# PHENOTYPIC AND MOLECULAR IDENTIFICATION OF BLAST RESISTANCE GENES IN RICE GERMPLASM

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**DECEMBER, 2019**

# PHENOTYPIC AND MOLECULAR IDENTIFICATION OF BLAST RESISTANCE GENES IN RICE GERMPLASM

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*A Thesis*
*Submitted to the Faculty of Agriculture*
*Sher-e-Bangla Agricultural University, Dhaka*
*in partial fulfilment of the requirements*
*for the degree of*

**MASTER OF SCIENCE**

**IN**

**PLANT PATHOLOGY**

**SEMESTER: JULY-DECEMBER, 2018**

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# *CERTIFICATE*

*This is to certify that thesis entitled "**PHENOTYPIC AND MOLECULAR IDENTIFICATION OF BLAST RESISTANCE GENES IN RICE GERMPLASM**" submitted to the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE in PLANT PATHOLOGY**, embodies the results of a piece of bona fide research work carried out by **MONTASIR AHMED** bearing Registration No. **08-02819** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

*I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.*

**Dated: 02 December, 2019**
**Dhaka, Bangladesh**

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# ABBREVIATIONS

| Full Word | Abbreviation |
|---|---|
| Accession | Acc. |
| and others | *et al.* |
| base pair | bp |
| before Christ | BC |
| Before Present | BP |
| Centimeter | cm |
| Degree Celsius | °C |
| Deoxyribonucleic acid | DNA |
| Gram | g |
| Hydrogen ion concentration | pH |
| Insertion Deletion | InDel |
| Kilobase | Kb |
| Liter | L |
| Location severity index | LSI |
| Microliter | µL |
| Milliliter | mL |
| Millimeter | mm |
| Millimolar | mM |
| Molar | M |
| Namely | *viz.* |
| Nanogram | Ng |
| Percent | % |
| Polymerase chain reaction | PCR |
| Polymorphic information content | PIC |
| Revolutions per minute | Rpm |
| Sequence-Tagged Site | STS |
| Single Nucleotide Polymorphism | SNP |
| That is | i.e. |
| Uniform Blast Nursery | UBN |
| Volt | V |

# *ACKNOWLEDGEMENTS*

*All the praises and gratitude are due to the omniscient, omnipresent and omnipotent Almighty Allah, who has kindly enabled the author to complete this research work and complete this thesis successfully for increasing knowledge and wisdom.*

*The author sincerely desires to express his cordial gratitude, deep sense of respect and enormous indebtedness to his research Supervisor,* ***Prof. Dr. F. M. Aminuzzaman,*** *Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for his scholastic supervision, incessant encouragement, positive suggestion, unvarying inspiration, co-operation and constructive criticisms throughout the entire period of research work and the preparation of this thesis.*

*The author expresses heartfelt gratitude and indebtedness to his Co-supervisor,* ***Dr. Tahmid Hossain Ansari****, Bangladesh Rice Research Institute, Gazipur for his constant guidance, helpful suggestions timely directions and inspirations on research for the successful completion of the research work and manuscript preparation.*

*The author also wishes to pay his deep respect to* ***Prof. Dr. Khadija Akhter****, Chairman, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for her keen interest, continuous effort and valuable advices throughout the study period. The author also wishes to express his sincere gratitude to all other* ***respected teachers*** *of the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for their valuable teaching and helping during the study period.*

*The author also likes to give special thanks to* ***Krishi Gobeshona Foundation (KGF)*** *for funding the research and granting the scholarship for MS study.*

*The Author also grateful to* ***Dr. Md. Khalequzzaman****, CSO and Head, GRS Division,* ***Dr. Md. Abdul Latif****, CSO and Head,* ***Dr. Md. Ashik Iqbal Khan****, PSO, Plant Pathology Division,* ***Md. Hasibur Rahaman Hera****, SO, R/S Barisal, Bangladesh Rice Research Institute for their support on research and valuable suggestion throughout the research period.*

*The author would like to expresses cordial thanks to all of the* ***staffs*** *and* ***labors*** *of Plant Pathology Division, BRRI for their support on official and research work.*

*The author expresses his massive thankfulness to all of them who supported and encouraged him to pursue higher education and regret for his inability for not to mention every one by name who also contributed in pursuing the research works.*

***The Author***

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# PHENOTYPIC AND MOLECULAR IDENTIFICATION OF BLAST RESISTANCE GENES IN RICE GERMPLASM

**BY**

**MONTASIR AHMED**

## ABSTRACT

Rice blast (*Magnaporthe oryzae*) is one of the most devastating rice diseases which can lead a complete failure of the crop under severe infection. Development of durable blast resistant variety is the best option to control the disease. A better option for such attempt is the use of native blast resistant germplasm. Therefore, experiment was conducted to explore the phenotypic reaction of native rice germplasm for selection of resistant source. Further, identification of major blast resistance gene(s) exist in these germplasm were detected using molecular markers and analyze the population structure on the basis of blast resistance genes. Forty-four native germplasm, collected from Bangladesh Rice Research Institute (BRRI) Genebank, were tested for phenotypic reaction in uniform blast nursery (UBN) of BRRI against blast disease during T. Aman 2018 and Boro 2018-19 season. Molecular screening was performed at the molecular laboratory of Plant Pathology Division, BRRI. Among the tested genotypes, Acc. 3080 having *Pita-2* gene was resistant (disease score 1). While nine accessions viz. Acc. 3058, Acc. 3060, Acc. 3068, Acc. 3071, Acc. 3073, Acc. 3975, Acc. 3076, Acc. 3083 and Acc.3085 were found moderately resistant (disease score 2-3). Accessions 3058 and 3075 having 6 genes (*Pit*, *Pib*, *Pi9*, *Pi40*, *Pita-2* and *Pita/Pita-2*) with disease score 2. Germplasm possessing blast resistance genes varied from 0 to 6 and gene frequency of resistance genes ranged from 2.27% to 59.09% among the tested germplasm. PIC values of eight markers varied from 0.04 to 0.37 at an average 0.29. Out of eight markers one marker Pita3 was found to be significantly associated ($p$=0.003, $R^2$=0.19) with blast disease. Population structure analysis based on genotypic and phenotypic against blast disease divided the germplasm into two distinct subgroups. Acc. 3076 having *Pit, Pi40 and Pita/Pita-2* genes with disease score 2 was detected in a separate group from other moderately resistant germplasm in cluster analysis. Therefore, Acc 3058, Acc 3075, Acc. 3076 and Acc. 3080 could be used for the development of durable blast resistant variety.

# CHAPTER 1

# INTRODUCTION

Rice (*Oryza sativa* L.) is one of the ancient cultivated crops (Islam and Catling, 2012; Qiu *et al.*, 2019) belong to the family Poaceae (former Gramineae) (USDA-NRCS, 2019). Over half of the world's population consume rice as a staple food (Muthayya *et al.*, 2014). Solely Asia produces and consumes more than 90% of world production (GRiSP, 2013). Bangladesh is one of the major rice-producing countries in the world. The country is the fourth-largest in the world based on rice production (CGIAR, 2019; FAO, 2018). In addition, Bangladesh is the most densely populated country and about 160 million people of this country consume rice as principal food (UN, 2019; UNFPA, 2019). Food security of this country mostly depends on rice production (Kashem and Faroque, 2013). Country's 75% of cultivable land is under rice production to produce 36.3 million tons of rice per annum (BBS, 2018). Though the rice production of Bangladesh is increasing each year, still 20.7% of rice yield gap is existing (Kabir *et al.*, 2016). Besides, several biotic and abiotic stress, due to climate change, becomes a further challenge for rice production. All these situations become a threat to the food security of Bangladesh.

Approximately 37% of rice yield is reduced per annum due to several biotic stress (IRRI, 2019; Mondal *et al.*, 2017). In Bangladesh, several diseases prevail in the rice field throughout the year. Among the diseases, rice blast becomes a major threat to rice production.

Rice blast is a fungal disease of rice, caused by *Magnaporthe oryzae* Couch (teleomorph) [anamorph: *Pyricularia oryzae* Cavara] (Couch and Kohn, 2002). It is one of the most devastating rice diseases all over the world (Ou 1985; Agrios 2005; Khan *et al.* 2016). Rice blast is probably one of the ancient diseases of rice and first recorded in China (in 1637) and then in Japan (in 1704) (Islam and

Catling, 2012; Ou, 1980). Recent studies suggested that the relationship between rice and *M. oryzae* began from the beginning of rice domestication (Saleh *et al.*, 2014). Rice-infecting *M. oryzae* isolates probably developed from the *Setaria* millet infecting lineage around 5000 BC (Webster and Weber, 2007). It is placed among the top ten fungal plant pathogens of the world due to its economic and scientific importance (Dean *et al.*, 2012). The fungal pathogen is distributed more than 85 rice-growing countries of the world (Wang *et al.*, 2014). Every year 10 to 30% of rice yield loss occurs only for rice blast (Dean *et al.*, 2012; Talbot, 2003). Blast disease outbreak is a regular phenomenon for all rice-growing regions and sometimes disease control is being very difficult (Valent and Chumley, 1991). Numerous blast epidemic outbreaks occurred in Bangladesh since 1980 (Shahjahan, 1994). Under low and medium pressure, the disease causes 11% and 46.4% yield loss respectively in Bangladesh (Shahjahan *et al.*, 1987).

*M. oryzae* is primarily dispersed through its air borne inoculum (Monsur *et al.*, 2016; Villari *et al.*, 2017). Conidia germinates within 1 hour after landing on leaf surface (Webster and Weber, 2007). Within 4 to 5 days, the fungus develops lesion (necrotrophic phase) on the host and begin sporulation. Under high relative humidity, numerous conidia can produce from a lesion that initiates a secondary infection cycle (Leung and Shi, 1994). Due to its capability to produce spore within a short time and also its polycyclic nature, *M. oryzae* can cause devastating outbreaks within a short period of time. Blast disease commonly occurs during the vegetative phase of the rice plant but its most devastating occurrence is neck blast during the reproductive and ripening phase of host plant (Bonman, 1989). Generally, fungus colonizes at the panicle base and adjacent tissues and inhibits the photosynthates flow to the developing grain, resulting sterile grain or empty panicles.

Sasaki first observed variation in virulence among different *M. oryzae* isolates in 1922 (Ou, 1985). Goto identified 13 races between 1960 to 1965 (Wang *et al.*,

2014). The *M. oryzae* isolates collected from Bangladesh were also widely diversified in virulence. A total of 267 races of rice blast pathogen were identified from Bangladesh among 331 blast isolates (Khan *et al.*, 2016). This report indicates that *M. oryzae* is a highly diversified fungus. This fungus can develop new races spontaneously by mutation, the parasexual cycle or heterokaryosis (Disthaporn, 1994). As a result, the fungus can easily overcome effective fungicide or resistance variety.

In the past few decades, T. Aman rice, especially fragrant rice, were susceptible to blast. But last few years, Bangladesh faces an outbreak of blast disease during Boro season. In Boro 2014-15, the highest 90% neck infection was found in some rice fields of the Rangpur region that even caused no harvest (Ansari *et al.*, 2015). Haor regions are also affected by blast disease in every year during Boro season including BRRI dhan28 and BRRI dhan29 (Chowdhury, 2018; Zaman, 2017). It is observed that moderately resistant varieties e.g. BRRI dhan28 (BRRI, 2019b) are now becoming highly susceptible due to change of pathogenic race and environmental condition. Besides, blast disease barely recognized by farmers at earlier symptom expression to take control measures and as a result the complete crop failure has occurred.

