GENETIC DIVERSITY ANALYSIS OF DEEP WATER RICE AND SCREENING OF RICE (*Oryza sativa* L.) GENOTYPES FOR BACTERIAL LEAF BLIGHT

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CERTIFICATE

This is to certify that the thesis entitled ***"GENETIC DIVERSITY ANALYSIS OF DEEP WATER RICE AND SCREENING OF RICE (Oryza sativa* L.*) GENOTYPES FOR BACTERIAL LEAF BLIGHT"*** *submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University (SAU), Dhaka for the partial fulfilment of the requirements for the degree of* ***MASTER OF SCIENCE (MS)*** *in Biotechnology, embodies the results of a piece of bona fide research work carried out by* ***KAZI MEFTAHUL JANNAT,*** *Registration no.* ***15-06528*** *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.

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DEDICATED

TO

MY BELOVED PARENTS

AND SISTERS

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The Author

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ABSTRACT

Genetic diversity analysis was performed to investigate polymorphism among deep water rice and molecular screening to identify bacterial leaf blight disease resistant gene containing rice genotypes. In the flooding region, most of the deep water rice genotypes is drown due to flood. Besides, rice genotype also affected by bacterial leaf blight disease which also causes vast yield loss. The experiment was carried out with 15 and 41 rice genotypes with five well known SSR and five gene specific markers amplification respectively. In case of 15 deep water rice, fifty nine alleles ranged from 7 to 16 alleles per locus and an average of 11.8 was detected. Forty seven polymorphic alleles were also identified. The Polymorphic Information Content (PIC) of SSR markers ranged from 0.103 (RM206) to 0.494 (RM252) with an average value of PIC 0.317. Three primers showed moderate diversity and two primers showed low diversity on the basis of PIC value. The RM252 marker can be considered as the best marker for giving highest number of band. The similarity among rice genotypes was computed from combined data for the 5 primers that ranged from 0.3729 to 0.9375 with an average of 0.7048. Dendrogram indicated the genetic distance of 15 deep water rice genotypes by grouping them into four main clusters. In case of gene specific markers *Xa4*, *xa5*, *Xa7*, *x13* and *Xa21* bacterial leaf blight disease resistant genotypes were identified carrying bacterial leaf blight resistant genes. Among 41 rice genotypes, two different genes were present in 10 genotypes (Dudhsor, Dula Bech, Dulai Aman, Kata Mukul, Anguli Aman, Katari, BR16, BR18, BRRI dhan29 and BRRI dhan58) and one separate gene was present in 12 genotypes. Phylogenetic tree represents the genetic relatedness of 41 rice genotypes by diving them into two main clusters with sub clusters. These results can be conveniently used for identifying diverse parent for the development of high qualitative and quantitative characteristics bearing genotypes in rice.

ABBREVIATIONS AND ACRONYMS

Full word	Abbreviation
2,4-Dichlorophenoxy acetic acid	2,4-D
Agricultural	Agril.
Agriculture	Agric.
American	Am.
Amplified Fragment Length Polymorphism	AFLP
And others (*et al*)	*et al*.
As for example	e.g.
Bangladesh Agricultural Rsearch Instution	BARI
Bangladesh Bureau of Statistics	B.B.S
Base pair	Bp
Biology	Biol.
Biotechnology	Biotech.
Botany	Bot.
Breeding	Breed.
Cetyl Trimethyl Ammonium Bromide	CTAB
Continued	Cont.
Cultivar	cv.
Culture	Cult.
Degree celsius	°C
Deoxyribonucleic acid	DNA
Distilled deionized water	ddH_20
Etcetera	etc.
Ethidium Bromide	Et-Br
Ethylene Diamine Tetra Acetic Acid	EDTA
Genetic Distance	GD
Genetics	Genet.
Gram per liter	g/L
Hectare	ha
International	Int.
Inter simple sequence repeat	ISSR
Journal	J.
Litre	L.
Marker assisted breeding	MAS
Marker-assisted backcross breeding	MABB
Metric ton	mt
Microliter	µl
Millilitre	ml
Millimetre	mm
Molecular	Mol
Namely	viz.
Negative Logarithm of Hydrogen ion concentration	pH

ABBREVIATIONS AND ACRONYMS

Full word	Abbreviation
Percent	%
Polymerase chain reaction	PCR
Polymorphic information content	PIC
Publication	Pub.
Random Amplified Polymorphic DNA	RAPD
Restriction Fragment length Polymorphism	RFLP
Research	Res.
Ribonucleic Acid	RNA
Rotation per minute	Rpm
Science	Sci.
Sequence Tagged Site	STS
Single nucleotide polymorphism	SNP
Simple Sequence Repeat	SSR
Sodium chloride	NaCl
Sodium Dodecyl sulphate	SDS
Species (plural)	Spp.
That is	i.e.
Thermophilus aquaticus	Taq
Tris Boric Acid EDTA	TBE
Tris EDTA	TE
Un-weighted Pair Group Method of Arithmetic Mean	UPGMA
Ultra violet	UV
Volt	V

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CHAPTER I

INTRODUCTION

GENETIC DIVERSITY ANALYSIS OF DEEP WATER RICE AND SCREENING OF RICE (*Oryza sativa* L.) GENOTYPES FOR BACTERIAL LEAF BLIGHT

CHAPTER I

INTRODUCTION

Rice (*Oryza sativa*, 2n=24) is the cereal crops of a semi aquatic grass that is cultivated extensively in warm climates in many countries for its edible grain. It belongs to the family Gramineae and subfamily Oryzoidae which is the most important food crop, providing the staple food for nearly half of the global population (Garris *et al*., 2005; Ramkumar *et al*., 2010). Historians believe that the area covering the foothills of the Eastern Himalayas (i.e. north-eastern India) was the first area where *Indica* variety of rice was first domesticated then stretching through Burma, Thailand, Laos, Vietnam and Southern China. Bangladesh is situated in the centre of the east-west belt where rice may originally have been domesticated in Asia.

In Asia almost 150 million hectares lands are used to cultivate over 90% of world's rice production. Rice accounts for about 50 percent of agricultural income in Asia and supplies almost 80 percent of the region's nutrition. The Food and Agricultural Policy Research predicted the rice demand will ascend as much as 555 million tons in 2035 (Oana, 2017). Asian cultivated rice (*Oryza sativa* L.) stands in a unique position among domesticated crop species in that it is both a critical staple food and the first fully sequenced crop genome.

