Molecular Characterization of *Pyricularia oryzae* Through Randomly Amplified Polymorphic DNA Marker

A THESIS

BY

STUDENT ID. MS-190806

A Thesis (Course No. PLP-6104) Submitted to Agrotechnology Discipline in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

IN

PLANT PATHOLOGY

AGROTECHNOLOGY DISCIPLINE
LIFE SCIENCE SCHOOL
KHULNA UNIVERSITY
KHULNA-9208

JUNE 2022

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Approved as to Style and Contents

By

.....	.....
Prof. Dr. Mst. Sabiha Sultana	**Prof. Md. Rejaul Islam**
Supervisor	**Co-Supervisor**

.....

(Chairman, Examination Committee

and

Head, Agrotechnology Discipline)

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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. Assessment of genetic diversity of pathogenic fungi provides a basis for development of future resistant crop variety. An experiment was conducted during the period from September 2020 - December 2021 in the Plant Protection Laboratory of Agrotechnology Discipline at Khulna University, Khulna, to determine the genetic variability among nineteen isolates of *Pyricularia oryzae*. The isolates were isolated from infected disease samples collected from different locations of South-Western rice growing regions of Bangladesh. Total ten arbitrary RAPD primers were used which generated clearly visible banding patterns. Only four primers (OPA-02, OPA-03, UBC-155 and UBC-173) produced a low number of bands on the PO-16 isolate. The other primers did not produce any bands on the PO-16 isolate, indicating that the PO-16 isolate is different from the other isolates. After cluster analysis two distinct clusters were observed. Cluster I contained only one isolates PO-16 that belonged to AEZ-13. Cluster II consisted of 18 isolates that were originated from different origin but having maximum 94% genetical similarity. The most genetic diversity was observed between PO-16 and PO-03 isolates belonging to the same location AEZ-13, i.e., showing 79% genetic distance, indicating that there is no direct relationship between genetic diversity and geographical origin. PCA analysis confirmed the clustering analysis and showed that PO-16 was a genetically distinct isolate.

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March 2022 **TheAuthor**

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Rice (*Oryza sativa*) is the main source of food grain for more than half of the world population (Yadav et al., 2015). Asia is the largest rice producing and consuming area where 60% of the world's people live (Khush and Jena, 2009). It is the staple food in the Asia providing 21% of global human per capita energy and 15% per capita protein (Akter et al., 2019). Rice is native to Tropical and Subtropical Southern Asia and Southeastern Africa.

Rice is the staple food in Bangladesh. It constituted about 85% of the total food grain production and covers about 76.86% of the total cultivable land in the country. The yield of rice in Bangladesh is 3.53t/ha (BBS, 2020).

Rice is often attacked by many pests and diseases causing huge amount of annual losses in the world. Among them rice blast, caused by *P. oryzae*, is significant in case of economic importance. Outbreaks of the disease is a serious and recurrent problem worldwide. There is a loss of about 28-36% average due to blast and in certain areas it could be as high as 80-100% (Hossain et al., 2017).

The disease was recorded on Boro and Transplanted Aman season all over Bangladesh. Some susceptible varieties are detected like BRRI dhan28, BRRI dhan29, BRRI dhan50, BRRI dhan61 and BRRI dhan63. The disease has the potentiality to destroy 70% of crop yields in worst-case in Bangladesh (Rayhanul et al., 2019).

Rice blast fungus can attack the rice plant at any growth stage causing severe leaf necrosis and impede grain filling, resulting in decreased grain number and weight. The pathogen can

infect all the above ground parts of rice plants at different growth stages, i.e., leaf, collar, nodes, internodes, base or neck and other parts (Ram et al., 2007). The drizzle brings down the spores on the leaf, node or on panicles. And then the spores start growing to produce conidiophores (a fungal hypha) and conidia (asexually produced spore) within 3-4 days (Rahman and Uddin, 2017).

Frequent and prolonged dew periods in the morning along with lower night temperature than day time is the most favorable environment for blast inoculation (Castilla et al., 2009). Continuous rains and average night temperatures between 18-25°C during the flowering stage of the crop followed by sunny, hot and humid days are the favorable climatic condition of this disease. The prolonged duration of the wetting period with 30°C occurs highest blast intensity (Chumley and Valent, 1989). Temperature, moisture, sunshine, humidity and wind speed play a major role in the infection and development of the disease (Couch and Kohn, 2002).

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). Genetic variability helps pathogens to break down host resistance. So to know the causes of the variability is very much important for developing resistant varieties. Molecular characterization helps breeders to perfectly determine the cause of variability and develop pathogen-resistant varieties.The present study was planned to investigate the diversity of *P.oryzae* from different geographical regions of south-western region of Bangladesh by using RAPD analysis

Objective

With the above facts the present piece of research work was under taken to fulfill the following objective:

- To find out the molecular variability among the isolates of *Pyricularia oryzae* using Random Amplified Polymorphic DNA (RAPD) marker

CHAPTER II

REVIEW LITERATURE

2.1 Rice blast disease

Rice blast disease, caused by the ascomycete fungus *Pyricularia oryzae*, is one of the most important and potentially devastating diseases of rice (Ou, 1985). It was first recorded as rice fever disease in China in 1637 and was later described as imochi-byo in Japan in 1704 (Wang, 2009). The pathogen is currently reported to be present in over 85 countries worldwide. The fungus can attack the aerial parts of the rice plant at any stage of growth, including leaves, sheaths, nodes, and panicles. Neck blast, where infection occurs on the node below the panicle, can cause breakage of the panicle or poorly developed grain (Webster et al., 1992). When the environment is favorable for disease development, neck blast can cause significant yield losses (Suzuki, 1975; Baker et al. 1997).

The disease was recorded on Boro season and Transplanted Aman in all over country. The government of Bangladesh is taking initiative to the extension of the Aus coverage. It means rice is grown with intensive care throughout the year. Thus the popular varieties are getting susceptible to some pest and diseases. Even the pests are getting the opportunities to change their races for better adaptation (Biswas, 2017).

2.2 The pathogen

The rice blast disease is caused by a ascomycete fungus named *Pyricularia oryzae* . It was first recorded in California in 1996. Earlier the perfect stage of *Pyricularia oryzae* was named as *Ceratosphaeria grisea (*Hebert, 1971). Then Yaegashi and Nishihara (1976) suggested the genus

Magnaporthe. Yaegashi and Udagawa (1978) finally proposed *M. grisea* as a perfect stage of *Pyricularia oryzae* instead of *Ceratosphaeria grisea*. It is filamentous ascomycetes that can reproduce both sexually and asexually. Sexual reproduction occurs when two strains of opposite mating types meet to form a fruiting structure known as perithecium in which ascospores is formed (Dean et al., 2005). The asexual life cycle begins when the hyphae of the fungus produces fruiting structures and sporulates to give conidia.

There are several epidemic outbreak of the disease Bangladesh since 1980 (Shahajahan et al., 1994) and the cultivar of rice including aromatic varieties, in both irrigated and rain feed low lands were highly susceptible to blast disease Khan et al. (2014).