Depends solely on chemical management of blast disease not only increase the cost of production but also cause environmental pollution. Improper application of fungicide can cause pest resurgence as a result host become more susceptible to disease. From the issue of environmental pollution and health hazard as well as cost reduction and durable resistance, blast-resistant variety development deserves high importance for disease management.

For blast disease management, host genetic resistance can be a potential option (Bonman *et al.*, 1992). The first blast resistance (R) gene, *Pia* gene, was identified from Aichi Asahi, a japonica variety, in 1967 (Wang *et al.*, 2014). After then several blast resistance genes have been identified form rice, most of them are from indica and japonica cultivars; some from wild rice species.

Rice is the most genetically and morphologically diversified crop (Islam and Catling, 2012). Bangladesh has affluent rice genetic diversity in the past. In 1982, a survey conducted by BRRI found that 12487 different rice varieties were still cultivated in different seasons (Biswas, 2012). Today BRRI has a collection of more than 8000 rice germplasms (BRRI, 2019a). Among them around 6000 are native germplasm that are not yet characterized against blast resistance. These native germplasm might have possessed novel genes for blast resistance due to its diversified genetic characteristics. Screening of these germplasm both phenotypically and with tightly linked marker may identify some novel blast resistance genes which can utilize for durable blast resistant variety. The present research was therefore carried out with the following objectives:

**Objectives:**

1. To investigate the phenotypic reaction of rice blast on native rice germplasm.
2. To identify major blast resistance gene(s) in native germplasm using molecular markers.
3. To find out the population structure of tested germplasm on the basis of disease reaction and blast resistance genes.

# CHAPTER 2

# REVIEW OF LITERATURE

The available literature of work done on molecular and phenotypic identification of blast resistance in rice have been reviewed in this chapter. The review of literature pertaining to this dissertation is presented in the following headings and sub-headings.

## 2.1 Rice and its economic importance

Rice (*Oryza sativa* L.) is under the family Poaceae (former Gramineae) (USDA-NRCS, 2019). It may be originated in a tropical humid belt in South-East Asia stretching from eastern India, through Burma and Indo-China where the richest diversity of cultivated forms has been recorded. This center of origin crosses the northern border of Bangladesh (Islam and Catling, 2012). It is one of the ancient cultivated crops and its cultivation began during 11,000 to 9000 BP with a full domestication of rice at around 10,000 BP (Islam and Catling, 2012; Qiu et al., 2019). It was most probably domesticated in India around 3000 BC and its cultivation spread north to east to reach China and Indochina, as well as south to Indonesia and to the Japanese islands (Shahjahan, 1994).

Today rice is the staple food of an estimated 3.5 billion people worldwide (Muthayya *et al.*, 2014). Rice production and consumption are among the highest in Asian populations. Rice is currently grown in over a hundred countries that produce more than 715 million tons of paddy rice annually (FAO, 2013). It is the main staple food in the Asia and the Pacific region, providing almost 39% of calories (Yaduraju and Rao, 2013). Bangladesh is the fourth-largest rice producer in the world (CGIAR, 2019; FAO, 2018). Besides, about 160 million people of Bangladesh consume rice as principal food (UNFPA, 2019). Almost all of the 13

million farm families of the country grow rice and about 75% of the total cropped area and over 80% of the total irrigated area is planted to rice (BRKB, 2019).

Besides, farmers lose an estimated average of 37% of their rice crop to pests and diseases every year (IRRI, 2019). About 15.6% yield loss due to disease in India (Mondal *et al.*, 2017). Rice yield loss due to pest and disease can range between 24% and 41% depending on the production situation (Sparks *et al.*, 2012).

### 2.2. Rice blast disease and its economic importance

Rice blast is one of the most important diseases of rice, caused by the fungus *Magnaporthe oryzae* Couch (Couch and Kohn, 2002).

One of the main limitations in production is rice blast disease caused by the fungus *Magnaporthe oryzae*. Annual rice losses caused by this fungus during 90's had been estimated at 35% of the worldwide production (Oerke and Dehne, 2004)

It is one of the most devastating rice diseases all over the world (Agrios 2005). Rice blast disease was described in China in 1637 and in Japan, M. Tsuchiya wrote about the disease in his book published in 1704 (Ou, 1985).

Blast disease causes yield losses from between 1- 100% in Japan (Kato, 2001), 70% in China, 21-37% in Bali Indonesia (Suprapta, 2012), and 30-50% in South America and Southeast Asia (Baker *et al.*, 1997). The fungus *Pyricularia oryzae* attacks at all stages of the crop and symptoms appear on leaves and nodes (Seebold *et al.*, 2004). The symptoms are more severe in case of neck blast that is characterized by the infection at the panicle base and its rotting (Bonman, 1989). Heavy yield losses have been reported in many rice growing countries. For example, 75, 85 and 60 percent grain loss may occur in India, Philippines and Thailand (IRRI, 2019). The extent of damage caused depends on environmental factors, but worldwide it is one of the most devastating cereal diseases, resulting in losses of 10–30% of the global yield of rice (Boddy, 2016). Annual rice losses caused by this fungus during 90's had been estimated at 35% of the worldwide

production (Oerke and Dehne, 2004). 10 to 30% of rice yield loss occurs in every year for rice blast (Dean et al., 2012). Blast disease outbreak is a regular phenomenon for all rice-growing regions (Valent and Chumley, 1991).

### 2.3. About *Magnaporthe oryzae*

*Magnaporthe oryzae* an ascomycete because it produces sexual spores (ascospores) in structures called asci, and is classified in the newly erected family Magnaporthaceae (TeBeest, 2007). It is haploid, with each nucleus containing about seven chromosomes (Valent, 1997). The asexual stage of *M. oryzae* is named as *Pyricularia oryzae* (TeBeest, 2007)

In tropical climates with up to three cropping seasons each year, the fungus can continuously infect fresh green foliage, whereas in Southern Europe it overwinters on rice stubble (Webster and Weber, 2007). A mature conidium swells upon hydration, and this causes the breakage of the wall at the tip of the spore, releasing a drop of mucilage stored in the periplasmic space. It attaches the spore firmly to the wax of the host cuticle or other hydrophobic surfaces (Hamer *et al.*, 1988). The secondary cycles are initiated by the spores produced by the lesions on the young seedlings, which can be repeated many times through the growing season. Thousands of spores can be produced from a single lesion in 15 days after infection (Wang *et al.*, 2014).

Spores of *M. grisea* germinates very fast and within two hours of landing on the leaf, a polarized germ tube is formed and then appressorium was developed (Bourett and Howard, 1990).

*M. oryzae* have large numbers of physiological races and first reported by Sasaki in Japan as early as 1922 (Sridhar and Singh, 2001). From 1950s to 60s differential rice lines resistant to races of *M. oryzae* were identified in Japan, the United States, India, the Philippines, and South Korea. In 1961, 18 physiological races of *M. oryzae* were identified with 12 differential rice varieties in Japan.

During that time, an international differential system using 8 rice varieties was established (Ling and Ou, 1969). A total of 267 races of rice blast pathogen were identified from Bangladesh (Khan *et al.*, 2016).

### 2.4. Blast resistance genes

Till date 99 blast R genes have been identified; in which 45% were found in japonica cultivars, 51% in indica cultivars, and the rest 4% in wild rice species (Wang *et al.*, 2014). More than 100 blast resistance loci or genes have been mapped to rice chromosomes (Fang *et al.*, 2016)

Two types of resistance genes are responsible for rice blast resistance: major resistance (R) genes that confer race-specific resistance and quantitative trait loci (QTLs) that control partial, nonrace-specific resistance (Skamnioti and Gurr, 2009)

Cloned R genes are distributed across all 12 chromosomes except chromosome 3 (QinZhong *et al.*, 2009). All of the cloned R genes except for *Pi-d2*, *pi21* and *Ptr* contain nucleotide-binding domain leucine-rich repeat (NLR) proteins (Zhao *et al.*, 2018).

R genes are highly specific to *M. oryzae* races, resistance of a single R gene is often rapidly overcome by the selection of compatible pathogen races (Hittalmani *et al.*, 2000). In response to the rapid evolution of *M. oryzae*, the rice genome has evolved R gene polymorphism, which confers multiple forms of race-specific resistance (Hayashi *et al.*, 2004).

### 2.5. Genetic association study

Genetic association study has recently been used for assessing associations between genetic markers and blast resistance in rice (Li *et al.*, 2019). It was first

used to identify genes underlying complex diseases in humans (Altshuler *et al.*, 2008). Genetic association modeling has a wider use (Liu *et al.*, 2016).

Genetic association has become a powerful approach for mapping agronomic traits of rice (Zhao *et al.*, 2018). Genetic association analysis was applied for QTL mapping using large germplasm collections (Huang *et al.*, 2010)

# CHAPTER 3

# MATERIALS AND METHODS

The materials used and methods applied or followed for conducting the experiments under the present study are described in this chapter.

## 3.1. Location of the experiment

The experiments were conducted at Uniform Blast Nursery (UBN) of Plant Pathology Division, Bangladesh Rice Research Institute (BRRI), Gazipur (23°59'26" N latitude 90°24'28" E longitudes) during September 2018 to October 2019. Molecular screening of resistance (R) genes from the rice germplasm were performed at the molecular laboratory of Plant Pathology Division, BRRI.

## 3.2. Screening of rice germplasm

### 3.2.1. Plant materials

Forty-four rice germplasm were collected from Genebank, BRRI (Table 1), which are native germplasm of Bangladesh. One additional genotype, US2 was included with this set as blast susceptible check.