Rice is central to Bangladesh's economy and agriculture, being the staple food of about 135 million people of Bangladesh. About 48% of rural employment, about two-third of total calorie supply and about one-half of the total protein intake of an average person in the country relies on it (http://www.knowledgebank-brri.org/riceinban.php). One cup (186g) of cooked, enriched, short-grain white rice supplies calories 242 kilocalories, fat 0.4g, sodium 0mg, carbohydrates 53.4g, fibre 0.6g, sugars 0g, protein 4.4g, manganese 0.7mg, iron 2.7mg, thiamine 0.3mg (USDA FoodData Central, 2022). Rice sector contributes one-sixth of the national income

and one-half of the agricultural GDP in Bangladesh. Rice is planted in 75% of the total cropped area and over 80% of the total irrigated area. Thus, rice plays a vital role in the livelihood of Bangladeshi people. Almost all of the 13 million farm families of the country grow rice on about 10.5 million hectares which has remained almost stable over the last three decades. Within next 20 years Bangladesh population may increase to 30 million but total rice area shrinks to 10.28 million hectares by this time. Thus, rice yield needs to be increased from the present 2.74 to 3.74 t/ha (www.knowledgebank-brri.org/riceinban.php**).**

In Bangladesh rice is grown as Boro, Aus and Aman crops in three overlapping seasons with large number of varieties that suits various agro-ecological and climatic niches. Aman (broadcast and transplanted) is generally cultivated in December-January, Boro in March-May and Aus in July-August cropping seasons. Aman season is very important for rice production as most of the low lands contains plenty of water which inhibit crop production and that lands become fallow land. (Banglapedia.org/index.php/Rice). There are many abiotic and biotic stress that causes a great loss of annual rice production.

Presently the focus is on both yield and quality aspects of major food crops to provide balanced diet to human beings. With changing climatic scenario, breeding of climate resilient varieties is becoming more important. The breeding of climate resilient varieties requires novel traits like tolerance towards potential new insect-pests and diseases, excess water level, extreme heat, extreme cold, and towards various air- and soil- pollutants. For ever-changing breeding goals, different genes need to be reserved in cultivated and cultivable crops species in the form of germplasm resources. Molecular screening within and between crop plant species permits the breeders to select superior germplasm either to be directly used as new variety or to be used as parent in hybridization programme for sustainable agriculture.

Flood and drought are the major abiotic barrier in case of crop production. The floodplains and deltas of rivers such as the Ganges and Brahmaputra of India and Bangladesh, the Irrawaddy of Myanmar, the Mekong of Vietnam and Cambodia, the Chao Phraya of Thailand, and the Niger of West Africa are the areas where deep

water rice was cultivated (Bouman *et al*., 2007). Yield loss due to floods ranges from 10 to 100 % depending on rice variety and flood duration (Ismail *et al*., 2013). In Bangladesh, deep water rice grows extensively at Maulovibazar, Cumilla, Noakhali, Kishoregonj, Faridpur, Habigonj, Natore etc. The numbers of deep-water rice cultivars are more than 2,000 in Bangladesh and more than 6,000 in Asia (www.greenpeace.org/india/en/story/13032/we-cant-afford-destruction-of-our-rice-heritage/). Therefore, it is high time to select potential rice cultivars for breeding program to develop submergence tolerant as well as flash flood resistant rice variety to reduce hunger problem overall the world.

During monsoon most of the rice grown in the low lying areas are floating rice, generally called as deep water rice, locally known as broadcast aman, jolidhan, poushdhan etc. It can be grown in more than 50 cm water for one month or longer during the cropping season. Deep water (floating) rice has three special adaptations: (i) the plant elongates with the rise of water levels; (ii) develop nodal tillers and roots from the upper nodes in the water; and (iii) the terminal part of the plant shows upward bending called 'kneeing' that keeps the reproductive parts above the water as the flood subsides. The endotypes are capable of developing roots and leaves in shallow water depths (Angaji *et al*., 2010). Miro and Ismail (2013) found that anaerobic respiration and starch degradation is the main traits associate in rice genotypes in case of flooding tolerance. The products of SNORKEL1 and SNORKEL2 then trigger remarkable internode elongation via gibberellin. Number of elongated internodes increases with the increase in water depths (Hattori *et al.*, 2009). Beside these, almost all the deep water cultivars are strongly photoperiod sensitive that fixes rice flowering time at a favourable point in the flooding period, enables the plant to escape the adverse effect, of low temperature in the reproductive phase, and usually crop matures as soon as floods have receded.

In rice one of the most serious biotic stress Bacterial blight disease caused by the bacterium *Xanthomonas oryzae*. It can cause significant yield losses in rice crops, leading to lower harvests and reduced income for farmers in Bangladesh, who rely heavily on rice cultivation for food and income. *Xanthomonas oryzae can* cause up to 5.8%-30.4% yield loss in Bangladesh field (Ansari *et al*., 2020). In order to control bacterial blight, farmers may need to invest in expensive fungicides and other

input that can increase their production costs and reduce their profits. This can lead to food shortages and increased food prices, making it harder for vulnerable households to access adequate nutrition. Efforts to prevent and control this disease are therefore critical to ensuring sustainable rice production and livelihoods in the country.

Molecular markers are used in many different areas such as genetic mapping, paternal tests, detect mutant genes which are connected to hereditary diseases, cultivars identification, marker assisted breeding of crops, population history, epidemiology and food safety, population studies (Hartl and Jones, 2005). Collard *et al*. (2005) studies that genetic marker used to identify genetic variation among individual species or organisms. Schulmann (2007) studies that genetic marker used to construct linkage maps and genetic diversity. The use of DNA markers has been suggested for precise and reliable characterization and discrimination of rice germplasms (Karkousis *et al*., 2003). DNA markers are extensively used because of their advantages of other markers as they are technically simple, time saving, highly informative and require small amount of DNA and independence from effects related to environmental conditions and the physiological stage of plant.