2.3 Dispersion and Host range

Hexing Qi et al. studied 103 *P. oryzae* and 20 *P. grisea* isolates, collected from seven species of plants, and analyzed their phylogeny, pathogenicity, and relationship with host preferences to investigate the differences among them from different hosts. Based on phylogenetic analysis of multilocus sequences, 16 isolates from crabgrass and four isolates from green bristlegrass were identified as *P. grisea* and another 103 isolates from crabgrass, green bristlegrass, goose grass, foxtail millet, wild millet, rice, and sedge belonged to *P. oryzae*. Results of pathogenicity tests by artificial inoculation demonstrated that six of 10 *P. oryzae* isolates from rice and three of 44 *P. oryzae* isolates from green bristlegrass showed cross-infectivity on green bristlegrass and rice, respectively. Taken together, the results demonstrated that isolates from green bristlegrass and crabgrass consist of both *P. oryzae* and *P. grisea* and that *P. oryzae* isolates showed cross-infectivity between rice and green bristlegrass, suggesting that host shifts may occur for *P. oryzae* and *P. grisea.*

The blast of rice has a significant effect in temperate, tropical, subtropical Asia, Latin America and Africa and distributed throughout the world in about 85 countries. This pathogen is also the key constraint for rice production in West Africa (Suprapta and Khalimi, 2012). Some members of the *Pyricularia grisea* can also infect other important cereal crops like wheat, rye, barley, and pearl millet causing diseases called blast disease or blight disease (Singh and Prasad, 2007).

2.4 Epidemiology of Rice Blast

Shafaullah et al. (2008) studied the effect of epidemiological factors (temperature, relative humidity and rain fall) on the incidence and severity of rice blast (*Pyricularia oryzae*), during the growing season 2008. The temperature and incidence of rice blast was negatively correlated i.e. -0.88, -0.80, -0.95, -0.84 respectively, this indicated that the disease incidence increases with the decrease of temperature. Humidity is positively correlated with Paddy blast i.e. 0.95, 0.90, 0.99, 0.89, 0.93 respectively that indicate increase in disease incidence as humidity increased. Rainfall was also positively correlated with incidence of disease i.e. 0.80, 0.90, 0.88, 0.93 and 0.84 respectively. However still more epidemiological studies are required to characterize the actual and critical factors to forecast and predict the blast disease of rice. This will help out to minimize the yield losses caused by rice blast disease.

2.5 Disease Cycle

The fungus infects the plant by the spore germinating and forming an apperssorium (a thick fungal cell) on the plant surface and then exerting a haustoria (feeding structure) into the plant cells. When the spores land on leaves and other aerials tissues of susceptible plant, they germinate and develop the appressorium which penetrate the plant cell by producing a penetration peg. Pressure in the appressorium increases and the structure explodes forcing the

penetration through the cell wall and into the cell (Dean et al., 2005). The fungus grows hyphae inter or intracellular within the leaf and form lesions. The initial infections occur on leaves usually around tillering and appear as diamond, football, or spindle shape lesion with pointed ends. Once it is established in the host plant the fungal hyphae sporulates and produce asexual spores (Kim, 1994). The pathogen completes the life cycle within one week. Each of the phases (sporulation, releases, germination and the penetration) play an important role during the blast epidemic and requires different environments. Sporulation phase is the first step that facilitate in building up the leaf blast epidemic as it provides the inoculum population (Webster and Gunnell 1992; Kim, 1994). The leaf blast phase occurs mostly between the seedling and late tillering stages. Lesions start as small water soaked areas on young leaves and enlarge quickly, under moist warm conditions, into diamond shape with a blue gray cast which are the fungal spores. However, under natural conditions, sporulation is greatly affected by the age of the crop and the size of the lesion together with the variety of rice (Kim, 1994). In case of sever or multiple infections, lesions may coalesce covering most of the leaf blast (Groth and Hollier, nd).

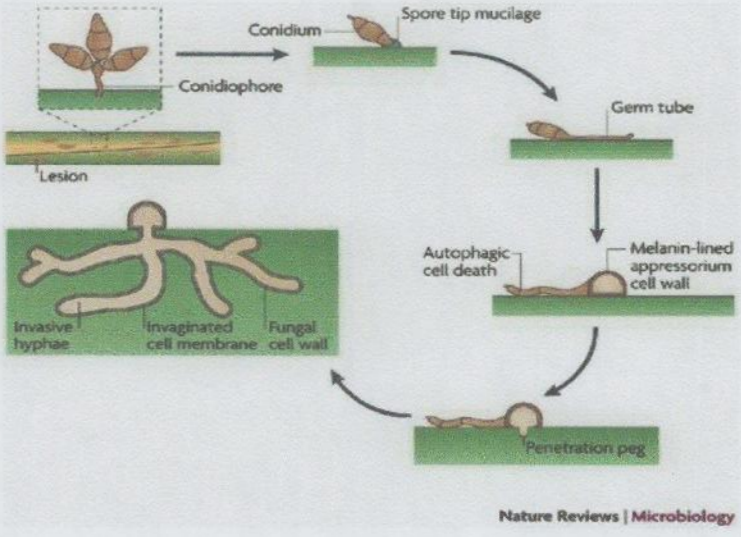

Figure 1. Disease cycle of *Pyricularia oryzae*

(Source: Researchgate.net)

The blast fungus sporulates quickly under high humidity and releases conidia in plenty. The conidia are usually transmitted by wind or rain splash to neighboring rice plants, starting another disease cycle (Hamer et al., 1988).

The blast is a trans-boundary disease, travels through the air from one region to the other, one country to another country. Infected seed also act as a carrier too. The drizzle brings down the spores and it starts growing (Bevitori and Ghini, 2015).

2.6 Yield loss of rice due to blast disease

The disease causes yield losses 30-50% in South America and Southeast Asia (Baker et al., 1997). The pathogen causes rice blast disease in rice cultivation areas worldwide. Annual rice losses caused by this fungus during 90's had been estimated 35% of the worldwide production (Oerke and Dehne, 2004).

The disease affects production statistics of rice in Pakistan (Jia et al., 2000). During the last two decades, rice blast is mostly found in districts of Faisalabad, Toba Tek Singh, Vehari and place like Gaggoo Mandi (Arshad et al., 2008).

This pathogen is the main constraint to production with yield losses ranging from 3-77% in In West Africa (Nutsugah et al., 2008). 75 percent, 50 percent and 40 percent grain loss may occur in India (Padmanabhan, 1965), Philippines (Ou, 1985) and Nigeria (Awodera and Esuruoso, 1975).

2.7 Morphology of *Pyricularia oryzae*

Morphological variation *Pyricularia* oryzae collected from different rice and grass hosts revealed that isolates showing rapid vegetative growth like gray green or gray white developed large numbers of spores than those with lower vegetative growth. Isolates from

grass hosts showed an irregular morphology of the spore that was longer, cylindrical, and pyriform are grayish cultures, single or fascicular conidiophores, simple, rarely branched and showing sympodious growth (Hawksworth, 1990).

Colony diameters of various isolates ranged from 68.40 to 83.50 mm. Blast fungi isolates developed ring shape colonies with irregular surface and smooth borders, grayish black in color (Gashaw et al.,2014).

Conidia are pyriform in shape and hyaline in color, produced acrogenously, one after another. Conidia are three celled, the middle cell being much wider and darker, and end cell germinates giving out germ tube. Conidia are rarely two celled or four celled. Conidia formed singly at the tip of the conidiophores at points arising sympodially and in succession, pyriform to obclavate, narrowed toward tip, rounded at the base, three celled rarely one or two celled, hyaline to pale olive, with a distinct protruding basal hilum (Hawksworth, 1990).The dimensions of conidia formed by *M. grisea* varied in length from 17.6 to 24.0 μm and width from 8.0 to 9.6 μm (Mijan, 2000).

The mycelium consists of septet, uninucleate, branched hypha. However, as the fungus gets older, the hypha become brown. Generally, growth of the pathogen is relatively more on upper surface making the spot more dark on upper side. Conidiophores are simple, septate, basal portion being relatively darker (Hawksworth, 1990).

Intercalary or terminal chlamydospores formation is common, which are globule, thick walled and olive brown. Chlamydospores often produced in culture, thick-walled, 5-12 μm diameter. Fungus produce sexual fruiting bodies called perithecia within 21 days. Perithecia are flask-shaped that carry asci containing ascospores, the products of meiosis. Ascospores are arranged

as unordered octads or as larger populations of randomly selected ascospores (Nicholas and Talbot, 2003).