**Table 1. Accession number and designation of rice germplasm used in the experiment.**

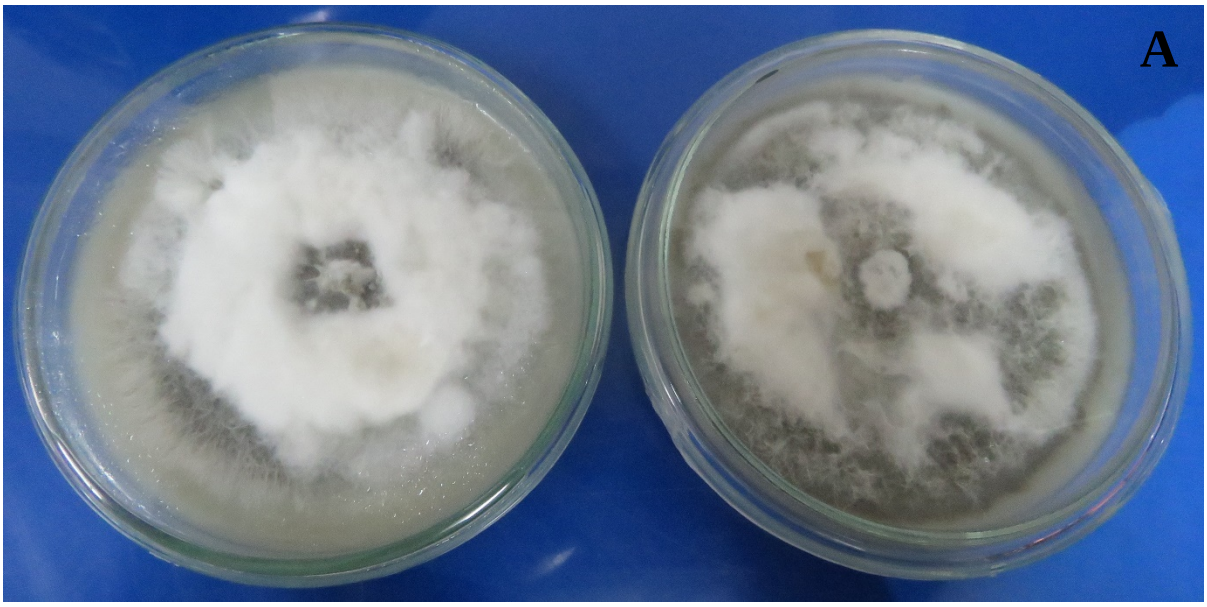
| Sl. No. | Accession No. | Designation | Sl. No. | Accession No. | Designation |
|---|---|---|---|---|---|
| 1 | 3051 | Patnai | 23 | 3076 | Patnai |
| 2 | 3052 | Patnai | 24 | 3077 | Patnai |
| 3 | 3053 | Patnai | 25 | 3079 | Patnai |
| 4 | 3054 | Patnai | 26 | 3080 | Patnai |
| 5 | 3056 | Patnai | 27 | 3081 | Patnai |
| 6 | 3058 | Patnai | 28 | 3082 | Patnai |
| 7 | 3059 | Patnai | 29 | 3083 | Patnai |
| 8 | 3060 | Patnai | 30 | 3084 | Patnai |
| 9 | 3061 | Patnai | 31 | 3085 | Patnai |
| 10 | 3062 | Patnai | 32 | 3086 | Patnai |
| 11 | 3063 | Patnai | 33 | 3087 | Patnai |
| 12 | 3064 | Patnai | 34 | 3089 | Patnai |
| 13 | 3065 | Patnai | 35 | 3090 | Patnai |
| 14 | 3066 | Patnai | 36 | 3091 | Patnai |
| 15 | 3067 | Patnai | 37 | 3093 | Patnai |
| 16 | 3068 | Patnai | 38 | 3094 | Patnai |
| 17 | 3069 | Patnai | 39 | 3095 | Patnai |
| 18 | 3070 | Patnai | 40 | 3096 | Patnai |
| 19 | 3071 | Patnai | 41 | 3097 | Nizer Sail |
| 20 | 3072 | Patnai | 42 | 3098 | Nizer Sail |
| 21 | 3073 | Patnai | 43 | 3099 | Nizer Sail |
| 22 | 3075 | Patnai | 44 | 3100 | Nizer Sail |

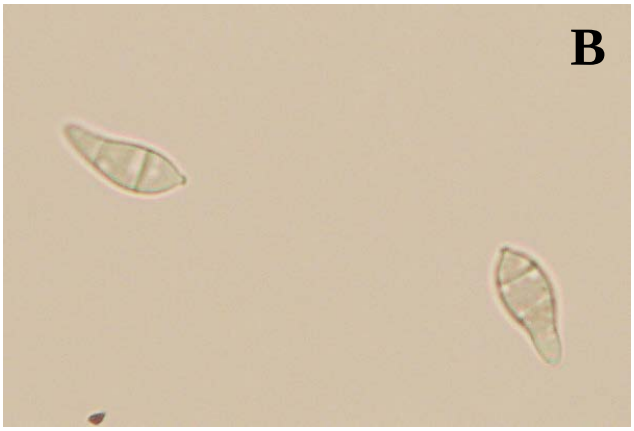
### 3.2.2. Phenotypic screening against leaf blast disease

Germplasm with susceptible check were screened for their reaction against leaf blast at UBN, BRRI, Gazipur. The screening was done two times in T. Aman 2018 and Boro 2018-19 season with four replications. Each entry was sown in rows in the nursery bed. Each row was 50 cm long and row to row distance was 10 cm. Susceptible check, US2, was sown after every ten entries as well as two rows in border around the rows (Appendix I). Though UBN was previously harbor of several isolates, additionally a virulent blast isolate Lal-1 (Figure 1), collected from Lalmonirhar district of Bangladesh, was inoculated at 21 days after seeding in both seasons. Disease reaction was recorded at 7 days after inoculation. Disease reaction was recorded visually following the scoring system

developed by Japan International Research Center for Agricultural Sciences (JIRCAS) (Hayashi *et al.*, 2009). Detailed scoring system is presented in Table 2. Infection type 0 to 1 was considered as resistance, 2 to 3 was considered as moderately resistant and infection type 4 to 5 was considered as susceptible. Disease scoring was done by considering the highest disease score among the replications. Additionally, the location severity index (LSI) was calculated to find the severity of blast disease using following formula (Wheeler, 1969):

$$\text{LSI} = \frac{\text{Sum of multiplication of entries and scales}}{\text{Total number of entries}}$$



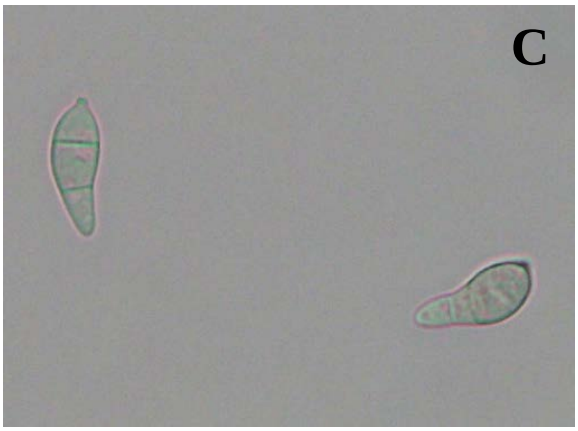




**Figure 1. Lal-1 isolate of *Magnaporthe oryzae*.**
**A.** Culture on oat meal agar media.
**B & C.** Conidia of isolate Lal-1 under compound microscope (60X).

**Table 2. Leaf blast scoring system of JIRCAS.**

| Disease score | Leaf blast symptom |
|---|---|
| 0 | No lesions. |
| 1 | Uniform or scattered brown specks. |
| 2 | Small lesions with distinct tan centers surrounded by a darker brown margin approximately 1mm in diameter. |
| 3 | Small eyespot lesions less than one and a half times of the interval between thin veins or less than 1.5mm in diameter surrounded by dark brown. |
| 4 | Intermediate size eyespot lesions less than twice the interval between thin veins or less than 2 mm in diameter. |
| 5 | Large eyespot lesions more than twice the interval between thin veins or more than 2 mm in diameter. |

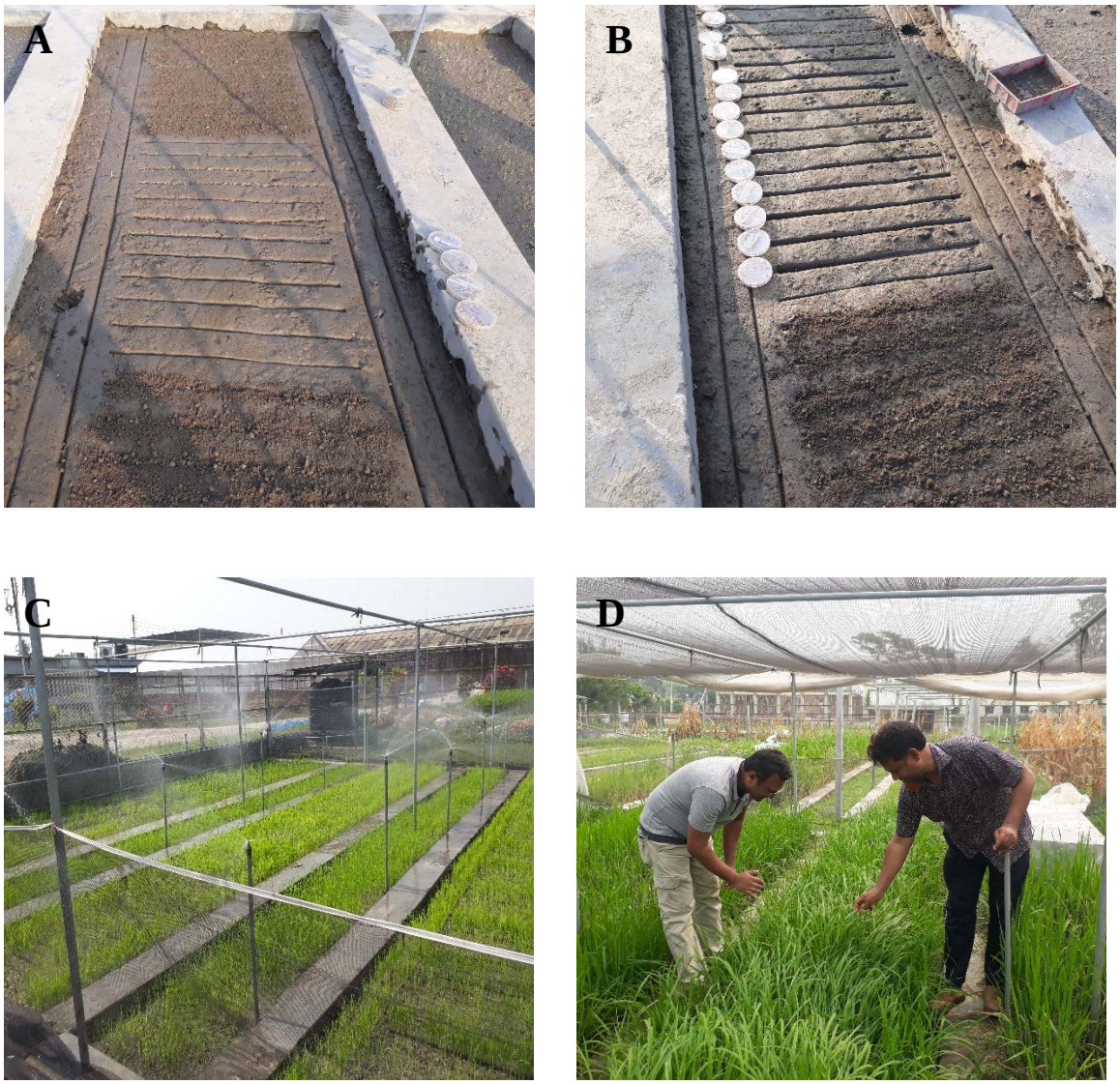



**Figure 2. Activities at uniform blast nursery (UBN).**

**A & B.** Sowing of germplasm.

**C.** Water spraying for maintaining disease favorable environment.

**D.** Disease observation.

### 3.3. Reagent Preparation

#### 3.3.1. 1M Tris-HCL, pH 8.0

For 1L solution preparation:

| Reagent | Amount |
|---|---|
| Tris, UltraPure (Invitrogen, USA) | 121.1 g |
| HCL, fuming 37% (Merck, Germany) | ~150 mL (until pH to 8.0) |
| $ddH_2O$ | Up to 1L |

#### 3.3.2. 0.5M EDTA, pH 8.0

For 1L solution preparation:

| Reagent | Amount |
|---|---|
| EDTA (VWR International, USA) | 186.12 g |
| NaOH (Merck, Germany) | ~20 g (until pH to 8.0) |
| $ddH_2O$ | Up to 1L |

#### 3.3.3. 5M NaCl

For 1L solution preparation:

| Reagent | Amount |
|---|---|
| NaCl (Duchefa Biochemie, Netherlands) | 292.2 g |
| $ddH_2O$ | Up to 1L |