The PCR-based marker has become the main tool for genetic analysis. A powerful technique for DNA fingerprinting is successful Polymerase Chain Reaction (PCR) amplification of tandem repeat sequences, which have long been known to be polymorphic and widespread in plant genomes referred to as Simple Sequence Repeats (SSR) or Microsatellite polymorphism (Cregan, 1992; Morgante and Olivieri, 1993). The use of Simple Sequence Repeats (SSRs) (Hearne CM *et al*., 1992) or microsatellites have many advantages over RFLP and other PCR based markers, like RAPD, AFLP, CAPS and SCAR markers. They are characterized by great abundance (Condit and Hubbell, 1991), high variability (Schug *et al.*, 1998), co-dominant inheritance, and locus-specificity and also map-based cloning of genes, controlling trait of interest (Xiao *et al.*, 1998 and Zou *et al.*, 2020). SSRs are increasingly useful for integrating the genetic, physical, and sequence-based maps of rice, and they simultaneously provide breeders and geneticists with an efficient tool to link phenotypic and genotypic variation.

Molecular screening of rice germplasm through gene specific markers can be an effective tool for identifying rice varieties that are resistant to bacterial blight, which in turn can help to reduce the incidence of this disease in rice cultivation. Through molecular screening, researchers can identify genes and lines that are naturally resistant to bacterial blight more efficiently and accurately. These lines can then be used in breeding programs to develop new, resistant varieties with improved resistance to the disease that can help to improve the resilience and productivity of rice crops, ultimately supporting the livelihoods of farmers and food security in Bangladesh Hence, research emphasis has been given on genetic diversity for microsatellite DNA markers in broadcasted deep water aman rice germplasm. Therefore, based on the above discussion we defined our following objectives.

OBJECTIVE(S)

The research work was formulated considering the following objectives:

- To assess the polymorphism and molecular characterization of fifteen Broadcasted Aman rice genotypes using SSR markers.
- To establish phylogenetic tree for classifying genotypes in different groups based on their genetic inheritance.
- To identify rice genotypes for carrying bacterial leaf blight disease resistance gene.

CHAPTER II

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Chen *et al.* (2000); conducted a study to improve the resistance of 'Minghui 63' to bacterial blight disease using molecular marker-assisted selection. Results showed that the use of molecular markers increased the frequency of the *Xa21* gene, leading to a higher level of resistance. This study contributes to the literature on the use of molecular markers in crop breeding**.**

Basavaraj *et al.* (2010); used Pusa1460 as the donor for introgressing BB resistance genes *xa13* and *Xa21* into Pusa6B and PRR78 using a marker-assisted backcross breeding program. RM6100, an STMS marker linked to fertility restorer gene (*Rf*), was used for marker-assisted selection of *Rf* gene in an improved version of PRR78. The extent of donor segments in the improved Pusa6B was estimated <0.97 to <2.15 Mb and <2.07 to <3.45 Mb in improved PRR78, the corresponding genomic regions. Improved lines showed yield advantages of up to 8.24 and 5.23%, respectively.

A genetic diversity was analysed using 30 microsatellite markers that detected a clear distinction between the *indica* and *japonica* varietal groups, with 80% of the landraces identified as tropical *japonica* and 20% *indica*. Fluorescent SSR genotyping by capillary electrophoresis revealed 166 alleles, ranging from two alleles up to 15 alleles per locus, with an average of 5.5 alleles across the study. The polymorphism information content (PIC) values ranged from 0.04 (RM271) to 0.87 (RM154), with an average of 0.45, and the mean heterozygosis was 0.03. On average, 62% of the 190 rice accessions shared a common major allele at any given locus **(Thomson *et al.*, 2009).**

Neeraja *et al.* (2005); aimed to characterize tall landraces of rice using 17 gene-derived simple sequence repeats (SSRs) and eight other genomic SSRs. The results revealed high levels of genetic diversity with 131 alleles detected across the 31 markers. Gene-derived SSRs (0.68) had lower polymorphism information content than the other SSRs (0.87). The use of gene-derived markers that target functional loci appears to be an effective strategy in characterizing landraces for optimizing choice of parents for hybridization programmes.

Nagaraju *et al.* (2002); provided valuable insights into the genetic diversity of Basmati and non-Basmati rice varieties, demonstrating the usefulness of ISSR-PCR and SSR markers in studying genetic variation of 56 rice varieties. The non-Basmati varieties were more diverse than both the traditional and evolved Basmati varieties.

Randive *et al*. (2019); screened 50 rice genotypes for biotic and abiotic stress tolerance using 19 trait linked SSR markers were screened. B 40 showed amplification of resistance/tolerance specific alleles for multiple traits, while IR 11 A 546, IR 11 A 581, IR 11 N 169, IRBB 2, IRBB 4, IRBB 5, IRBB 13, IRBB 64, IRBB 12, DS-GMEI-22, IR 64, IRBB 55, IRBB 62, IRBB 14, IRBB 50, IR 11 N 223, IR 64, IRBB 55, IRBB 62, IRBB 14, IRBB 50, IR 11 N 223, IR 64, IRBB 55, IRBB 62, IRBB 14, IRBB 50, IR 11 N 223, IR 64, IRBB 55, IRBB 62, IRBB 14, IRBB 50, IR 11 N 223, IR 64, IRBB 55, IRBB 62, IRBB 14, IRBB 50,

Cho *et al.* (2000); identified 1,669 microsatellites from three rice genomic libraries and 768 microsatellites from Gen-Bank sequences. The authors also investigated the level of polymorphism among the microsatellites by genotyping 48 rice varieties. They found that 57% of the microsatellites from genomic libraries and 67% of the microsatellites from Gen-Bank were polymorphic, indicating that they could be useful for genetic mapping and diversity studies in rice.

The bacterial leaf blight (BLB) resistance gene *xa-5* was shown to be tightly connected to microsatellite and sequence-tagged site (STS) markers in this investigation. When it was discovered that *xa-5* co-segregated with two RFLP clones, RZ390 and RG556, they were transformed into STS markers. The range of allelic diversity for each of the microsatellite loci associated with *xa-5* was determined by a germplasm survey of different lines bearing BLB resistance genes. In attempts to link the recessive *xa-5* gene with other BLB resistance genes, PCR-based markers are practical, affordable, and simple to utilize **(Blair and McCouch, 1997)**

Chakravarthi *et al.* (2006); aimed to use SSR (Simple Sequence Repeats) markers for DNA fingerprinting and diversity analysis of 16 rice varieties from different regions of India and analysed them with 15 SSR markers. The SSR analysis produced 69 alleles, out of which 67 (97.10%) were polymorphic. The researchers also performed a cluster analysis using the SSR data, which resulted in the formation

of two main groups of rice varieties. The high level of genetic diversity found in the rice varieties suggests that these varieties could be a valuable resource for rice breeding programs.

