-	-	-
18	OPC 15	GACGGATCAG	7	4	57.1	.57
19	OPO 08	GTGTGCCCCA	9	5	55.6	.54
20	OPY 15	AGTCGCCCTT	10	6	60	.71
21	OPY 18	GTGGAGTCAG	-	-	-	-
22	OPY 14	GGTCGATCTG	-	-	-	-
23	OPY 09	AGCAGCGCAC	-	-	-	-
24	OPF 14	TGCTGCAGGT	-	-	-	-
25	OPF17	AACCCGGGAA	-	-	-	-
		Total	193	106	54.92	

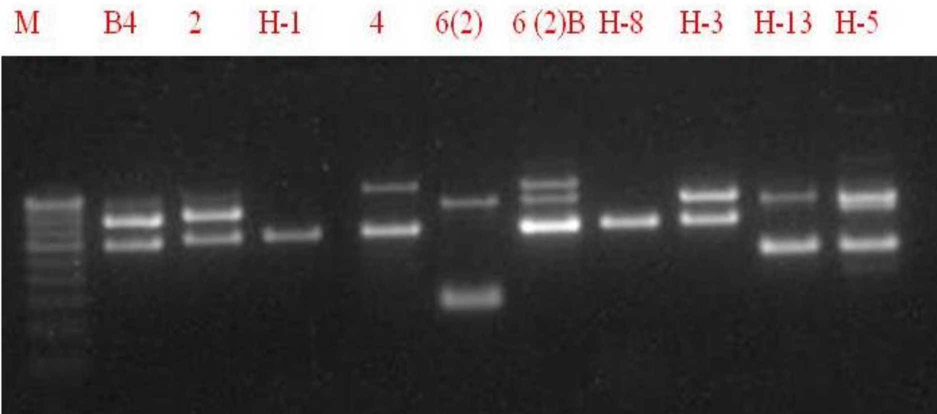

Fig. 1: Amplification of *P. oryzae* genomic DNA with primer OPB18, M: 100 bp ladder (B4 to H5 *P. oryzae* isolate)

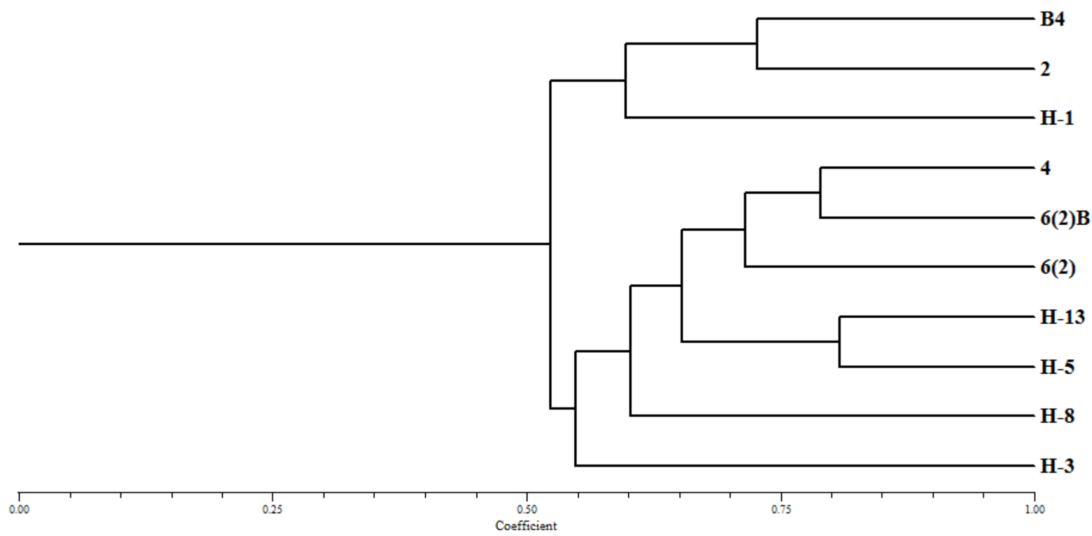

Fig .2: Dendrogram showing the relationship between the ten *P. oryzae* isolates derived from cluster analysis of the RAPD profile.