### 3.3.4. 2X Cetyl trimethylammonium bromide (CTAB)

For 1L solution preparation:

| Reagent | Concentration | Amount |
|---|---|---|
| Tris, pH 8.0 | 1M | 100.0 mL |
| EDTA, pH 8.0 | 0.5M | 40.0 mL |
| NaCl | 5M | 280.0 mL |
| Polyvinylpyrrolidone (PVP-40) (Bio Basic Inc., Canada) | | 10.0 g |
| CTAB (Bio Basic Inc., Canada) | | 20.0 g |
| dd$H_2O$ | | Up to 1L |

### 3.3.5. Chloroform: Iso-amyl alcohol: Phenol (CIP)

For 1L solution preparation:

| Reagent | Amount |
|---|---|
| Chloroform (DAEJUNG Co. Ltd., Korea) | 912.0 mL |
| Isoamyl Alcohol (AMRESCO, USA) | 38.0 mL |
| Phenol (Wako Pure Chemical Industries, Ltd., Japan) | 50.0 mL |

### 3.3.6. TE buffer

For 1L buffer preparation:

| Reagent | Concentration | Amount | Final Concentration |
|---|---|---|---|
| Tris, pH 8.0 | 1M | 10 mL | 10 mM |
| EDTA, pH 8.0 | 0.5M | 2 mL | 1 mM |
| dd$H_2O$ | | Up to 1L | |

### 3.3.7. 10X and 1X TBE buffer

For 1L 10X TBE buffer preparation:

| Reagent | Amount |
|---|---|
| Tris (MP Biomedicals, USA) | 108 g |
| Boric Acid (Bio Basic Inc., Canada) | 55 g |
| EDTA (VWR International, USA) | 9.3 g |
| dd$H_2O$ | Up to 1L |

1X TBE buffer was prepared from 10X TBE following the formula,

$V_1S_1=V_2S_2$

Where, $S_1$= Concentration of stock solution (10X)

$S_2$= Desired concentration of solution (1X)

$V_1$= Volume of stock solution (10X)

$V_2$= Final volume of buffer with desired concentration

## 3.4. Genomic DNA extraction

Genomic DNA were extracted from leaves of each germplasm following the CTAB method (Ferdous *et al.*, 2012) with minor modification.

### 3.4.1. Protocol for genomic DNA extraction

Young leaves were collected from three-week-aged seeding of each genotype/germplasm. Then the leaves were cut into small pieces (around 1-1.5 cm) and were placed into a separate 2 mL microcentrifuge tube (Axygen, Inc., USA) for each genotype. A zirconium oxide ball (3mm) was put into each tube and 300 µL of 2X CTAB was added into the tube and the tubes were placed into Mixer Mill MM400 (RETSCH, Germany) and crashed the leaves at 30 rpm for 2 minutes. 300 µL CIP was added into the tubes after crashing the leaves and were

vortex mixer (Whirlimixer, Fisher Scientific, UK). After vortex, tubes were centrifuged (Microfuge 20R, Beckman Coulter, Inc., Germany) at 15000 rpm for 10 minutes. 200 µL supernatant was taken and put into a new 1.5 mL microcentrifuge tube (Axygen, Inc., USA). Additionally, 200 µL cold isopropanol (Molecular Biology Grade, Fisher BioReagents, USA) was mixed with the supernatant. The tubes were inverted for few minutes and kept at room temperature for 10 minutes. Again, the samples were centrifuged at 15000 rpm for 10 minutes. Finally, Supernatants were removed and wash the pellet with 70% ethanol (TEDIA, USA).

DNA pellets were dried on the Dri-Block heater (Techne, UK) at 55°C. After complete drying, the pellets were resuspended with 100 µL ultra-pure water (PURELAB Option-Q, ELGA LabWater, UK) and that solution were used as stock DNA solution. The quantity and quality of purified genomic DNA were estimated on Genova Nano micro-spectrophotometer (Jenway, UK). The DNA samples were later diluted with ultra-pure water to the concentration of 20 ng/µL for polymerase chain reaction (PCR).

### 3.5. Molecular screening for rice blast R genes

Collected germplasm were genotyped for the presence of 8 (eight) blast resistance genes viz. *Pit, Pib, Piz, Pi9, Pi5, Pita, Pita-2, Pi40*. Eight molecular markers tightly linked with those R genes were used for molecular screening (Table 3). All molecular markers were synthesized by Integrated DNA Technologies, Inc., USA.

#### 3.5.1. PCR amplification

Each DNA sample was run into PCR Thermal Cycler (SimpliAmp, Applied Biosystems, Thermo Fisher Scientific, USA) to amplify the targeted sequence for a specific gene/allele/QTL using specific marker.

### 3.5.2. PCR reaction volume preparation

A 20 µL PCR reaction volume was prepared for each PCR sample. Each PCR sample containing 20 ng template DNA, 1.0 µL of each primer (0.4 µmol/L), 10 µL of Taq PCR Master Mix (Bio Basic Inc., Canada) and nuclease-free water to final volume of 20 µL.

### 3.5.3. PCR condition

PCR program was set up as follows: initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, primers annealing at varying temperatures (Table 3) for 45 seconds, and extension at 72°C for 2 minutes, followed by a final extension at 72°C for 10 minutes.

### 3.5.4. Gel electrophoresis and gel documentation

The amplified PCR products along with 100 bp and 1kb DNA ladder (Promega Corporation, USA) were separated using gel electrophoresis (multiSUB Maxi, Cleaver Scientific Ltd., UK) in 2% UltraPure agarose (Invitrogen, USA) gel in 1X TBE buffer at 90V for 2 to 2.5 hours. After electrophoresis, the gel was stained with UltraPure ethidium bromide (0.5 µg/mL) (Invitrogen, USA) and visualized. The picture was taken in a gel documentation system (BioDocAnalyze, Biometra, Germany).

All PCR reactions of each sample were repeated twice to confirm the results.

**Figure 3. Some activities of PCR and gel electrophoresis.**

**Table 3. List of molecular markers used for detection of blast resistance genes in rice germplasm.**

| Gene | Chr | Primers | Primer Sequence (5' to 3') | Types of Marker | AT (°C) | Expd. Size (bp) | References |
|---|---|---|---|---|---|---|---|
| *Pit* | 1 | t311 | F: CGTGAACCCAAGGCACCAGTATTA<br>R: CATGTAGTTCTGGATGTTGTAGCTACTC | SNP | 60 | 287 | (Fukuta *et al.*, 2009; Hayashi *et al.* 2006) |
| *Pib* | 2 | b28 | F: GACTCGGTCGACCAATTCGCC<br>R: ATCAGGCCAGGCCAGATTTG | SNP | 60 | 388 | (Fukuta *et al.*, 2009; Hayashi *et al.*, 2006; Yadav *et al.*, 2017) |
| *Piz* | 6 | Z56592 | F: GGACCCGCGTTTTCCACGTGTAA<br>R: AGGAATCTATTGCTAAGCATGAC | SNP | 60 | 292 | (Hayashi *et al.*, 2006; Yadav *et al.*, 2017) |
| *Pi9* | 6 | 195R-1 | F: ATGGTCCTTTATCTTTATTG<br>R: TTGCTCCATCTCCTCTGTT | STS | 56 | 2000 | (Qu *et al.*, 2006) |
| *Pi40* | 6 | MSM6 | F: TGCTGAGATAGCCGAGAAATC<br>R: GCACCCTTTTCGCTAGAGG | Codominant | 55 | 256 | (Gavhane *et al.*, 2019) |
| *Pi5* | 9 | 40N23r | F: TGTGAGGCAACAATGCCTATTGCG<br>R: CTATGAGTTCACTATGTGGAGGCT | InDel | 55 | 700/480 | (Jeon *et al.*, 2013) |
| *Pita-2* | 12 | Pita3 | F: AGTCGTGCGATGCGAGGACAGAAAC<br>R: GCATTCTCCAACCCTTTTGCATGCAT | SNP | 59 | 861 | (Hayashi *et al.*, 2006) |
| *Pita/Pita-2* | 12 | YL155/YL87 | F: AGCAGGTTATAAGCTAGGCC<br>R: CTACCAACAAGTTCATCAAA | Dominant | 55 | 1042 | (Yadav *et al.*, 2017) |

*Chr-Chromosome, AT-Annealing Temperature, SNP-Single Nucleotide Polymorphism, STS-Sequence-Tagged Site, InDel-Insertion Deletion

### 3.6. Statistical Analysis

#### 3.6.1. Allele scoring and diversity analysis

The amplified PCR product were scored for the presence of absence of each marker allele. Binary matrix was created on the basis of scoring '1' for the presence and '0' for the absence of the allele. The genetic distance and similarity coefficients were estimated using binary matrix of 8 markers. Major allele frequency, gene diversity and polymorphism information content (PIC) value of each marker were estimated using Powermaker Ver3.25 software (Liu and Muse, 2005). Genetic similarities were estimated by constructing a cladogram through unweighted Pair Group Method with Arithmetic Means (UPGMA) to their genetic relationship (Sneath and Sokal, 1975) using TASSEL 4.3 software (Bradbury *et al.*, 2007).

#### 3.6.2. Association analysis

The hypothesis of genetic association between blast resistance genes and blast disease was tested using general liner model (GLM) function in TASSEL 4.3 software (Bradbury *et al.*, 2007). The GLM of TASSEL 4.3 software was conducted with permutation of 1000.

#### 3.6.3. Population structure

Analysis of population structure of 44 native germplasm based on 8 markers and blast disease score was carried out using STRUCTURE version 2.3.4 software (Pritchard *et al.*, 2000). The number of subgroups (K) in the population was estimated by the program at different K values from K=1 to K=10, with 5 independent iterations per K using the admixture model and correlated allele frequencies with 100000 burn-in period and 100000 Markov Chain Monte Carlo (MCMC). The peak value of ΔK was estimated by using STRUCTURE HARVESTER (Earl and vonHoldt, 2012).

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1. Phenotyping for blast resistance

Based on the screening scores of native germplasm in UBN, only one germplasm Acc. 3080 (R: 2.27%) was found resistant with the disease score 1. Nine germplasm (MR: 20.45%) exhibited moderately resistant reaction (disease score 2-3) and thirty-four germplasm (S: 77.27%) were susceptible with the disease score >3 (Table 4). The location severity index (LSI) of two seasons was found 4.0, which confirm the adequate disease pressure in the study location. Native germplasm in the same group like Patnai varied in their phenotypic reaction against blast inoculation due to their evolutionary change in the genetic characters.

## 4.2. Identification of blast resistance genes

Gel electrophoresis results of 44 native germplasm for 8 linked markers are shown in Figure 5a and Figure 5b. The results of cataloguing of germplasm based on gel electrophoresis of different markers with the highest disease score against blast disease are present in Table 5. From the analysis of eight tightly linked markers, it was found that the genetic frequency of different blast resistance genes ranged from 2.27% to 59.09%. Among the germplasm, maximum number of genes (6) found in five germplasm (Acc. 3052, Acc. 3058, Acc. 3060, Acc. 3068 and Acc. 3075). Four of these germplasm found moderately resistant to blast in UBN while Acc. 3052 found susceptible. Total 13 germplasm contained more than half of the genes studied (Table 5). Six germplasm (Acc. 3053, Acc. 3064, Acc. 3089, Acc. 3091, Acc. 3094, Acc. 3095) found having no studied genes and all these germplasm were susceptible to blast.