Bhuyan *et al.* (2007); identified the genetic diversity of traditional lowland rice varieties in Assam, India using both RAPD (Random Amplified Polymorphic DNA) and SSR (Simple Sequence Repeats) markers. The results showed high levels of genetic diversity among the varieties producing 138 bands, out of which 128 (92.8%) were polymorphic by RAPD and SSR analysis produced 48 alleles, out of which 45 (93.75%) were polymorphic. Both the marker perform cluster analysis forming aromatic and non-aromatic rice varieties. The findings can be used to develop strategies for conservation and breeding of traditional rice varieties in the region.

Buu and Lang (1999); concluded molecular markers are useful tools for studying the genetic diversity of rice, allowing for the identification of genetic variations at the DNA level. They can be used to create genetic maps, study gene flow, and identify QTLs (quantitative trait loci) associated with important agronomic traits to determine the relationships between different rice varieties. The use of molecular markers has led to the discovery of a great deal of genetic diversity within rice

The researchers used gene-specific markers to identify genes associated with resistance against bacterial leaf blight in rice, The researchers identified several genes, including *Xa21*, *Xa26*, and *Xa33*, which were found to be associated with resistance against bacterial leaf blight. They also validated molecular markers for these genes, which can be used for marker-assisted selection of resistance against bacterial leaf blight in rice breeding programs (**Tiwari *et al.*, 2016).**

Siwach *et al.* (2004); assessed allelic diversity and prepare a DNA fingerprint database of 24 rice genotypes. 229 alleles were detected at 50 SSR loci and 49 alleles were present in only one of the 24 varieties. Polymorphism information content (PIC) values ranged from 0.0 to 0.78, with an average of 0.62 per marker. Traditional and cross-bred Basmati rice varieties amplified different alleles than those in the *indica* and *japonica* rice varieties. These results have implications for genotype identification, monitoring purity and adulteration, and plant variety protection.

Singh *et al.* (2014); conducted a study to map the genetic locus associated with bacterial blight resistance in rice (*Oryza sativa L.*) using a population of 232 F2 individuals derived from a cross between a resistant *indica* rice cultivar and a susceptible japonica rice cultivar. The results showed that the xa8 gene was located on chromosome 4 between the SSR markers RM205 and RM418, covering a genetic distance of 0.9 cM. This provides valuable insights into the genetic basis of bacterial blight resistance and could contribute to the development of improved rice varieties with enhanced resistance to this important disease.

Ranjith *et al.* (2016); reviewed the use of marker-assisted backcross breeding (MABB) to improve Basmati rice varieties for resistance to blast and bacterial blight diseases. MABB involves the selection of suitable parental lines, the use of molecular markers to identify and track the introgressed genes, and the backcrossing process to transfer the resistance genes from the donor parent to the recurrent parent. Several studies have demonstrated the success of MABB, with the resulting lines showing high levels of resistance to blast without affecting the grain quality and other desirable traits of the variety.

Neelam *et al.* (2020); The article provides a detailed description of the genetic mapping process, including the use of molecular markers and a large mapping population to identify the location of the resistance gene. The author also present data on the performance of the new variety, including disease resistance, yield potential, and grain quality. The article is likely to be of interest to plant breeders and researchers working on developing new crop varieties with improved disease resistance and agronomic performance.

Mohanty *et al.* (2015); discusses the pyramiding of three bacterial blight resistance genes, *Xa21*, *xa13*, and *Xa33*, into the deep water rice variety, Jalmagna. . The authors used a combination of conventional breeding and marker-assisted selection to develop Jalmagna lines with the three resistance genes from Swarna BB pyramid line. The resulting lines showed high levels of resistance to bacterial blight under both laboratory and field conditions, indicating that the pyramiding of the three genes resulted in broad-spectrum resistance. The results have important implications for improving the productivity and sustainability of rice production, particularly in regions where bacterial blight is a major problem.

Rahman *et al.* (2019); conducted a comprehensive analysis of the genetic diversity and population structure of 70 deep water rice landraces in Bangladesh. The results showed that the average number of alleles per locus was 6.5, and the average observed heterozygosis was 0.55. The authors suggested that the observed genetic diversity could be due to the long history of cultivation and adaptation to local environmental conditions. The study identified several landraces with unique genetic characteristics, which could be conserved and used for future breeding programs.

Yadav *et al.* (2020); used SSR markers to assess the molecular and genetic diversity of deep water rice genotypes from 31 rice accessions from different regions of India. Results showed high levels of genetic diversity, with an average polymorphic information content (PIC) value of 0.68. The authors discussed the importance of deep water rice for food security in Asia and the challenges posed by flooding for its cultivation. This paper provides valuable information for breeding programs aimed at improving its adaptation to flooding conditions.

Dhakal *et al.* (2021); conducted a study on the genetic diversity of 40 deep water rice accessions collected from Nepal using 20 SSR markers. The results showed a high level of genetic diversity, with an average number of alleles per locus was 4.15 and heterozygosis ranging from 0.11 to 0.92. A cluster analysis was performed to classify the accessions into different groups based on their genetic similarities. The accessions were grouped into two main clusters, with the first cluster consisting of accessions from the Terai region of Nepal and the second cluster from the hilly and mountainous regions of Nepal. This could be useful for future breeding programs aimed at improving the yield and quality of deep water rice varieties in Nepal.

In this experiment, phenotyping for BB resistance was carried out in 210 rice germplasm from eastern and north-eastern India. The most frequent gene was *Xa1*, followed by *Xa7 > Xa4 > Xa10 > Xa11*. The findings indicated that *Xa1*, *Xa7*, and *Xa11* had been frequently selected in breeding programmes, and the frequency of *Xa5, Xa8, xa13* and *Xa21* should be increased in released varieties to achieve durable resistance **(Banerjee *et al.*, 2018)**

Aye *et al.* (2019); conducted a study to investigate the genetic diversity and population structure of 95 deep water rice landraces in Myanmar with the amplification of 24 SSR primers. The results showed that the SSR markers had a

high level of polymorphism, with an average of 9.3 alleles per locus. The genetic diversity of the accessions was also high, with a mean expected heterozygosity (He) of 0.741. The population structure analysis revealed two major groups, which corresponded to the geographical origin of the accessions. The study also identified several accessions with unique alleles that could be used for conservation and breeding programs.