2.8 Genetic variability

Srivastava D. et al. (2014) evaluated ten isolates of *Pyricularia oryzae* causing rice blast disease for their morphological, pathogenic, virulence and genetic characterization using RAPD marker. They analyzed the molecular polymorphism among isolates by means 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. Cluster analysis by unweighted pair group method with arithmetic average (UPGMA) differentiated the isolates into two major groups. 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.

Cristina M. et al. (2002) tested a set of 87 isolates on 32 rice genotypes including eight international differentials. Analysis of *P. grisea* isolates using rep-PCR with two primer sequences from Pot2 generated fingerprint profiles of one to nine bands. Cluster analysis revealed the occurrence of six fingerprint groups with similarities ranging from 0.09 to 1. There was no straight relationship between virulence of the isolates based on reaction pattern on 32 genotypes and grouping based on Pot2 rep-PCR analysis of *P. grisea* isolates collected from 'Metica-1'.

Shahriar et al. (2020) found high possibility in *Pyricularia oryzae* to cross over and produce fertile strains from rice, and from other grasses. Such fertile strains enhance genotypical variability of the pathogen where progeny can have new capacity to infect various rice cultivars.

Sahu C. et al. (2018) evaluated twenty isolates of blast isolates with two ITS primers. The sequence analysis of the OD-2 blast isolate showed maximum similarity with Malaysia isolate. In phylogenetic analysis, the blast isolates were grouped into two major clusters. The Odisha isolate (OD-2) along with Chhattisgarh, are in one cluster whereas, blast isolates from China and Japan are in another cluster. They found high degree of morphological and genetic variation among the isolates collected from different locations. The finding of this study would enhance the practical application of research in the field of epidemiology, disease control and developing breeding strategies for blast disease.

2.9 Random Amplified Polymorphic DNA (RAPD)

The RAPD technique uses a single arbitrary sequence primer which is usually 10 bases long (Ross, 1995). RAPD was initially one of the more popular markers used in population diversity studies due to the simplicity of the approach as no sequence information of the organism was needed. There were many reports of the use of RAPD technique in *P. oryzae* population genetics studies (Ross, 1995; Sharma et al. 2002; Rathour et al., 2004; Chadha and Gopalakrishna, 2005; Sirithunya et al., 2008). However, the RAPD approach has subsequently been shown to have problems with reproducibility.

RAPD markers are genetically reliable and have commonly been used in plant's (Grimmer et al., 2007) and other organism's (Abd El-Salam, 2004) genetic mapping. The technique is easy, sensitive and quick to detect, distinguish, and determine the phylogenetic relationship between isolates of multiple pathogen species (Narayanasamy, 2001).

Shamim et al. (2014) evaluated ten isolates of *Pyricularia oryzae* for their morphological and genetic characterization using RAPD marker. 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.

Noman et. al. (2021) evaluated twenty-four monoconidial isolates of *Pyricularia oryzae* representing four major districts, namely Chuadanga, Meherpur, Kustia and Jhenaidah of Bangladesh using eight RAPD and four ISSR primers for genetic diversity assay producing a total of 94 bands of which 85% were polymorphic.

CHAPTER III

MATERIALS AND METHODS

3.1 Experimental Site

The following experiment was performed in the Plant Protection laboratory of Agrotechnology Discipline, Khulna University, Khulna during the period from September 2020 - December 2021.

3.2 Collection of Diseased Sample

Typical leaf blast diseased leaves/neck of rice were collected from the South-Western rice growing regions Bangladesh during March, 2020 to September, 2020.

Figure 2. Collected diseased samples showing blast symptom

Table 1. Blast disease leaf sample collection site with designation and pathogenic reaction

AEZ No.	Zones	Location	No of isolates	Isolate Designation	Pathogenic reaction
AEZ-11	High Ganges River Floodplain	Bagherpara- Jeshore, Sharsha-Jeshore	2	PO-2, PO-11	+
AEZ-12	Low High Ganges River Floodplain	Lohagara-Narail,	1	PO-1	+
AEZ-13	Low Ganges River Floodplain	Satkhira Sadar, Dumuria-Khulna, Samnagar- Satkhira, Batiaghata-Khulna, Debatta-Khulna, Terokhada- Satkhira, Morrelganj- Khulna, Rupsha- Khulna, Tala-Satkhira, Chitalmari-Bagerhat, Dasmina-Patuakhali, Mollahat- Bagerhat, Banaripara-Barishal, Boalmari-Faridpur, Asasuni-Satkhira, Kathalia-Jhalokathi	16	PO-3, PO-4, PO-5, PO-6, PO-7, PO-8, PO-9, PO-10, PO-12, PO-13, PO-14, PO-15, PO-16, PO-17, PO-18, PO-19	+