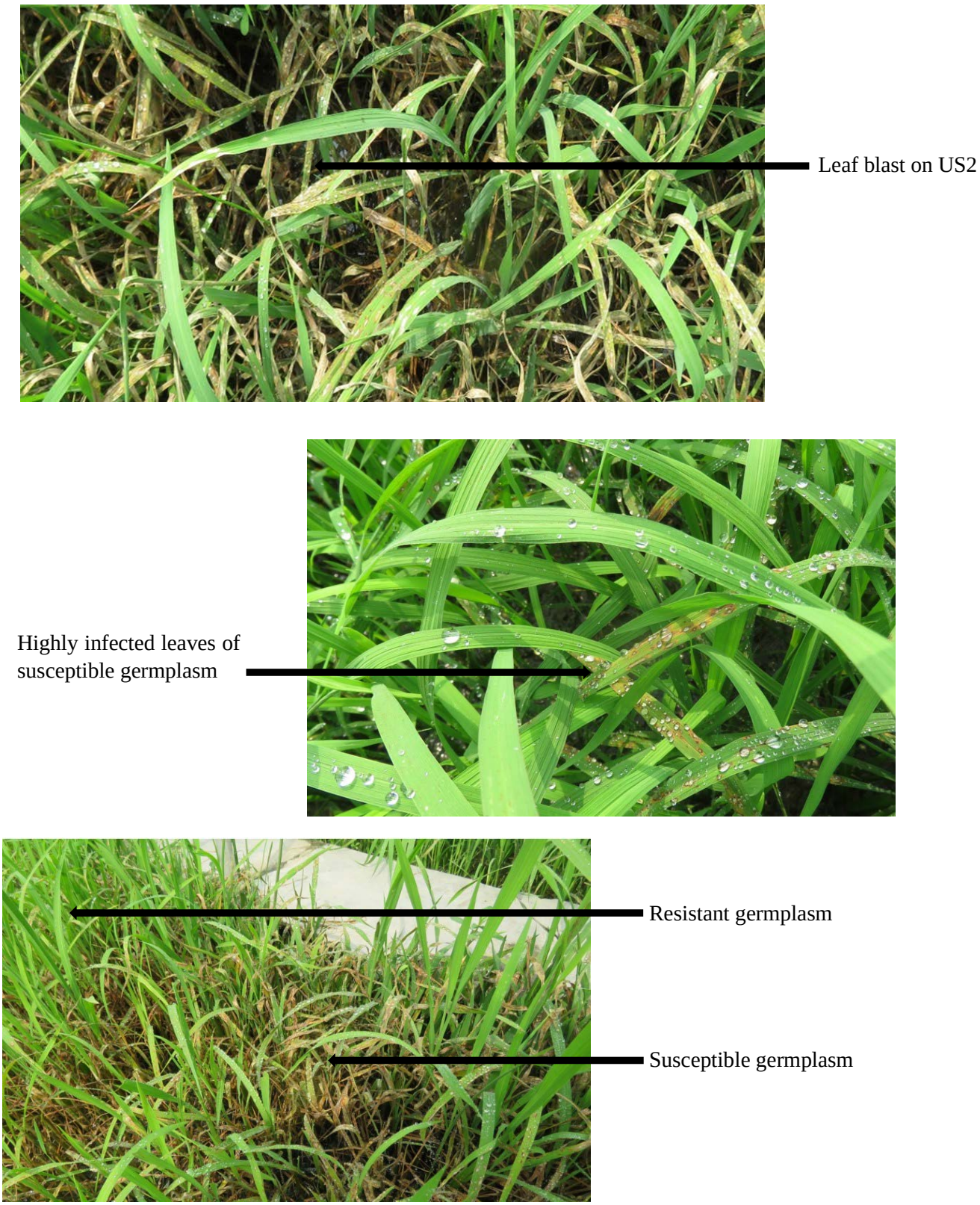



**Figure 4. Blast disease at uniform blast nursery (UBN).**

## Table 4. Evaluation of native germplasm for leaf blast resistance.

| Sl. No. | Accession No. | Designation | Disease Score | | | Disease Reaction |
|---|---|---|---|---|---|---|
| | | | T Aman2018 | Boro2018-19 | Highest Score | |
| 1 | Acc. 3051 | Patnai | 4 | 4 | 4 | S |
| 2 | Acc. 3052 | Patnai | 5 | 5 | 5 | S |
| 3 | Acc. 3053 | Patnai | 2 | 4 | 4 | S |
| 4 | Acc. 3054 | Patnai | 5 | 5 | 5 | S |
| 5 | Acc. 3056 | Patnai | 4 | 4 | 4 | S |
| 6 | Acc. 3058 | Patnai | 2 | 2 | 2 | MR |
| 7 | Acc. 3059 | Patnai | 3 | 4 | 4 | S |
| 8 | Acc. 3060 | Patnai | 2 | 3 | 3 | MR |
| 9 | Acc. 3061 | Patnai | 5 | 5 | 5 | S |
| 10 | Acc. 3062 | Patnai | 5 | 5 | 5 | S |
| 11 | Acc. 3063 | Patnai | 5 | 5 | 5 | S |
| 12 | Acc. 3064 | Patnai | 5 | 5 | 5 | S |
| 13 | Acc. 3065 | Patnai | 5 | 5 | 5 | S |
| 14 | Acc. 3066 | Patnai | 4 | 4 | 4 | S |
| 15 | Acc. 3067 | Patnai | 5 | 5 | 5 | S |
| 16 | Acc. 3068 | Patnai | 3 | 3 | 3 | MR |
| 17 | Acc. 3069 | Patnai | 5 | 5 | 5 | S |
| 18 | Acc. 3070 | Patnai | 4 | 4 | 4 | S |
| 19 | Acc. 3071 | Patnai | 3 | 3 | 3 | MR |
| 20 | Acc. 3072 | Patnai | 5 | 5 | 5 | S |
| 21 | Acc. 3073 | Patnai | 2 | 2 | 2 | MR |
| 22 | Acc. 3075 | Patnai | 2 | 2 | 2 | MR |
| 23 | Acc. 3076 | Patnai | 2 | 1 | 2 | MR |
| 24 | Acc. 3077 | Patnai | 4 | 4 | 4 | S |
| 25 | Acc. 3079 | Patnai | 5 | 0 | 5 | S |
| 26 | Acc. 3080 | Patnai | 1 | 0 | 1 | R |
| 27 | Acc. 3081 | Patnai | 3 | 5 | 5 | S |
| 28 | Acc. 3082 | Patnai | 5 | 5 | 5 | S |
| 29 | Acc. 3083 | Patnai | 3 | 3 | 3 | MR |
| 30 | Acc. 3084 | Patnai | 5 | 5 | 5 | S |
| 31 | Acc. 3085 | Patnai | 2 | 2 | 2 | MR |
| 32 | Acc. 3086 | Patnai | 5 | 5 | 5 | S |
| 33 | Acc. 3087 | Patnai | 5 | 5 | 5 | S |
| 34 | Acc. 3089 | Patnai | 5 | 5 | 5 | S |
| 35 | Acc. 3090 | Patnai | 5 | 5 | 5 | S |
| 36 | Acc. 3091 | Patnai | 5 | 5 | 5 | S |
| 37 | Acc. 3093 | Patnai | 5 | 5 | 5 | S |
| 38 | Acc. 3094 | Patnai | 5 | 5 | 5 | S |
| 39 | Acc. 3095 | Patnai | 5 | 5 | 5 | S |
| 40 | Acc. 3096 | Patnai | 5 | 5 | 5 | S |
| 41 | Acc. 3097 | Nizer Sail | 4 | 4 | 4 | S |
| 42 | Acc. 3098 | Nizer Sail | 5 | 5 | 5 | S |
| 43 | Acc. 3099 | Nizer Sail | 5 | 5 | 5 | S |
| 44 | Acc. 3100 | Nizer Sail | 5 | 5 | 5 | S |
| 45 | - | US2 (Sus. Ck.) | 5 | 5 | 5 | S |
| | LSI | | 4.0 | 4.0 | | |

*R= Resistant, MR= Moderately Resistant and S= Susceptible

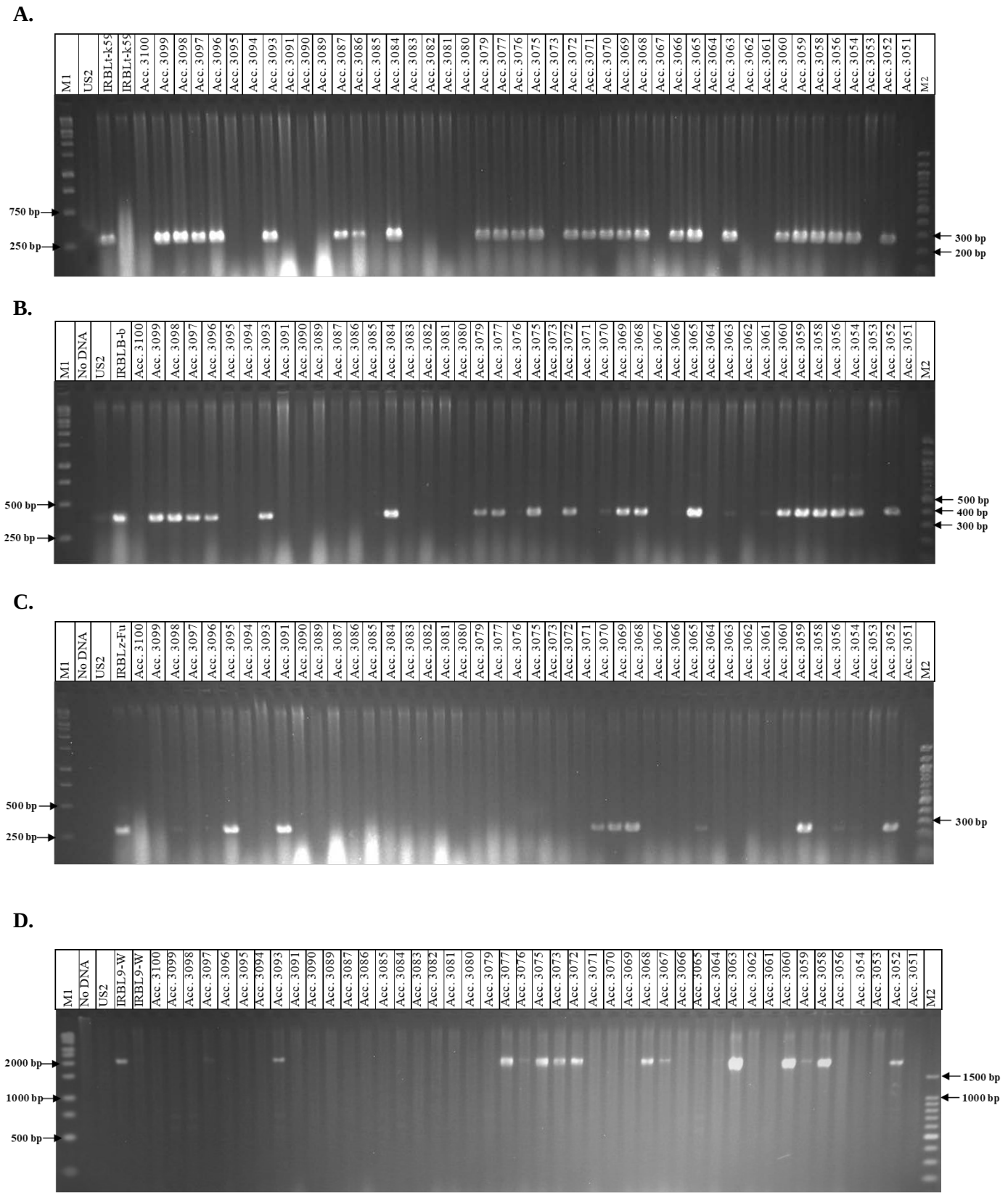
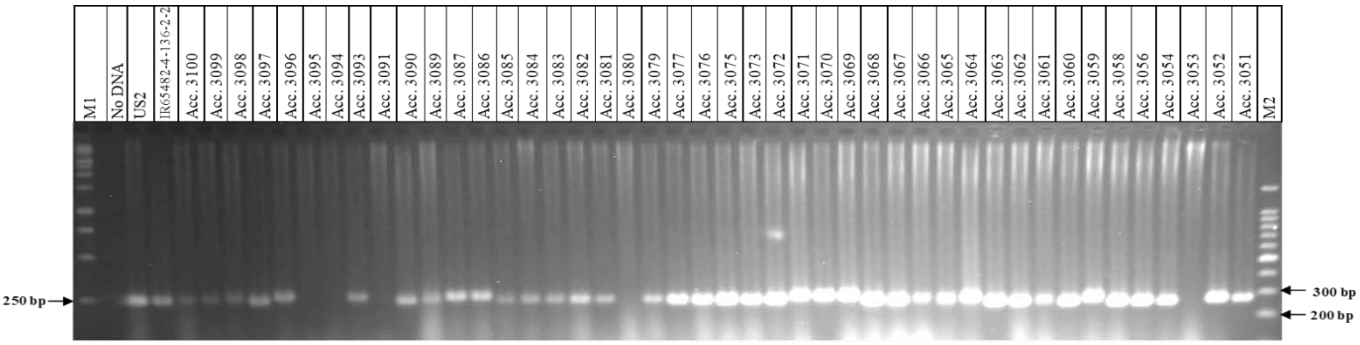