Rohilla *et al.* (2020); investigated the genetic diversity architecture of 94 deep-water rice genotypes of Assam and used genome-wide association studies (GWAS) to identify 20 significant genes associated with AG (anaerobic respiration)-related traits. Two genes, *OsXDH1* and *SSXT*, were found to be associated with anaerobic response index and *Xanthine Dehydrogenase 1* indicating a comparable allele for AG tolerance. These genes are involved in purine catabolism pathway and act as a scavenger of reactive oxygen species in plants. The average heterozygosity rate for all 50 K SNPs was 2.51, with 1% of variation found among population, 35% within individuals, and 64% among individuals. Overall, large variances (44.07%) were seen across all coordinates, demonstrating the diversity and genetic separation of deep-water rice genotypes.

Khannetah *et al*. (2021); assessed the potentiality of BB resistance and molecular characterization of 100 rice accessions for four major BB resistance genes, viz. *Xa4*, *xa5*, *xa13*, and *Xa21*. Four accessions were resistant, 34 were moderately resistant, 49 were moderately susceptible, and 13 were susceptible. Phylogenetic tree grouped the rice germplasm into four major clusters.

The genetic diversity of 42 Indian elite rice varieties was evaluated using three types of DNA markers and parentage analysis. 153 bands (91%) were polymorphic, and the average genetic similarity coefficient was 0.70. Cluster analysis revealed a poor correlation between dendrograms and molecular marker genetic similarities, which can be attributed to selection pressure, genetic drift, sampling of loci and unknown relationships (**Davierwala *et al*., 2000**)

Gopalakrishnan *et al.* (2022); combined non-basmati bacterial blight resistant donor lines IRBB55 with 'Pusa Basmati 1' using mapped microsatellite markers employing backcross pedigree strategy. IRBB55 responsible for *Xa13* and *Xa21* genes and recurrent parent PB1 allele for intermediate amylose content and

maintainer allele at fertility restorer locus in BC1F5. Recombination with recurrent parent genome is 86.3% with quality traits. The extent of non-Basmati donor chromosome segments in superior selection was estimated to be <7.8Mb and <6.7 Mb in the *Xa13* and *Xa21* respectively. The backcross pedigree breeding strategy associated three quantitative trait loci identified by associated mapping. The elite selection of Pusa 1460-01-32-6-7-67 gave resistant reaction against BB and yield of 11.9% over the best check in multiplication agronomic trial that is 'Improved Pusa Basmati 1'.

Sixty-five rice accessions were analysed to evaluate genetic polymorphism and identify diverse parents. A total of 52 alleles were detected by 19 polymorphic markers showing highly polymorphic across all accessions with an average of 2.7 alleles per polymorphic marker. Nineteen polymorphic markers detected 52 alleles, with RM-84 and RM-481 producing maximum 4 alleles. PIC ranged from 0.032 to 0.588, with RM231 being the most appropriate marker. Cluster analysis showed nine clusters, with highest dissimilarity coefficient between LC-4 and IR-82635-B-B-47- and between OR-1946-2-1 and UPLRI-7 cultivars (**Rashmi *et al.*, 2017**)

Sanchez *et al.* (2000); studied improved IR65598-112 and IR65600-42 rice lines with three bacterial blight (BB) resistance genes, *xa5, xa13,* and *Xa21* which successfully transferred to the NPT lines via a marker-aided backcrossing procedure. Sequence tagged site (STS) markers for the two resistance genes were developed based on DNA sequences of their linked restriction fragment length polymorphism (RFLP) markers. Fifty-nine BC3F2 near-isogenic lines (NILs) in the three NPT backgrounds containing one to three BB resistance genes in various combinations were developed through marker-assisted selection (MAS) for the resistance genes and phenotypic selection for the NPT. Results showed that MAS reached an accuracy of 95 and 96% of identifying homozygous resistant plants for xa5 and xa13.

This study evaluated 41 rice genotypes with different drought tolerance from different geographic locations. 68 alleles were detected, with an average of 4.71 alleles per locus. The polymorphic information content (PIC) values ranged from 0.07 to 0.80. Genetic similarity coefficients of pair wise comparisons ranged from 0.23 to 0.91. The primers RM20A, RM302, RM212 and RM286 could be useful for

selecting drought tolerant lines through MAS approach. The results indicated the ability of SSR markers to identify the allelic diversity and genetic variation among the studied genotypes (**Freeg *et al*., 2016)**

Anik *et al.* (2022); explored disease reaction status of 792 landraces against three highly virulent strains of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) to identify bacterial blight resistant reaction responsible genes using six STS (Sequence Tagged Site) markers and morphological variability of potential bacterial blight resistant germplasm using quantitative traits. Maximum number of gene combination was 4 carried by 10 germplasm where G3 genotype (Acc. No. 4216; highly resistant) having *Xa4*, *Xa7*, *xa13*, *Xa21* and G43 genotype (Acc.No. 1523; resistant) having *Xa4*, *xa5*, *xa13* and *Xa23* gene. Multiple desired traits was identified in cluster-III and cluster-VIII through UPGMA dendrogram and heat map analysis.

Siddique *et al*. (2016); assessed genetic diversity and molecular characterization of 96 aman rice landraces using eight SSR markers. Total 159 alleles were found where (RM60, RM239) shows lowest 13 alleles per locus and highest 34 alleles in case of (RM163) with an average 19.88. Highest variation was assessed through highest PIC value 0.95(RM163) germinated seed was sown in earthen pot for further growth and sample DNA extraction. Principal coordinate analysis (PCoA) support the cluster analysis support the cluster analysis but different from six cluster bearing.

Ravi *et al.* (2003); studied to assess the genetic diversity among 40 cultivated varieties and five wild relatives of rice. After amplification with 36 decamer primers and 38 SSR primer pairs, SSR markers determine polymorphic and also monomorphic genotype relationship among accessions. The Polymorphism Information Content (PIC) value ranged from a low of zero (RM 115) to a high of 0.890 (RM 202) with an average of 0.578. In case of RAPD markers, it was difficult to separate close related accessions producing 499 RAPD markers with a polymorphism percentage of 90.0. To compare the similarity matrices the Mantel matrix correspondence test was done and the correlation coefficient was 0. 582 as against the minimum required value was 0.800 indicated that clusters produced based on RAPD and SSR markers were not conserved.