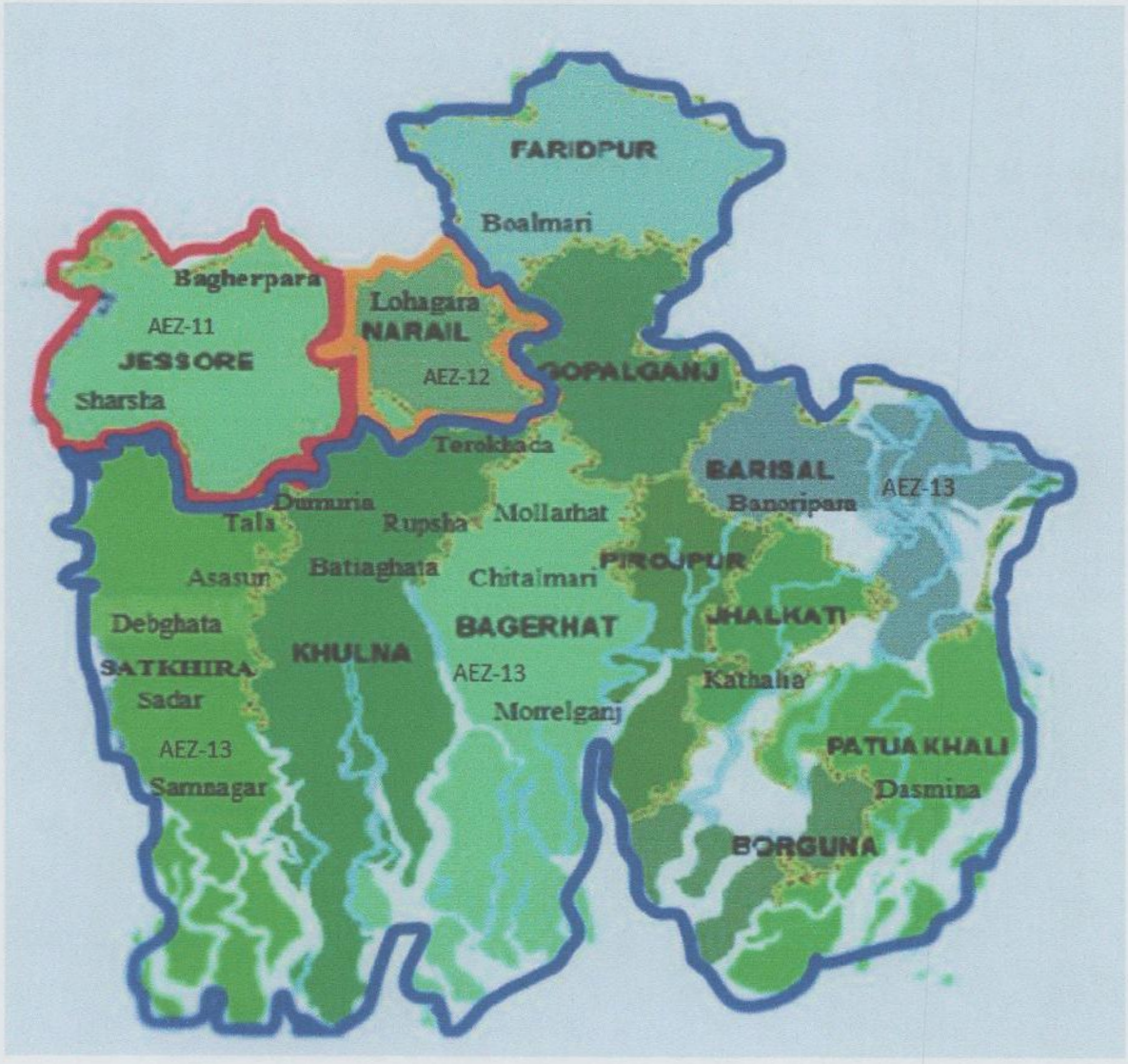

Figure 3. Sample collection area map

3.3 Isolation of the Pathogen

The collected plant parts were used for isolation of the pathogen. Diseased samples were cut into small pieces around the infected area including the edge of the lesion (1-2cm) surface sterilized with 1% sodium hypochlorite. These samples were kept inside laminar air flow on moist blotting paper to enhance sporulation for 2-3days.

After incubation, these infected plant pieces were examined under electrical microscope to confirm the typical shape of *Pyricularia oryzae* spores. Single spore was picked up with a glass needle and incubated on PSA media for about one week. The isolates were identified based on the morphological and cultural characteristics of pathogen after confirming microscope examination.

3.4 Morphological identification

Characteristics features of the germinated spore were observed cautiously by preparing temporary slides with glycerin solution. Morphological descriptions given by Barnett and Hunter (1972) were followed to identify *Pyricularia oryzae* conidia. Carl Zeiss compound microscope (40X) was used in this process (Carl Zeiss, Germany).

3.5 DNA isolation

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. (Hamer and Givan 1990). Invitrogen™ PureLink™ Pro 96 Genomic DNA Purification Kit was used for DNA isolation according to the manufacturer's protocol.

3.6 Storage of DNA

Genomic DNA of 19 isolates was extracted and DNA solution containing eppendorf tube was stored at -20°C. To confirm the presence of DNA materials test electrophoresis in 1% agarose gel was run. Picture of DNA residues was seen under UV light in Gel documentation system.

3.7 DNA quantifications

Multiskn GO Microplate Spectrophotometer (Thermo Fisher Scientific, Germany) was used to quantify purified DNA at a wave length of 260 nm. The final concentration of the template DNA for PCR (Kandan, 2013) was adjusted to 50 ng (μL)-1 and stored at -20° C.

3.8 TAE buffer preparation

At first 250 ml 50X TAE buffer was prepared. In this case 60.5 g Tris base, 4.75 g EDTA salt was weighted with a digital balance. Then EDTA salt was mixed with 100 ml sterilized Mili-Q water with a magnetic hot stirrer. After getting clean disodium EDTA solution, 60.5 g tris base was added and mixed. Then 14.5 ml glacial acetic acid was added. Volume was adjusted to 250 ml with sterilized Mili-Q water (Brown and Lorenz, 2016).

Finally 980 ml sterilized Mili-Q water and 20 ml 50X TAE buffer was mixed to prepare 1X TAE buffer (Rebecca et al., 2011).

3.9 PCR amplification

Polymerase Chain Reaction was performed in a 0.2 mL thin walled PCR tube. Amplifications were performed in thermos cycler (Biometra 4, Germany) using the following protocols: 94°C for 4 minutes, followed by 40 cycles of 94°C for 30 seconds, 30°C for 1 minute and final extension at 72°C for 5 minutes. Then cool downed to 8°C.

Table 2. Polymerase chain reaction chemicals for the molecular characterization

PCR reaction Chemicals	Concentration	Quantity (µL)
Genomic DNA	50 ng μL^{-1}	5.0
Reaction buffer without $MgCl_2$	10 X	1.0
dNTPs	10 mN	0.2
Tag DNA polymerase	5 U µL-1	0.2
$MgCl_2$	25mN	1.2
Molecular water	18 M	15.4
RAPD primer	100 M	1.0

3.10 Agarose gel electrophoresis

Amplified products were separated by electrophoresis on 1% agarose gels with TAE running buffer and stained with ethidium bromide (at 0.5 µg mL-1). High voltage switch option was pressed to supply electricity in Tris Acetate EDTA buffer solution (1.0 X TAE) for 45 minutes. Bioneer gel electrophoresis machine was used.

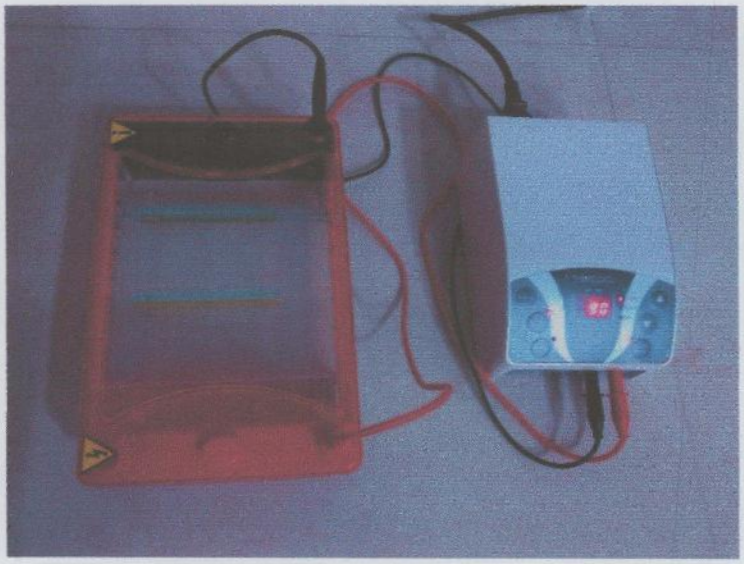

Figure 4. Bioneer gel electrophoresis machine

3.11 Gel documentation

The electrophoresis gels were scanned and photographs were taken with Biodoc Analyze 2.2 version computer program attached in gel documentation system (Biometra gel documentation, A Biodoc Analyse 2.2 version, Germany). A molecular weight marker 1kbp (Direct load, Sigma Aldrich, USA) was used for sizing of the amplicons.

3.12 PCR product analysis

Using the gel documentation system, RAPD-PCR bands were detected. For their presence (1) or absence (0), bands were scored. All scored band was considered as single allele/locus. Sizes of the bands were determined using standard 1kb ladder/marker. Ten RAPD primers were used to produce band patterns among them four primers showed clear banding patterns. Some parameters such as polymorphism percentage, polymorphic information content, resolving power of the marker and marker index were calculated to check the informativeness and discriminatory power of RAPD primers used in this study. Then binary data were subjected to similarity correlation analysis by sample matching coefficient (Sokal and Michener, 1958) and then a UPGMA dendrogram was constructed by cluster analysis. Further Principal Component Analysis was performed to calculate eigenvalue and eigenvector then a 3D plot was made using NTSYSpc (Numerical Taxonomy and Multivariate Analysis System version 2.01e) portable software (Rohlf, 2002).

3.13 Calculation of Polymorphism percentage:

It was calculated by dividing the number of polymorphic bands by the total number of bands and multiplied by 100.

$$\text{Polymorphism percentage} = \frac{\text{Number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

CHAPTER IV

RESULTS AND DISCUSSION

4. Results

4.1 DNA fingerprint analysis

There was genetic variation detected among 19 isolates of *Pyricularia oryzae* by using RAPD markers. Ten primers, used in the experiment, resulted in amplification of distinct and reproducible bands in the present investigation. The fingerprints were evaluated for overall clearness of the banding pattern. Amplified maximum number of DNA fragments was 57. Primer OPA-02 generated greater numbers of amplified fragments and give distinct amplification.