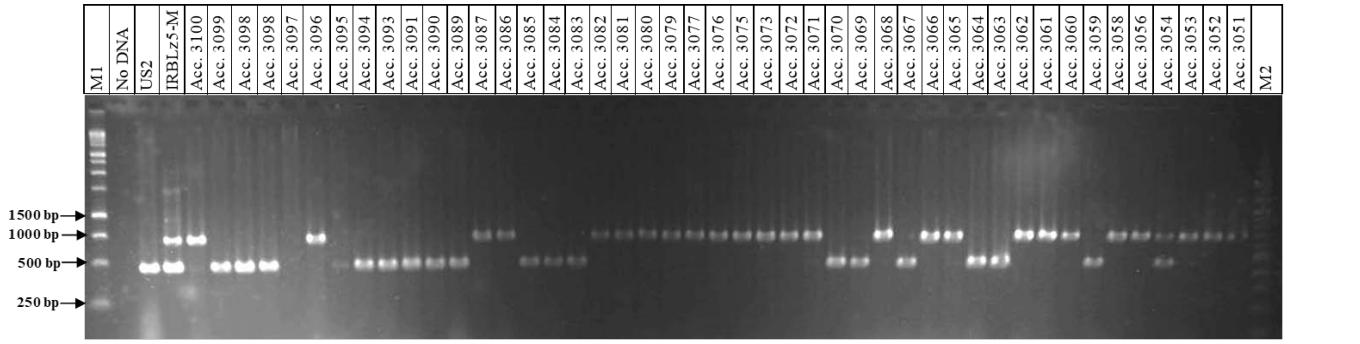

**Figure 5a. Agarose gel picture of PCR product of 44 native germplasm.**

**A.** resistance gene *Pit* amplified with primer t311(287 bp). **B.** resistance gene *Pib* amplified with primer b28 (388 bp). **C.** resistance gene *Piz* amplified with primer Z56592 (292 bp). **D.** resistance gene *Pi9* amplified with primer 195R-1 (2000 bp).
M1 and M2 denotes for 1kb and 100bp DNA ladder respectively

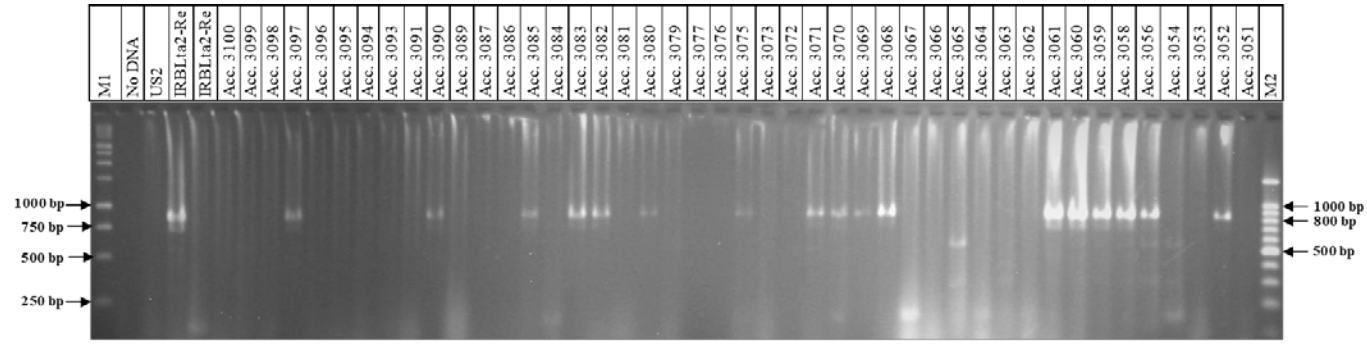
**E.**



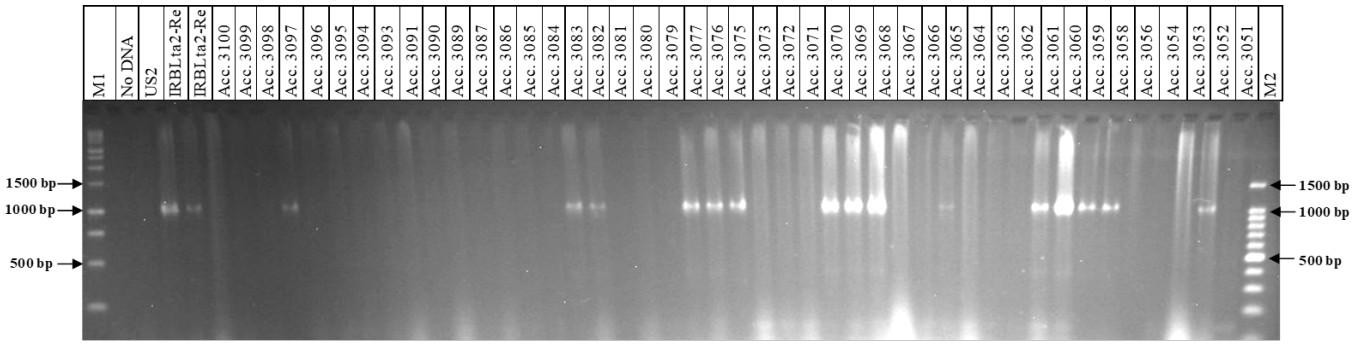

**F.**




**G.**




**H.**




## Figure 5b. Agarose gel picture of PCR product of 44 native germplasm.

**E.** resistance gene *Pi40* amplified with primer MSM6 (256 bp). **F.** resistance gene *Pi5* amplified with primer 40N23r (700/480 bp). **G.** resistance gene *Pita-2* amplified with primer Pita3 (861 bp). **H.** resistance gene *Pita/Pita-2* amplified with primer YL155/YL87 (1042 bp).
M1 and M2 denotes for 1kb and 100bp DNA ladder respectively.

## Table 5. Details of screening of rice germplasm for different blast resistance genes using linked marker based on expected product size.

| Accession no. | Blast score[#] | *Genes** | | | | | | | | No. of Gene |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ***Pit*** | ***Pib*** | ***Piz*** | ***Pi9*** | ***Pi40*** | ***Pi5*** | ***Pita-2*** | ***Pita/ Pita-2*** | |
| | | **t311** | **b28** | **Z56592** | **195R-1** | **MSM6** | **40N23r** | **Pita3** | **YL155/ YL87** | |
| Acc. 3051 | 4 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Acc. 3052 | 5 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 6 |
| Acc. 3053 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Acc. 3054 | 5 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 4 |
| Acc. 3056 | 4 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 4 |
| Acc. 3058 | 2 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 6 |
| Acc. 3059 | 4 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 5 |
| Acc. 3060 | 3 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 6 |
| Acc. 3061 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 2 |
| Acc. 3062 | 5 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Acc. 3063 | 5 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 3 |
| Acc. 3064 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Acc. 3065 | 5 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| Acc. 3066 | 4 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Acc. 3067 | 5 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 2 |
| Acc. 3068 | 3 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 6 |
| Acc. 3069 | 5 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 5 |
| Acc. 3070 | 4 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 4 |
| Acc. 3071 | 3 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 3 |
| Acc. 3072 | 5 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 4 |
| Acc. 3073 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 2 |
| Acc. 3075 | 2 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 6 |
| Acc. 3076 | 2 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 3 |
| Acc. 3077 | 4 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 5 |
| Acc. 3079 | 5 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 3 |
| Acc. 3080 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| Acc. 3081 | 5 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Acc. 3082 | 5 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 3 |
| Acc. 3083 | 3 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 3 |
| Acc. 3084 | 5 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 3 |
| Acc. 3085 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| Acc. 3086 | 5 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Acc. 3087 | 5 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Acc. 3089 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Acc. 3090 | 5 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 2 |
| Acc. 3091 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Acc. 3093 | 5 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 4 |
| Acc. 3094 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Acc. 3095 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Acc. 3096 | 5 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 3 |
| Acc. 3097 | 4 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 3 |
| Acc. 3098 | 5 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 3 |
| Acc. 3099 | 5 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| Acc. 3100 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Total no. of gene** | | 26 | 19 | 7 | 11 | 21 | 1 | 17 | 13 | |
| **Gene frequency (%)** | | 59.09 | 43.18 | 15.91 | 25.00 | 47.73 | 2.27 | 38.64 | 29.55 | |

[#] highest disease score. *0: Absent, 1: Present

Among 34 susceptible germplasm, maximum six genes found in Acc. 3052, Acc. 3058, Acc. 3060, Acc. 3068 and Acc. 3075.

The presence of *Pit* gene was estimated by visualization of amplicons of 287bp fragment using SNP marker along with positive control IRBLt-k59. The gene frequency of *Pit* gene among the studied germplasm was found 59.09%, which is the highest among the studied genes. *Pib* gene was estimated by presence of 388 bp amplicons using SNP marker b28. IRBLB-b was used as positive control for *Pib* gene detection and the gene frequency was found to be 43.18%. SNP primer Z56592 was used to detect *Piz* gene among the germplasm at 292 bp amplicon. Monogenic line IRBLz-Fu was used as positive control for *Piz*. Only seven germplam contain *Piz* gene and genetic frequency was 15.91%. *Pi9* gene was estimated by using STS primer named 195R-1 with a positive control IRBL9-W. Eleven germplasm contained *Pi9* gene and genetic frequency was 25.00% among the germplasm. Twenty-one germplasm with 47.73% genetic frequency among the germplasm were detected with *Pi40* gene. Primer MSM6 was used for estimate Pi40 gene with a positive control IR65482-4-136-2-2. *Pi5* gene was found only one germplasm detected using 40N23r marker with a resistant check IRBLz5-M. genetic frequency of *Pi5* gene among the studied germplasm found 2.27%. Gene frequency was found 38.64% and 29.55% respectively for Pita3 and YL155/YL87 markers. *Pita-2* and *Pita/Pita-2* were detected by using Pita3 and YL155/YL87 markers respectively. All the genes ere amplified using US2 as a negative control.