This study explored the genetic resources regarding the presence and absence of BLB resistance genes *Xa4*, *xa5*, and *Xa21* through DNA marker technology. DNA

fingerprinting results indicated the presence of *Xa4* gene in 41 entries, while 14 lines were positive for xa5 gene. Only one local line was carrying *Xa21* gene along with *Xa4* (**Sabar *et al*., 2016)**

Kanawapee *et al*. (2011); estimated genetic diversity of thirty rice cultivars after amplification with 20 random decamer primers and 20 SSR primer pairs. RAPD primers revealed 161 alleles indicating 68.94% polymorphism and mean genetic similarity coefficient was 0.82. RAPD marker based cluster was analysed and grouped into four depending on the levels of salinity tolerance. SSR markers determine 89.47% polymorphism exposing 190 alleles which indicates high genetic diversity and low average genetic similarity with a value 0.70 among cultivars. SSR markers separate similar genotype clusters based on their genetic relatedness which did not correspond to salinity tolerance level. The data obtained through genetic distance also with salt tolerance ability enables a breeder to choose suitable genetically dissimilar parents establishing salt tolerance characters.

Rahman *et al*. (2012); characterize and discrimination among 21 types of rice, 34 microsatellite marker was used. Per locus average allele value was 4.18 while the value ranged from 2 to 11 across 34 loci and 14 Rice varieties produced 42 unique alleles at 20 loci. Polymorphic information content (PIC) value ranged from 0.157 to 0.838 with an average 0.488 while RM401 might be the best marker for identification and diversity estimation as it serves the highest PIC value. Five clusters with a similarity coefficient 0.50 was determined through UPGMA cluster dendrogram. Specific alleles was identified among aromatic varieties through eight SSR markers that could be useful for molecular identification and background selection for breeding purpose.

Samal *et al*. (2012); analysed 47 rice genotypes using nine SSR primers to assess genetic diversity and fingerprinting. A total of 25 alleles were detected by using nine Simple Sequence Repeats (SSRs). The number of alleles per locus ranged from 2 to 4, with an average 2.7. Results showed distinct polymorphism among the varieties studied, cluster analysis showed 8 classes, and Principal component analysis and UPGMA showed similar results. This information can be used in background selections during backcross in breeding programs.

On the basis of genetic distance 53 rice genotypes are grouped into 11 groups resulting maximum inter cluster value 32.96 and 32.90 between cluster I and X followed by I and IV respectively. Maximum intra cluster distance was identified in cluster VII (24.62) followed by cluster XI (22.15) rice lines. Among the studied traits grain yield contributed maximum divergence (50.87%) followed by days to 50 % flowering (15.02%), total grains per panicle (10.52%) and plant height (10.23%) together contribute 86.62% divergence. Highest inter varietal cluster value, maximum intra varietal cluster value and evaluating yield attributed characters highest divergence value provide information for exploiting hybrid vigour, maximum covering areas for developing any desirable trait and for selecting segregating population for further hybridization programme respectively **(Banumathy *et al*., 2010)**

Chakhonkaen *et al*. (2012); examined 43 Thai and 57 IRRI germplasm using 19 SSR markers. A total of 127 alleles for all loci, with a mean of 6.68 alleles per locus, and a mean Polymorphic Information Content (PIC) 0.440 were identified. Genetic diversity of Thai rice were 0.3665, 0.4479 and 0.3972 for improved cultivars, breeding lines, and landraces, respectively, while genetic diversity of IRRI improved and breeding lines were 0.3272 and 0.2970, respectively. Cluster, structure, and differentiation analyses showed six distinct groups concluded that *Oryza* species had the highest genetic diversity, followed by Thai rice lines and IRRI germplasm.

Sonkar *et al*. (2016); assessed genetic diversity is important in rice breeding for selection, conservation and proper utilization. These rice genotypes were grouped into two main clusters that is cluster I and II with similarity coefficient (0.00). 36 rice genotypes were studied for diversity studies using polymorphic SSR markers, with highest similarity between cultivars URG-5 and URG-8 followed by NDR-359 and Pusa-6-B. Akshaya dhan and URG-24 were the most diverse cultivars.

Thomson *et al.* (2007); evaluated genetic diversity among 330 rice accessions, including 246 Indonesian landraces and 63 Indonesian improved cultivars that representatives the classical subpopulations of cere, bulu and gundil rices. After amplification with 30 fluorescently labelled simple sequence repeats microsatellite markers 394 alleles was detected with an average 13 alleles per locus across all accessions. Average polymorphism information content value was 0.66 indicating

Indonesian landraces characterized as 68% *indica* referring gene diversity value 0.53 and 32% tropical *japonica* with genetic diversity value 0.56 that formed a separate clusters through high quality Indonesian varieties including Rojolele. He mentioned average genetic diversity value of improved varieties was 0.46 and sampled as *indica*. All these information provides a valuable source for breeding and association mapping.

Choudhary *et al.* (2013); estimated genetic diversity among 100 rice genotypes in India between 1970 to 2000 using 64 hyper variable microsatellite marker. Fifty two rice genotypes shown polymorphism (81.25%) with a total of 184 alleles were identified ranging from 3 to 7 and with an average of 3.6 alleles per locus. Highest PIC values were estimated ranging between 0.67(RM16416) and 0.97(RM14735) with an average of 0.87. More than 40% of the loci showed frequency of 0.7 to 0.8. Highest genetic distance was observed between the high yielding varieties of the 1990s (0.2715) with lowest value between decades 1990s and 2000s (0.0905).

Luce *et al.* (2010); evaluated 95 inbred *indica* rice cultivars in South China in 1949-2000 amplifying with 300 SSR markers among which 236 were polymorphic. A total of 776 alleles were detected ranging from 2 to 12 with an average 3.29 alleles per locus. The polymorphic information content value ranged from 0.041 to 0.0790 with an average 0.42. Genetic variation among six decades were significant ($p<0.001$) accounting 3.77% of total genetic variance and genetic distance gradually decreased. Cluster analysis formed five groups with Genetic similarity 0.685 indicating only several core parents are responsible for improving inbreed rice in different decades.