The PCR amplified products using RAPD primers are shown in Figure 5(a) to 5(j). It is noteworthy to mention that most of the primers produced more than 80% polymorphic fragments and six of them showed 100% polymorphism.

There were two types of bands found among the amplified DNA products- monomorphic and polymorphic. The bands, present in all individuals, are monomorphic and those present in one or more individuals were polymorphic bands, but not in all individuals (Mehetre et al., 2004).

Polymorphism percent (Table 3) were 69.23%, 60.00%, 100.00%, 100.00%, 100.00%, 100.00%, 71.42%, 100.00%, 100.00%, 60.00% for OPA-02, OPA-03, OPA-12, OPA-13, OPA-14, OPA-18, OPB-05, UBC-155, OPF-04, OPH-09, UBC-173 primers respectively. Different polymorphism percent show efficiency of each marker to separate and segregate in studied genotypes.

Monomorphic and polymorphic bands were detected from 500 bp to 5000bp. The monomorphic bands are more prominent near 500 bp, 1000 bp and 2000 bp .

Only four primers (OPA-02, OPA-03, UBC-155 and UBC-173) produced a small number of bands on the PO-16 isolate. The other primers did not produce any bands for PO-16.

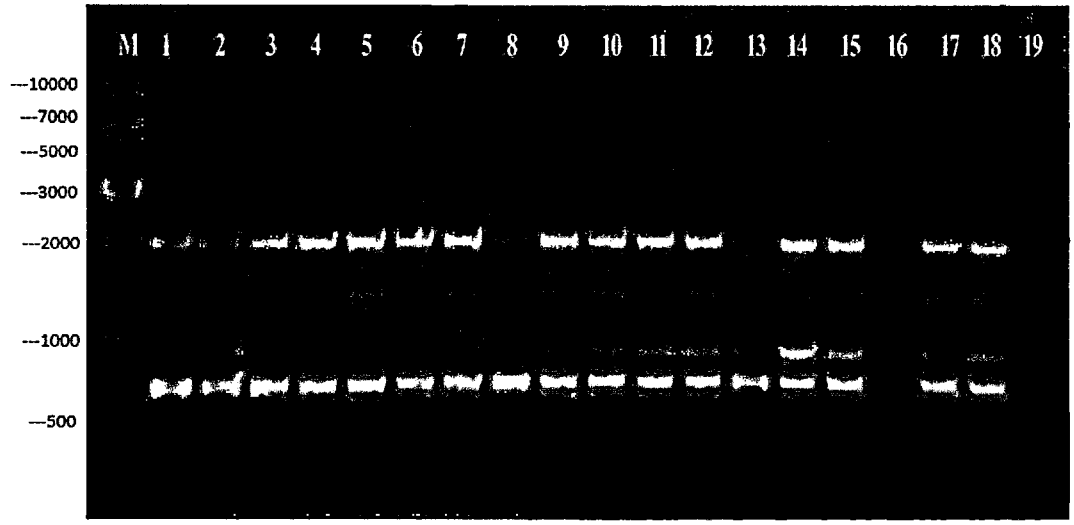

Figure 5 (e). RAPD profile of *Pyricularia oryzae* generated by using OPA-18 M- Kilo base molecular weight ladder.

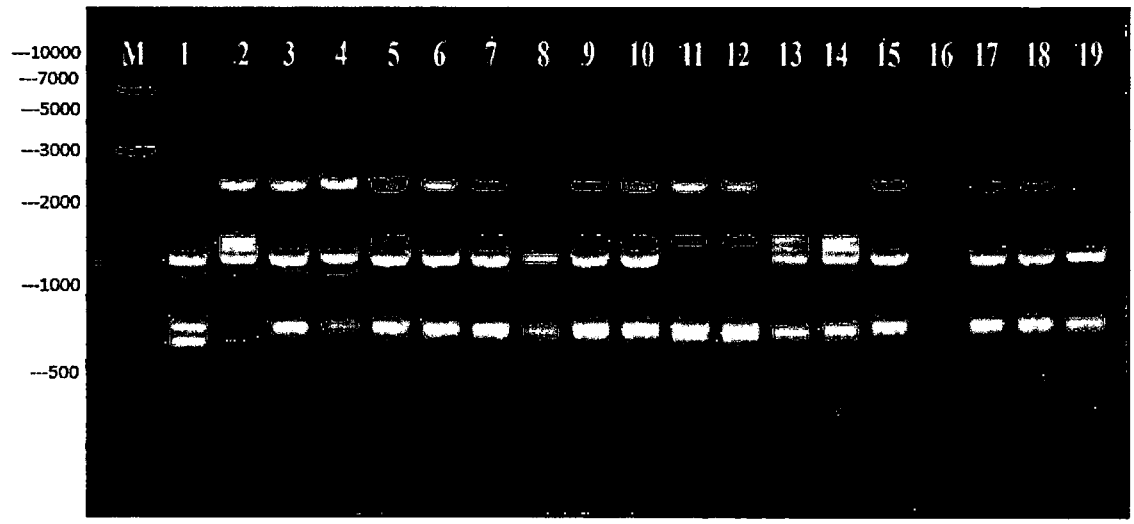

Figure 5 (f). RAPD profile of *Pyricularia oryzae* generated by using OPB-05 M- Kilo base molecular weight ladder.

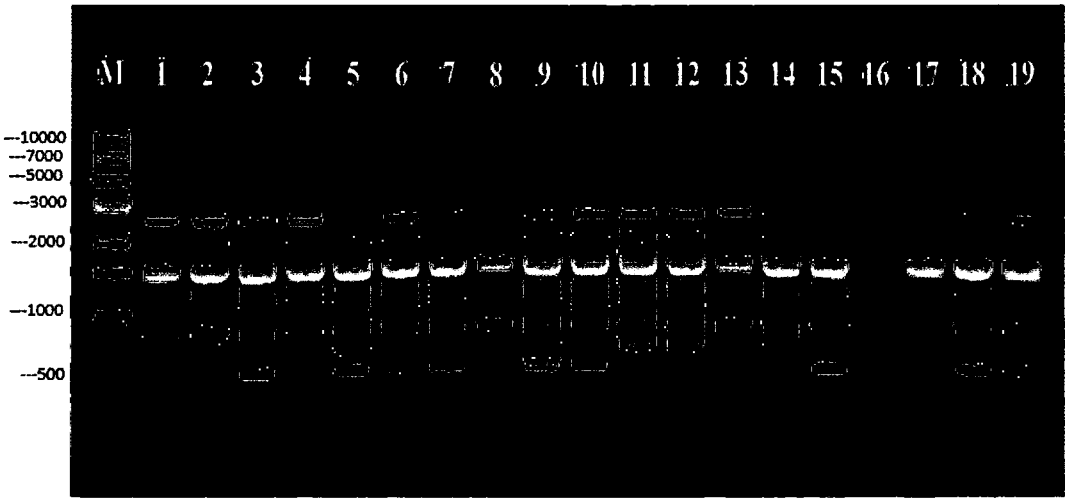

Figure 5(g). RAPD profile of Pyricularia oryzae generated by using OPF-04 M- Kilo base molecular weight ladder.