Resistant germplasm Acc. 3080 was contain only one gene *Pita-2*, which is indicate that *Pita-2* was the most effective gene against blast disease among the studied genes. Khan *et al.* (2016) also found that IRBLta2-Re (monogenic line contains *Pita-2* gene) was given a resistance reaction against most of the pathotypic races of Bangladesh. Yadav *et al.* (2019) also found some germplasm in his study that contain low number of genes but not showed resistance reaction against blast. Another fourteen germplasm also contained *Pita-2* genes. Among

them seven germplasm were moderately resistant. Surprisingly another seven germplasm among fourteen were susceptible to blast though they were possessed *Pita-2* gene. These germplasm may be possessed *Pita-2* gene but that gene may not functional. Among the studied germplasm nineteen germplasm possessed *Pib* gene but this gene showed no effect of blast reaction. Khan *et al.* (2016) support that information. In their study, they found IRBLb-B (monogenic line contains *Pib* gene) gave susceptible reaction against most of the Bangladeshi blast isolates. Acc. 3058, Acc.3060, Acc 3068 and Acc.3075 contained the maximum number of genes (6) and showed moderately resistant reaction. Besides, some germplasm (Acc. 3052, Acc. 3069, Acc.3077) contained 5 to 6 different genes but showed susceptible reaction. Similar type of result has been reported by Ingole *et al.* (2014). This result might be explained by the allele type of resistance gene in these germplasm changed due to mutation or other complete set of functional gene may not present.

The broad-spectrum *Pita/Pita-2* gene present in 13 germplasm (29.55%). There was no work done in Bangladeshi accession but Singh *et al.* (2015), Imam *et al.* (2014) and Shikari *et al.* (2014) found presence of *Pita/Pita-2* gene in 19.29%, 6.25% and 27% respectively among their studied Indian landraces.

In this study there was no strong relationship found among the known resistance gene with blast disease reaction except *Pita-2* gene.

### 4.3 Genetic diversity of blast resistance genes

The numbers of allele per locus for eight markers 2 in average whether allele frequency ranged from 0.5227 to 0.9773. For the measurement of the information content of a genetic marker, polymorphic information content (PIC) was used. The PIC value of eight markers ranged from 0.04 (40N23r) to 0.37 (MSM6 and b28) and the average PIC value was 0.2978. Marker MSM6 (*Pi40*) and b28 (*Pib*)

were more informative to study the genetic diversity due to their highest PIC value (Table 6).

In this study eight linked marker generate 2 alleles per locus indicates a low diversity level among the studied germplasm. Average PIC value was 0.2978 which indicate low level of polymorphism among markers as only eight markers were used. This result supported by Anupam *et al.* (2017). They found low level of polymorphism in their study as they used only some selective markers.

**Table 6. Estimation of major allele frequency, genotype number, allele number, gene diversity and PIC in germplasms.**

| Marker | Major allele frequency | Genotype number | Allele Number | Gene diversity | PIC |
|---|---|---|---|---|---|
| t311 | 0.5909 | 2 | 2 | 0.4835 | 0.3666 |
| b28 | 0.5682 | 2 | 2 | 0.4907 | 0.3703 |
| Z56592 | 0.8409 | 2 | 2 | 0.2676 | 0.2318 |
| 195R1 | 0.7500 | 2 | 2 | 0.3750 | 0.3047 |
| MSM6 | 0.5227 | 2 | 2 | 0.4990 | 0.3745 |
| 40N23r | 0.9773 | 2 | 2 | 0.0444 | 0.0434 |
| Pita3 | 0.6136 | 2 | 2 | 0.4742 | 0.3618 |
| YL155/YL87 | 0.7045 | 2 | 2 | 0.4163 | 0.3297 |
| **Mean** | **0.6960** | **2** | **2** | **0.3813** | **0.2978** |

*PIC= polymorphic information content

## 4.4. Cluster Analysis

Cluster analysis grouped forty-four native rice germplasm into two main clusters (cluster I and II). The cluster I was a major cluster comprised of 37 germplasm. Cluster I subdivided into two sub cluster IA and IB and comprised of 25 and 12 germplasm respectively. Further sub cluster IA divided into two subgroup sub cluster IA-1 and IA-2. Sub cluster IA-1 comprise of 24 germplasm having 1 resistance and 3 moderately resistant germplasm. Sub cluster IA-2 comprised of only 1 germplasm (Acc.3076) and that was moderately resistant.

Besides sub cluster IB grouped into two subgroup IB-1 and IB-2 and contained seven and five germplasm respectively. All of the germplasm in cluster IB-1 were susceptible except 1 moderately resistant. Cluster IB-2 comprised of three susceptible and two moderately resistant germplasm.

Similarly, cluster II was divided into two subgroup IIA and IIB. Sub cluster IIA comprised of five germplasm among them two were moderately resistant and three were susceptible. Sub cluster IIB contained only two germplasm and both were susceptible to blast (Figure 6).

In this study most of the resistant and susceptible population were not formed distinct cluster. This may be happened due to some germplasm may contain maximum number of genes but susceptible to blast disease. It is also observed in this study that some germplasm were resistant but contain only few or single gene. From the cluster analysis, Acc. 3076 formed a distinct cluster and moderately resistant to blast. Besides, disease score of this germplasm was 2, which indicates that this germplasm contains some functional resistance genes may have relation against blast.

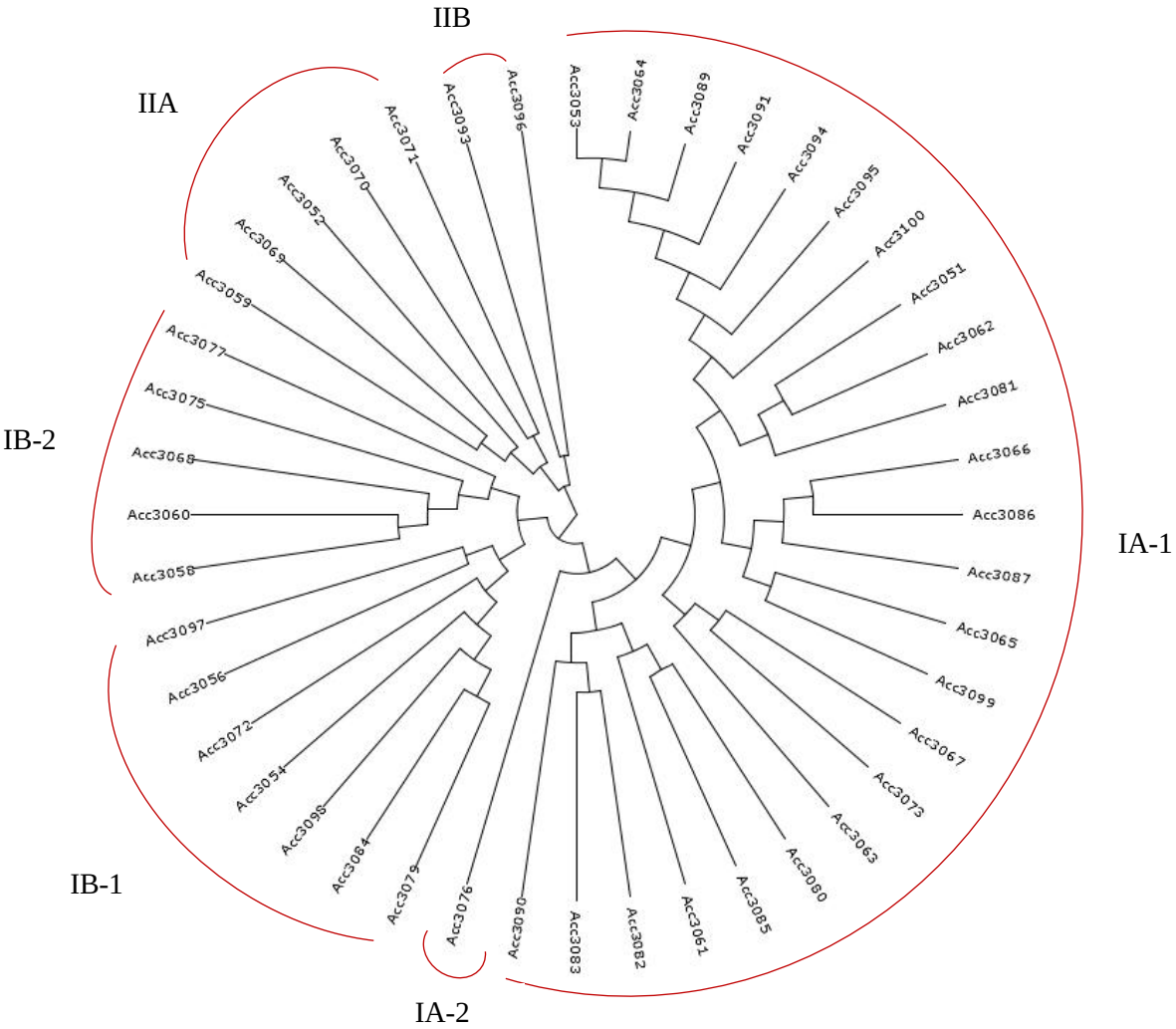



**Figure 6. Cluster analysis of forty-four native germplasm based on R genes.**

## 4.5. Genetic association of blast resistance genes

Among eight markers, only one marker (Pita3) for one R gene (*Pita-2*) showed a significant association to the blast disease (Table 7). The phenotypic variance of Pita3 marker was found 18.5%. Other seven marker representing seven resistance genes did not exhibits any significant association among them. Genetic association is the key to detect novel genes for important traits and identify the potential donor for variety improvement (Choudhury *et al.*, 2014). However, present study did not describe the complete association between blast disease and resistance gene markers. Several numbers of markers linked with resistance gene

should be tested against the germplasm set for understanding complete association between marker and disease reaction. From this study result, Pita3 marker was established as associated marker with blast disease. This marker can be used for genotyping of rice blast resistance gene (*Pita-2*). This result is supported with the finding of Yadav *et al.* (2017). In their study they found Pita3 marker have significant association with blast disease among Indian germplasm.

**Table 7. Genetic association of blast resistance genes with rice blast disease in 44 native germplasm.**

| Sl. No. | Blast R Gene | Marker | *p*-value | Marker_$R^2$ |
|---|---|---|---|---|
| 1 | *Pit* | t311 | 0.16941 | 0.04529 |
| 2 | *Pib* | b28 | 0.02017 | 0.12389 |
| 3 | *Piz* | Z56592 | 0.04778 | 0.09160 |
| 4 | *Pi9* | 195R1 | 0.37647 | 0.01902 |
| 5 | *Pi40* | MSM6 | 0.14315 | 0.05125 |
| 6 | *Pi5* | 40N23r | 0.53086 | 0.0096 |
| 7 | *Pita-2* | Pita3 | 0.00376* | 0.18577 |
| 8 | *Pita/Pita-2* | YL155/YL87 | 0.16042 | 0.04721 |

* significant at 5% level on the basis of Bonferroni adjustment.