This study screened 12 rice genotypes for submergence, drought and bacterial leaf blight disease. PCR markers Sub1BC2, RM431 and four bacterial leaf blight resistance genes were used to select the tolerant or resistant genotypes. RYC743, Purnendu and FR13A were identified as submergence tolerant, while Vaidehi, Dudhi and Birar had drought grain yield QTLqDTY1.1 (**Sinha *et al.*, 2018**)

Shahriar *et al.* (2014); assess the genetic diversity of advanced rice (*Oryza sativa L.*) using three SSR markers viz., RM147, RM167 and RM215. All three primers showed polymorphism, with 29 alleles detected and a polymorphism information content (PIC) ranging from 0.47 to 0.88. A dendrogram was constructed based on

total microsatellite polymorphism and 34 genotypes were grouped into four major clusters at 0.36 based on similarity co-efficient.

conducted a study to understand the pattern of genetic variability in deep-water rice accessions based on molecular markers. Randomly Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeats (ISSR) markers are superior to morphological markers for analysis of genetic diversity and variety identification (**Barooah and Sarma, 2008**)

In the study **Babu *et al*. (2014):** characterized 82 rice genotypes from different parts of Asia using 39 microsatellite loci. Population structure analysis suggested four subpopulations, while phylogenetic analysis grouped them into three populations. Genetic diversity analysis yielded 182 polymorphic alleles, with an average of 4.8 alleles per locus. Seven highly polymorphic SSR loci were identified, which can be used in molecular breeding programs and QTL mapping studies. SSR analysis resulted in a more definitive separation of clustering of genotypes.

Akter *et al.* (2022); used pathogenicity testing and molecular screening in the current work to identify hybrid rice parental lines that were resistant to BB. The 66 maintainers and 200 top restorer lines were tested against BB using three virulent *Xoo* races, *BXo93*, *BXo822*, and *BXo887*. 13 restoration lines that possessed the Xa4 gene but weren't resistant to all three races of BB were chosen from a group of 31 restorer lines to test for the presence of important bacterial blight resistance genes. The most efficient genotypes for developing bacterial blight resistance were those with *Xa21*, *Xa7* + *Xa21*, or *Xa4*+ *Xa21*. These genotypes might be employed as parental lines to create hybrid varieties resistant to bacterial blight.

The study assessed the genetic diversity and molecular characterization of 83 rice landraces in Bangladesh using nine SSR markers. The highest number alleles were identified by RM336, while the lowest was 13 by RM262. The polymorphism information content (PIC) value ranged from 0.951 to 0.766, and marker RM336 was found to be the most appropriate marker to discriminate among the rice genotypes. The Phylogenetic tree based on Nei's genetic distance classified the landraces into 5 clusters with a similarity coefficient of 0.6 (**Hoque *et al*., 2021**)

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The experiment's supplies and procedures were the main topics of the chapter. In this chapter, the specifics of the various tools and procedures used for the study have been covered.

3.1 The experiment's location and duration

At the Department of Biotechnology Laboratory, Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh (location code: 1207), the experiment was conducted. The experiment took place between January, 2022 and December, 2022.

3.2 Pre-treatment of seed and collection of leaf sample

Good quality, disease free, healthy rice seeds were collected from Bangladesh rice research institute (BRRI), Gazipur, Cumilla and Gaibandha district. Germplasm seeds were dried in the sun light for 2-3 days then soaked in water for one day. Tissue was placed on the Petridis and spreading the soaked seeds on it maintaining space, temperature and air for proper germination. After three to four days seed germinated depending on rice cultivars and germinated seeds were sown in individual pots. Soil and pots were collected from nearby nursery. Young green leaves were collected from 15 to 20 days aged rice plants. About 4 cm long leaf tips were collected from the plants and immediately preserved in ice containing box. Then leaf samples were stored in the chamber of -20°C freezer.

Plate 1. Germination after soaking in water for 36 hours

3.3 Study materials source and title

Forty one different rice varieties were employed in the experiments (Table 1). Germplasms was gathered from the Bangladesh Rice Research Institute (BRRI) in Gazipur and different districts of Bangladesh.

Table 1. List of the rice germplasm collected from different region

Sl. No.	Germplasm Name	Collected from	Cropping Season
1	Mota Kartik Sail	BRRI	Deep water rice (Aman)
2	Manik Gira		
3	Laxmi Digha		
4	Anguli Aman		
5	Dudhsor		
6	Dula Bech		
7	Kartik Sail		
8	Kartik Jhul		
9	Dulai Aman		
10	Kata Mukul		
11	Ijule Khoma	Cumilla	
12	Lali Khoma		
13	Dholi Khoma		
14	Hira	Gaibandha	
15	Atash		
16	Katari		
17	BR18	BRRI	Boro
18	BR16		
19	BR8		Boro, Aus
20	BR7		
21	BR19		Boro
22	BRRI dhan28		
23	BRRI dhan35		
24	BRRI dhan45		
25	BRRI dhan47		
26	BRRI dhan50		
27	BRRI dhan81		
28	BRRI dhan84		
29	BRRI dhan99		
30	BRRI dhan100		
31	BRRI dhan58		
32	BRRI dhan36		
33	BRRI dhan97		
34	BRRI dhan29		
35	BRRI dhan55		Aus, Boro
36	IRBB2277		
37	IRBB5		
38	IRBB24		
39	IRBB60		
40	IRBB64		
41	IRBB65		

3.4 Extraction of genomic DNA

The leaf sample's genomic DNA was extracted using the proper phenol with various modifications, the chloroform-isoamyl alcohol technique was described by Islam *et al.* (2013). Using 1% agarose gel, the DNA's quality was evaluated by electrophoresis. Total genomic DNA was isolated using the following tools and procedures.

3.4.1 Equipment's required

1. A mortar and pestle is an essential piece of equipment.
2. A warm bath
3. Centrifugation
4. Vortex combination
5. Ice machine
6. A micro oven
7. PCR machine
8. Electrophoresis system
9. Gel documentation system
10. Micropipette (10, 100, 1000 µl)
11. Microfuge tube (1.0 and 1.5 ml) etc.