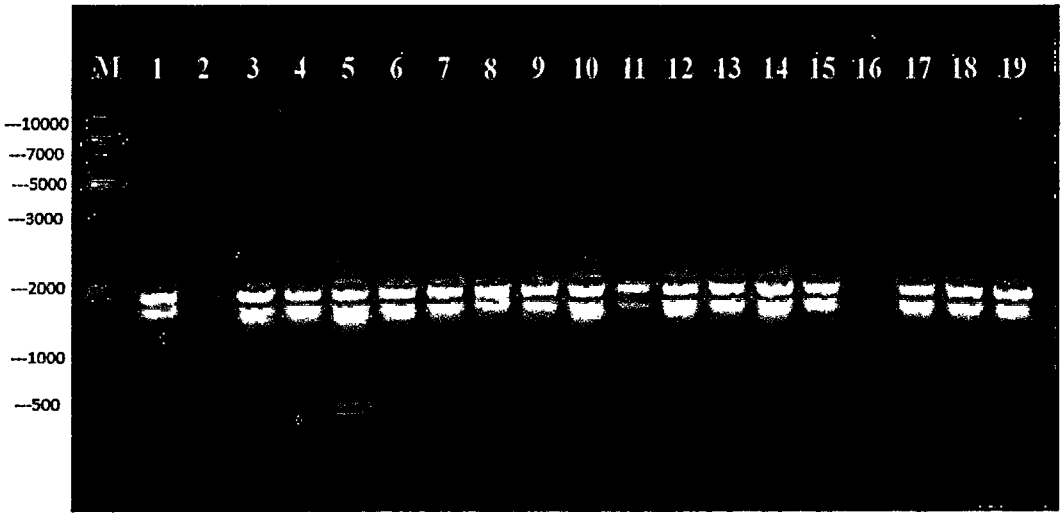

Figure 5 (h). RAPD profile of Pyricularia oryzae generated by using OPH-09 M- Kilo base molecular weight ladder.

Figure 5 (i). RAPD profile of Pyricularia oryzae generated by using UBC-155 M- Kilo base molecular weight ladder.

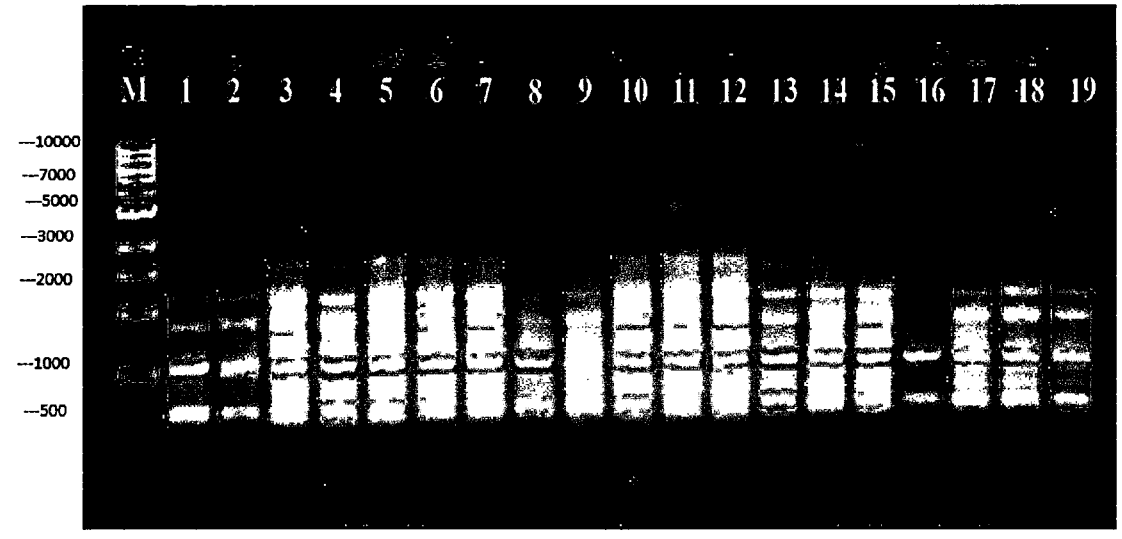

Figure 5 (j). RAPD profile of Pyricularia oryzae generated by using UBC-173 M- Kilo base molecular weight ladder

4.2 Cluster analysis

The dendrogram was constructed with the data generated from the amplifications of the 19 isolates from 10 primers, which exhibits two very distinct clusters (Figure 6). Cluster II consists of 18 isolates, cluster I contain only one isolates (PO-16). The cluster II was further subdivided into two sub-clusters. Cluster IIA contains 16 isolates and cluster IIB contains only two isolates.

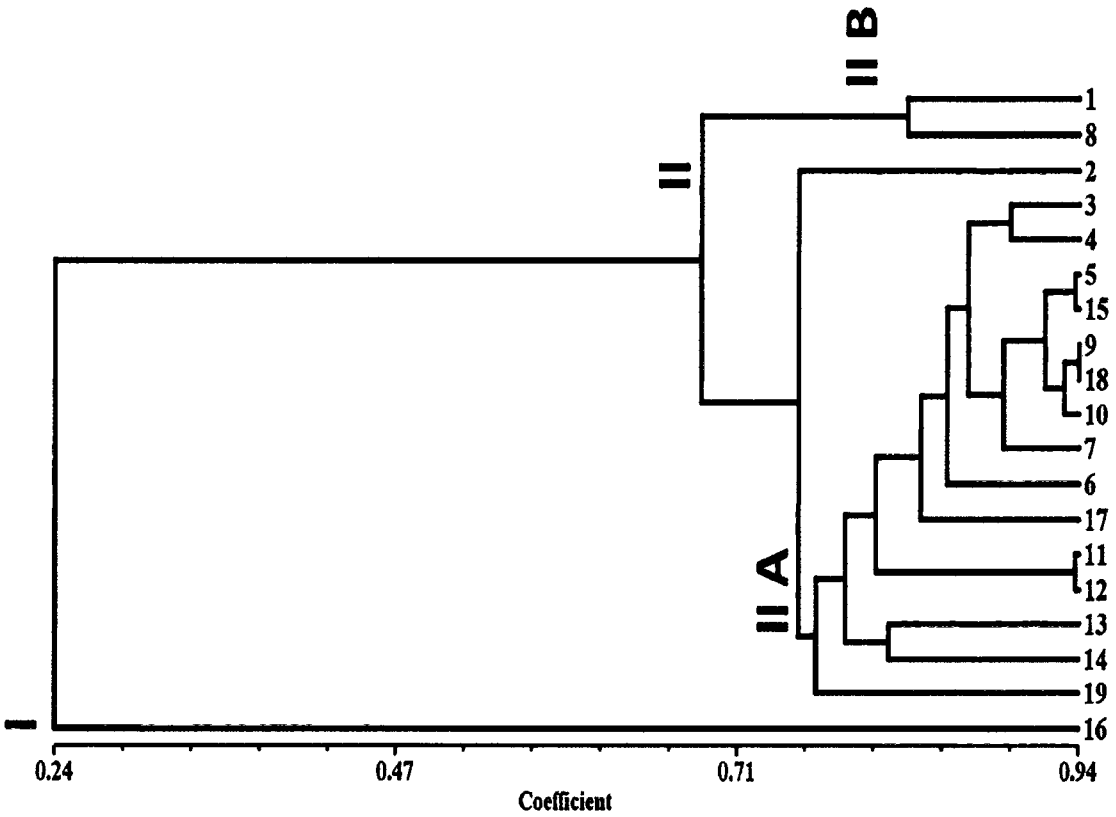

Figure 6. Dendrogram constructed based on combined data obtained from using 10 primers in RAPD analysis of 19 isolates of *Pyricularia oryzae*

4.3 Pair wise genetic similarity

Analyzing the matrix constructed with the amplification data, it was observed a coefficient of similarity between the pairs maximum similarity was 94% between the isolates PO-11 and PO-12; which belonged to AEZ-11 and AEZ-13 and minimum pair wise similarity was only 21% between the isolates PO-16 and PO-03, they are originated from AEZ-13 (Table 4).