### 4.6. Population structure analysis

All 44 native germplasm were evaluated for estimation of population structure for blast disease based on eight markers corresponding to eight blast resistance genes. The peak plateau of adhoc measure ΔK was found to be K=2 (Figure 7). That indicated the entire germplasm population were distributed into two subgroups (G1 and G2). Based on the ancestry threshold >60%, all germplasm were classified into two subgroup with one admixture (Figure 7, Table 8). Acc. 3076 has inferred ancestry value of 58.5% and 41.5% for Q1 and Q2 and so it classified as admixture (AD). G1 was consisting 22 germplasm (50.0%) of which 5 germplasm were moderately resistant. In contrast, in G2 subgroup 21

germplasm were present among them three germplasm were moderately resistant. The only one resistant germplasm (Acc. 3080) was in G2 subgroup.

Acc. 3076 was moderately resistant to blast and also formed different group in cluster analysis.

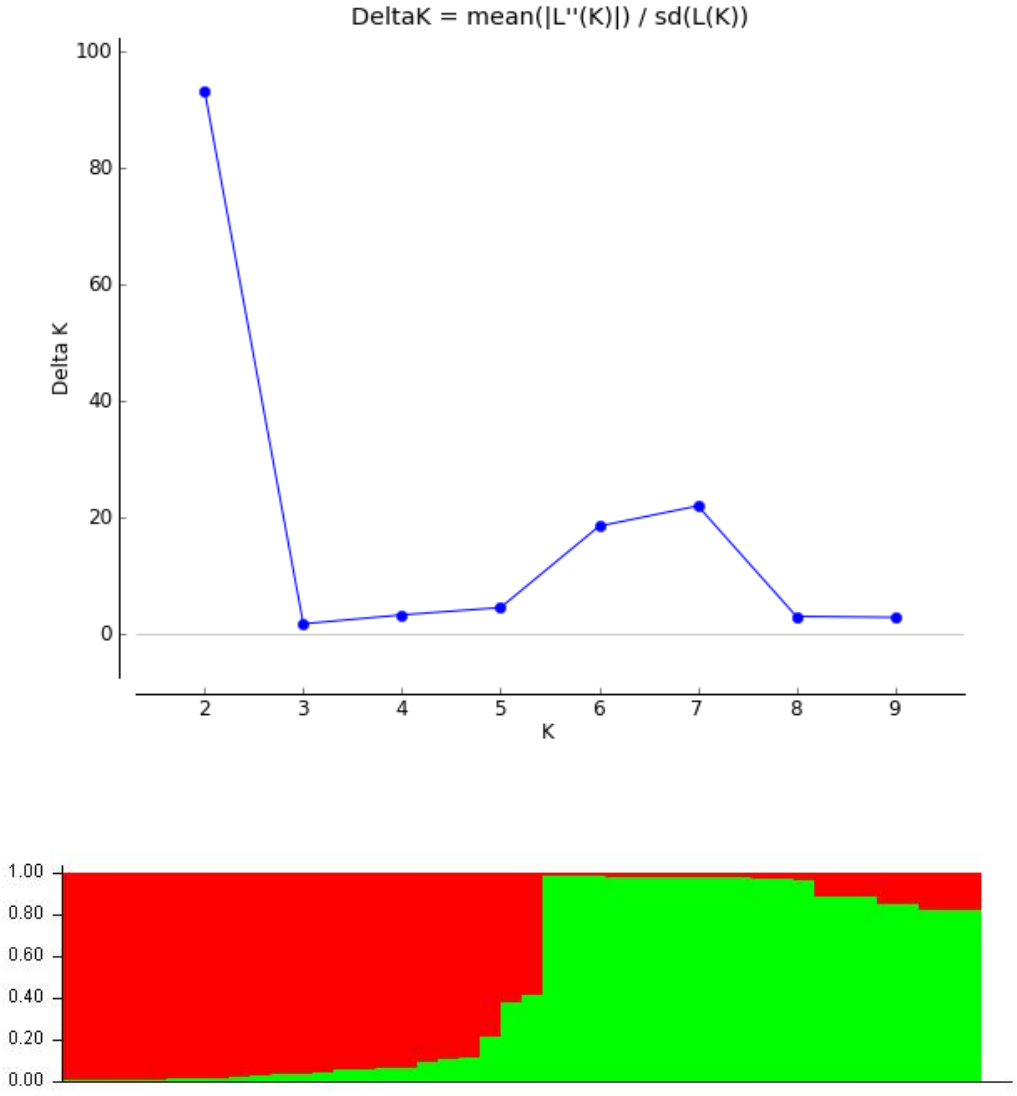



**Figure 7. Population structure of 44 native germplasm based on blast score and 8 markers (K=2) and graph of estimated membership fraction for K=2.**

## Table 8. Population structure group of 44 native germplasm based on inferred ancestry values.

| Sl. No. | Accession Name | Designation | Inferred Ancestry | | Structure group | Group based on blast Score* |
|---|---|---|---|---|---|---|
| | | | Q1 | Q2 | | |
| 1 | Acc. 3051 | Patnai | 0.019 | 0.981 | G2 | S |
| 2 | Acc. 3052 | Patnai | 0.989 | 0.011 | G1 | S |
| 3 | Acc. 3053 | Patnai | 0.015 | 0.985 | G2 | S |
| 4 | Acc. 3054 | Patnai | 0.964 | 0.036 | G1 | S |
| 5 | Acc. 3056 | Patnai | 0.962 | 0.038 | G1 | S |
| 6 | Acc. 3058 | Patnai | 0.987 | 0.013 | G1 | MR |
| 7 | Acc. 3059 | Patnai | 0.984 | 0.016 | G1 | S |
| 8 | Acc. 3060 | Patnai | 0.987 | 0.013 | G1 | MR |
| 9 | Acc. 3061 | Patnai | 0.109 | 0.891 | G2 | S |
| 10 | Acc. 3062 | Patnai | 0.018 | 0.982 | G2 | S |
| 11 | Acc. 3063 | Patnai | 0.621 | 0.379 | G1 | S |
| 12 | Acc. 3064 | Patnai | 0.015 | 0.9854 | G2 | S |
| 13 | Acc. 3065 | Patnai | 0.892 | 0.108 | G1 | S |
| 14 | Acc. 3066 | Patnai | 0.174 | 0.826 | G2 | S |
| 15 | Acc. 3067 | Patnai | 0.112 | 0.888 | G2 | S |
| 16 | Acc. 3068 | Patnai | 0.987 | 0.013 | G1 | MR |
| 17 | Acc. 3069 | Patnai | 0.983 | 0.017 | G1 | S |
| 18 | Acc. 3070 | Patnai | 0.881 | 0.119 | G1 | S |
| 19 | Acc. 3071 | Patnai | 0.784 | 0.216 | G1 | MR |
| 20 | Acc. 3072 | Patnai | 0.970 | 0.030 | G1 | S |
| 21 | Acc. 3073 | Patnai | 0.114 | 0.886 | G2 | MR |
| 22 | Acc. 3075 | Patnai | 0.987 | 0.013 | G1 | MR |
| 23 | Acc. 3076 | Patnai | 0.585 | 0.415 | AD | MR |
| 24 | Acc. 3077 | Patnai | 0.983 | 0.017 | G1 | S |
| 25 | Acc. 3079 | Patnai | 0.936 | 0.064 | G1 | S |
| 26 | Acc. 3080 | Patnai | 0.024 | 0.976 | G2 | R |
| 27 | Acc. 3081 | Patnai | 0.019 | 0.981 | G2 | S |
| 28 | Acc. 3082 | Patnai | 0.146 | 0.854 | G2 | S |
| 29 | Acc. 3083 | Patnai | 0.145 | 0.855 | G2 | MR |
| 30 | Acc. 3084 | Patnai | 0.934 | 0.066 | G1 | S |
| 31 | Acc. 3085 | Patnai | 0.024 | 0.976 | G2 | MR |
| 32 | Acc. 3086 | Patnai | 0.177 | 0.823 | G2 | S |
| 33 | Acc. 3087 | Patnai | 0.178 | 0.822 | G2 | S |
| 34 | Acc. 3089 | Patnai | 0.014 | 0.986 | G2 | S |
| 35 | Acc. 3090 | Patnai | 0.033 | 0.967 | G2 | S |
| 36 | Acc. 3091 | Patnai | 0.015 | 0.985 | G2 | S |
| 37 | Acc. 3093 | Patnai | 0.977 | 0.023 | G1 | S |
| 38 | Acc. 3094 | Patnai | 0.015 | 0.985 | G2 | S |
| 39 | Acc. 3095 | Patnai | 0.014 | 0.986 | G2 | S |
| 40 | Acc. 3096 | Patnai | 0.956 | 0.044 | G1 | S |
| 41 | Acc. 3097 | Nizer Sail | 0.942 | 0.058 | G1 | S |
| 42 | Acc. 3098 | Nizer Sail | 0.933 | 0.067 | G1 | S |
| 43 | Acc. 3099 | Nizer Sail | 0.900 | 0.100 | G1 | S |
| 44 | Acc. 3100 | Nizer Sail | 0.014 | 0.986 | G2 | S |

*R= Resistant, MR= Moderately Resistant, S= Susceptible, G1= Subgroup 1, G2= Subgroup 2, AD= Admixture.

# CHAPTER 5

# SUMMARY AND CONCLUSION

Phenotypic screening of forty-four native germplasm against rice blast disease and molecular identification of resistance genes help to identify the potential germplasm for blast resistance. The present study gives an overview of genetic diversity of blast resistance among the selected native germplasm of rice. These rice germplasm were collected from BRRI genebank and were screened against blast disease at UBN, BRRI, Gazipur during T. Aman 2018 and Boro 2018-19 season. Molecular screening for blast resistance gene(s) was conducted at the molecular laboratory of plant pathology division, BRRI. From the study, Acc. 3080 was detected as resistant to blast disease. Nine moderately resistant native germplasm viz. Acc. 3058, Acc. 3060, Acc. 3068, Acc. 3071, Acc. 3073, Acc. 3975, Acc. 3076, Acc. 3083 and Acc.3085 were also identified. Furthermore, among the germplasm, 59.09%, 43.18%, 15.91%, 25.00%, 47.73%, 2.27%, 38.64% and 29.55% germplasm possessed *Pit, Pib, Piz, Pi9, Pi40, Pi5, Pita-2* and *Pita/Pita-2* gene, respectively. Pita3 marker was found significantly associated with blast disease ($p$=0.003, $R^2$=0.19). This marker could be used for the selection of parent materials and development of resistance breeding lines for identification of *Pita-2* gene. Population structure analysis showed two distinct subgroups among the studied germplasm. Acc. 3076 was admixture among the population and was formed different cluster from other susceptible and resistant group. Acc. 3058, Acc. 3075, Acc. 3076 and Acc. 3080 could be used for the development of durable blast resistant variety. However, further studies are needed for identification of more resistance gene possessed and validating these results.

# CHAPTER 6

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# CHAPTER 7

# APPENDIX

## Appendix I. Sample layout of nursery bed in uniform blast nursery (UBN).