4.4 Evolutionary relationship among the isolates

Neighbour Joining (NJ) tree demonstrate the evolutionary relationship among the isolates. To construct the NJ tree PO-16 as common emerging point (ancestor) (Figure 7). Here two distinct groups were found. Cluster I contains only one isolates and rest of the isolates belonged to cluster II. Isolate PO-08 was the first generation from evolutionary relationship among the 18 isolates.

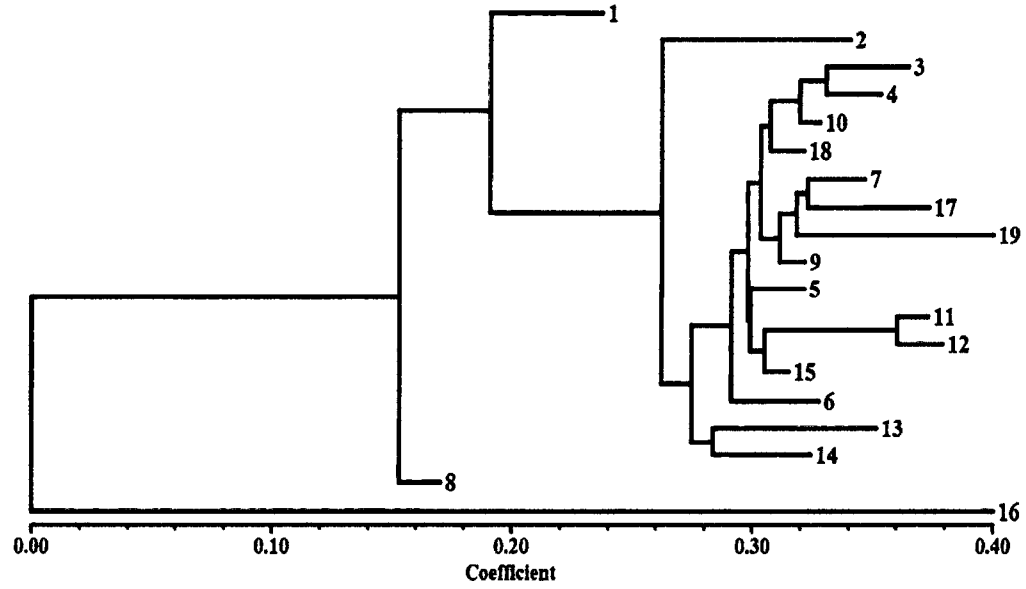

Figure 7. Evolutionary relationships among 19 isolates of *Pyricularia oryzae* through neighbor-joining method

Table 4. Pair wise genetic similarity of 19 isolates of *Pyricularia oryzae* based on DNA polymorphism:

Isolate No.	PO1	PO2	PO3	PO4	PO5	PO6	PO7	PO8	PO9	PO10	PO11	PO12	PO13	PO14	PO15	PO16	PO17	PO18	PO19
PO1	-																		
PO2	0.67	-																	
PO3	0.73	0.74	-																
PO4	0.75	0.76	0.89	-															
PO5	0.74	0.78	0.84	0.86	-														
PO6	0.71	0.76	0.82	0.80	0.89	-													
PO7	0.66	0.70	0.83	0.84	0.86	0.81	-												
PO8	0.82	o.67	0.70	0.75	0.70	0.68	0.63	-											
PO9	0.67	0.78	0.84	0.86	0.92	0.86	0.90	0.68	-										
PO10	0.71	0.78	0.92	0.90	0.92	0.86	0.90	0.68	0.92	-									
PO11	0.64	0.78	0.77	0.78	0.81	0.78	0.80	0.60	0.84	0.81	-								
PO12	0.62	0.73	0.75	0.74	0.83	0.80	0.81	0.59	0.83	0.83	**0.94**	-							
PO13	0.66	0.75	0.74	0.79	0.74	0.79	0.76	0.70	0.75	0.75	0.74	0.69	-						
PO14	0.70	0.74	0.73	0.78	0.80	0.82	0.83	0.66	0.81	0.81	0.77	0.79	0.81	-					
PO15	0.72	0.76	0.86	0.88	0.94	0.88	0.88	0.69	0.94	0.90	0.86	0.84	0.76	0.82	-				
PO16	0.28	0.25	**0.21**	0.24	0.22	0.24	0.21	0.32	0.22	0.22	0.22	0.22	0.26	0.23	0.22	-			
PO17	0.66	0.70	0.80	0.85	0.80	0.78	0.86	0.69	0.88	084	0.73	0.72	0.80	0.80	0.82	0.22	-		
PO18	0.73	0.77	0.86	0.88	0.90	0.88	0.88	0.66	0.94	0.94	0.83	0.81	0.76	0.86	0.92	0.21	0.86	-	
PO19	0.67	0.65	0.71	0.72	0.78	0.79	0.80	0.63	0.82	0.75	0.68	0.70	0.71	0.78	0.80	0.25	0.77	0.80	-

4.5 Principal component analysis

PCA converts a set of correlated variables to a new set of uncorrelated variables. The PCA simply shows components that are close to the original variables but arranged in decreasing order of variance. The cluster analysis was mostly confirmed by the PCA.

Genetically similar sixteen isolates PO-02, PO-03, PO-04, PO-05, PO-06, PO-07, PO-09, PO-10, PO-11, PO-12, PO-13, PO-14, PO-15, PO-17, PO-18 and PO-19 which formed their own group in cluster analysis and the PCA analysis (Figure 8) gave the evidence i.e. no notable genetically variation was found among the sixteen isolates. In this analysis, similarities were also found between isolates PO-01 and PO-08. PCA analysis revealed that PO-16 was a genetically distinct isolate, which was found prior to the clustering analysis.

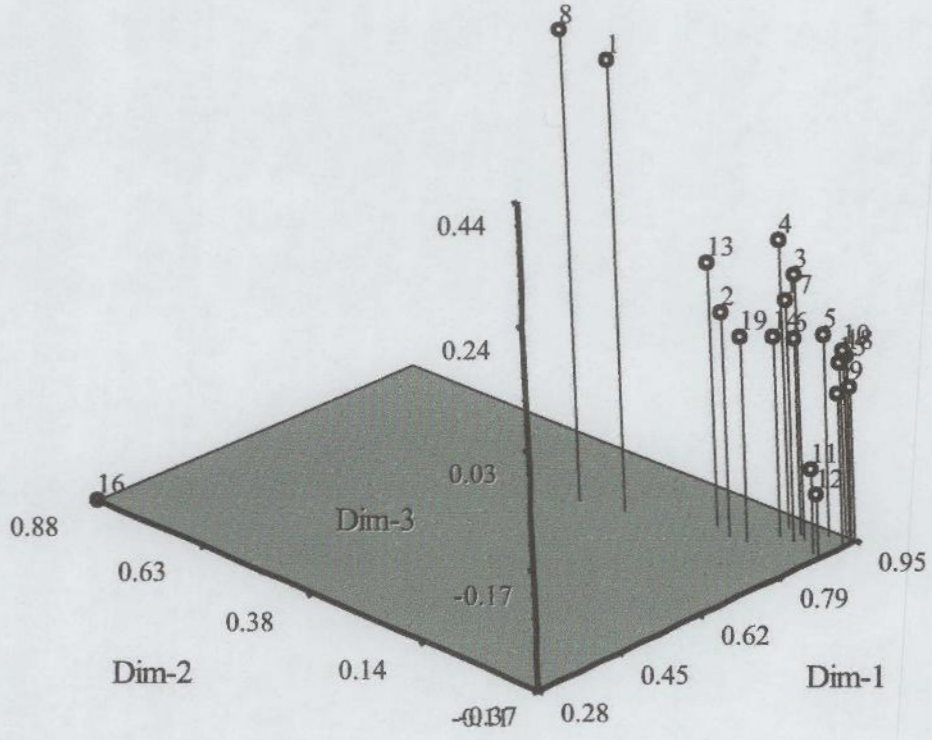

Figure 8. Three-dimensional plot of PCA showing relationships among the 19 isolates *Pyricularia oryzae* using genetic traits

4.6 Discussion

Shahriar et al. (2020) reported high possibility in *Pyricularia oryzae* to cross over and produce fertile strains from rice, and from other grasses. Such fertile strains enhance genotypical variability of the pathogen where progeny can have new capacity to infect various rice cultivars.

Use of molecular techniques particularly PCR technique has revolutionized the analysis of the diversity of the plant pathogens and helped to reveal population structure and genetic diversity (Banerjee et al., 2014). Higher genetic variation was detected among the isolates of *Pyricularia oryzae* in this experiment. Primer OPA-02 generated greater numbers of amplified DNA fragments and showed distinct polymorphism. The findings showed the applicability of random PCR primers for the study of characterization and evaluation of intra-specific polymorphisms among the isolates of *Pyricularia oryzae*. Srivastava D. et al. (2014) evaluated ten isolates of *Pyricularia oryzae* causing rice blast disease based on genetic characterization using RAPD marker. Powell et al. (1996) reported the level of polymorphism revealed the degree of genome coverage. The molecular profiles generated by RAPD markers showed presence of some common fragments in the isolates. However common amplification product might be useful in generating diagnostic marker (Bala and Vikal, 2015). After cluster analysis two distinct clusters were observed. Cluster I contains only one isolate PO-16 which was collected from AEZ-13. Cluster II consists of 18 isolates that were originated from different origin named AEZ-11 and AEZ-13 but having maximum 94% genetical similarity. Shamim et al. (2014) analyzed ten isolates to test molecular polymorphism by RAPD-PCR. 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. Sahu C. et al. (2018) evaluated twenty isolates of blast isolates with two ITS primers. The sequence analysis of the OD-2 blast isolate showed maximum similarity

with Malaysia isolate. In phylogenetic analysis, the blast isolates were grouped into two major clusters. High degree of morphological and genetic variation among the isolates was found collected from different locations. Noman et. al. (2021) evaluated twenty-four monoconidial isolates of *Pyricularia oryzae* representing four major districts, namely Chuadanga, Meherpur, Kustia and Jhenaidah of Bangladesh using eight RAPD and four ISSR primers for genetic diversity assay producing a total of 94 bands of which 85% were polymorphic. The UPGMA dendrogram differentiated the isolates into two main clusters having similarity ranged from 64 to 93%. Principal coordinate analysis showed congruent result with cluster analysis. Molecular variance analysis indicated higher level of variation (96%) in the population present within districts while a relatively low proportion (4%) of the variation was detected among districts. Principal component analysis showed similarities with the dendrogram. Isolate PO-16 has been detected as a genetically distinct isolate. Isolates PO-02, PO-03, PO-04, PO-05, PO-06, PO-07, PO-09, PO-10, PO-11, PO-12, PO-13, PO-14, PO-15, PO-17, PO- 18 and PO-19, the sixteen isolates were similar to each other and formed their own groups in the cluster analysis. There was also similarity between isolates PO-01 and PO-08 in the PCA analysis, which was consistent with the cluster analysis. In gel electrophoresis, monomorphic and polymorphic bands were detected from 500 bp to 5000 bp. The monomorphic bands were more prominent around 500bp, 1000bp and 2000bp. Only four primers (OPA-02, OPA-03, UBC-155 and UBC-173) produced a low number of bands on the PO-16 isolate. The other primers did not produce any bands for PO-16. This indicates that the isolates of PO-16 are different from the other isolates. From the pairwise distance matrix, PO-11 and PO-12 belonging to AEZ-11 and AEZ-13 had the highest similarity, while PO-16 and PO-03 had the lowest pairwise similarity of 21%, i.e., 79% dissimilarity between them, even though they both came from the same AEZ-13 (Table 4). This implies that geographic diversity does not only generate genetic distances between isolates. The high polymorphism

observed was also among *Pyricularia oryzae* isolates, but its variability was independent of their geographical origin, because genetic diversity of a population is not established based on a single trait (e.g., location), and it may be influenced by many other factors. The results of this experiment suggest that high genetic diversity exists in southwestern Bangladesh, but it is not directly related to genetic diversity and geographic origin.

CHAPTER V

SUMMARY AND CONCLUSION

5.1 Summary

The experiment was conducted to detect genetic variation among nineteen isolates of *Pyricularia oryzae*. The blast infected plant materials were collected from various locations of the South-Western rice growing regions Bangladesh. Nineteen isolates of *Pyricularia oryzae* were isolated following standard procedure in Plant Pathology Laboratory of Agrotechnology Discipline at Khulna University, Khulna. Mycelium was harvested for each isolate to obtain DNA by using DNA purification kit. For RAPD analysis a total of ten arbitrary RAPD primers were used. Amplified maximum number of DNA fragments was 57. Primer OPA-02 generated greater number of amplified fragments and give distinct amplification. Different parameters on primers were calculated for showing efficiency of each marker to separate and segregate in studied genotypes.

Maximum similarity was 94% between the isolates PO-11 and PO-12 which belonged to AEZ-11 and AEZ-13 and minimum pair wise similarity was only 21% between the isolates PO-16 and PO-03 that means dissimilarity between them was 79% though they were originated from the same location AEZ-13(Table 4).

The dendrogram, constructed with the data generated from the amplifications of the 19 isolates from 10 primers, exhibited two very distinct clusters (Figure 6). Cluster I contained only one isolate (PO-16) from AEZ-13 and cluster II consists of 18 isolates from different locations (AEZ-11 and AEZ-13). The cluster II was further subdivided into two sub-clusters. Cluster IIA contains 16 isolates and cluster IIB contains only two isolates.

There were two types of bands- monomorphic and polymorphic. The bands were detected from 500 bp to 5000bp. The monomorphic bands are mostly present near 500 bp, 1000 bp and 2000 bp. In case of PO-16 isolate only 4 primers (OPA-02, OPA-03, UBC-155 and UBC-173) generated low number of bands. Other six primers did not produce any band.

Neighbour Joining (NJ) tree demonstrated two distinct groups. Cluster I contained only one isolates belonging to AEZ- 13 and rest of the isolates belonged to cluster II. Isolate PO-08 was the first generation from evolutionary relationship among the 18 isolates.

5.2 Conclusion

1. Among nineteen isolates maximum similarity was 94% between the isolates PO-11 and PO-12 belonging to different location (AEZ-11 and AEZ-13). Maximum 79% genetical distance was observed between PO-16 and PO-03 isolates belonging to same location AEZ-13.
2. UPGMA dendrogram exhibited two very distinct clusters. Cluster I contained only one isolates (PO-16) from AEZ-13. Cluster II consisted of 18 isolates from different locations-AEZ-11 and AEZ-13. The cluster II was further subdivided into two sub-clusters. Cluster IIA contained 16 isolates and cluster IIB contained only two isolates. Principal component analysis showed similarities with the dendrogram.
3. Ten primers, used in the experiment, generated clear banding pattern for all the isolates except PO-16. Four primers (OPA-02, OPA-03, UBC-155 and UBC-173) produced a lower number of bands of PO-16 isolate and the rest six primers did not produce any band. It indicated that PO-16 isolate was different from other isolates.
4. Most of the primers produced more than 80% polymorphic fragments. So notable genetic variability exists among the species

CHAPTER VI

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