3.4.2 Ingredients needed

pH 8.0 extraction buffer

Composition of extraction buffer is as follows:

- 1M Tris HCl
- 0.5 M EDTA (Ethylene diamine tetra-acetic Acid) (pH =8.0)
- 5 M NaCI
- Distil H_20
- 10% SDS (Sodium Dodecyl Sulphate)
- PVP (Polyvinylpyrrolidone)

2. Phenol: Chloroform: Isoamyl Alcohol = 25: 24: 1

3. TE (Tris-EDTA) buffer, pH =8.0

Composition of extraction TE buffer are as follows:

- 1 M Tris HCI
- 0.5 M EDTA

- Distil H_20

4. Isopropanol

5. 0.3 M Sodium Acetate

6. Absolute (100%) ethanol

7. Ethanol (70%)

8. RNase

9. Ethidium Bromide Solution

3.4.3 Reagent preparation for DNA extraction

3.4.3.1 DNA extraction buffer stock solution (1000 ml)

- 100 ml of 1M Tris HCl (pH 8.0) was measured in a measuring cylinder.
- 40 ml of 0.5 M EDTA were then added.
- The mixture was combined with 100 ml of 5 M NaCl.
- The final step was to add sterilized ddH_2O to get the capacity to 1000 ml.
- Following thorough mixing, the mixture was autoclaved.

3.4.3.2 250 ml Tris-HCI stock solution (pH 8.0)

- First, 100 ml of ddH_2O was added to 30.28 g of Tris in a 500 ml volumetric flask.
- HCl was added in order to get the pH to 8.0.
- The volume was then increased to 250 ml by the addition of sterilized ddH_2O.
- The remedy underwent autoclaving.

3.4.3.3 Stock solution for 250 ml 0.5 M EDTA (pH 8.0)

- Initially, 46.53 g EDTA. $2H_2O$ was taken in a volumetric flask (500 ml) with 100 ml ddH_2O.
- 4 g of NaOH was then added.
- NaOH was used to bring the pH level to 8.0, and then sterilized ddH_2O was added to bring the volume to 250 ml.
- The remedy underwent autoclaving.

3.4.3.4 5 M NaCl stock solution (250 ml)

Initially, 250 ml of ddH_2O and 73.05 g of NaCl were added.

- It was then thoroughly combined and autoclaved.
- The remedy underwent autoclaving.

3.4.3.5 SDS Stock Solution 10%

- In a 250 ml beaker, 100 ml of extraction buffer solution was mixed with 10 g of SDS.
- SDS is dangerous, thus the liquid was thoroughly mixed using a hot top magnetic stirrer without using an autoclave.
- 80 ml of ddH_2O and 20 ml of the 10% solution in a 250 ml beaker.

3.4.3.6 TE buffer stock solution, 100 ml

- A volumetric flask was filled with 1 ml of Tris HCl (pH 8.0). (250 ml).
- 0.2 ml of EDTA (pH 8.0) was then added.
- To bring the volume up to 100 ml, 98.8 ml ddH_2O was added.

3.4.3.7 The components of a 10x TBE buffer (1 litre)

- 55 g of boric acid and
- 108 g of tris-HCl
- 9.35 g of EDTA
- A pH of 8.3

Sterile ddH_2O were added to bring the volume to 1000 ml.

I added these chemical sequentially after completely dissolving the previous one.

3.4.3.8 TBE buffer 1X

900 ml of ddH_2O were added to 100 ml of 10X TBE buffer.

Phenol: Isoamyl Alcohol: Chloroform = 25: 24: 1 (100 ml)

- At initially, a volumetric flask containing 50 ml of phenol was used (250 ml).
- 48 ml of chloroform were then added.
- 2 ml of isoamyl alcohol was also added and thoroughly combined.
- The solution was kept at 4°C for storage.

3.4.3.9 RNAse

- One millilitre of ddH_2O was mixed with 10 mg of RNase.
- It was then completely dissolved using the required heat (at 65°C, 30 minutes in a bath of water).

3.4.3.10 70% ethanol (1000 ml)

- 700 ml of 100% ethanol and 300 ml of ddH_2O were combined with 1000 ml.

3.4.3.11 Sodium acetate 0.3M

- 2.05 g of sodium acetate is dissolved in 50 ml of sterile deionized water to provide 0.3 M sodium acetate.

3.5 Genomic DNA extraction protocol

The total genomic DNA was extracted using following procedures

1. Leaf tissues from 41 different rice hybrids that were strong, young, and actively growing were taken in order to isolate genomic DNA.

2. Youngest, healthy leaves were first thoroughly cleaned with tap water, then washed with deionized water, and finally dried on tissue paper.

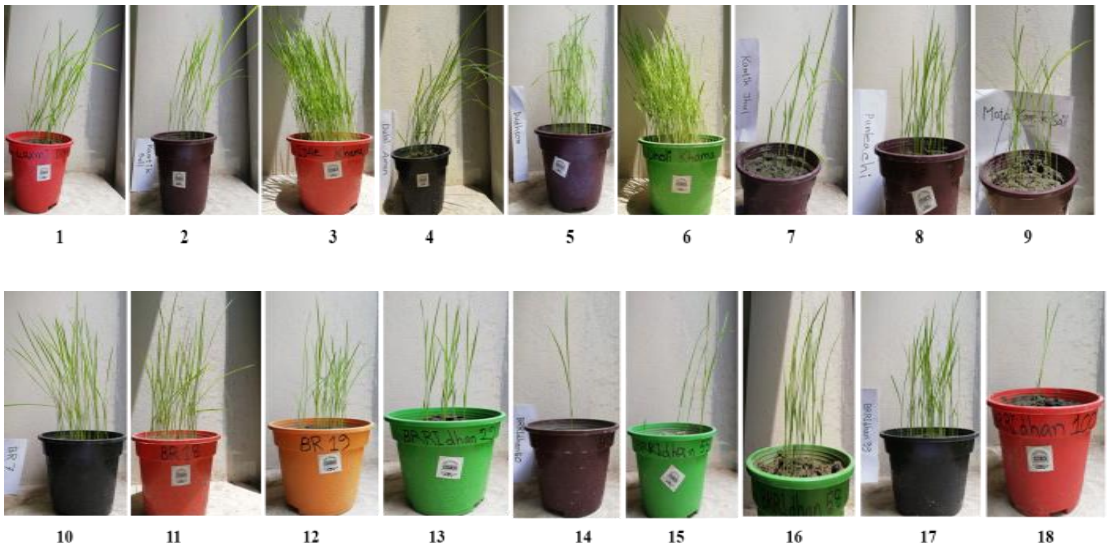

Plate 2. Representative sample of rice seedlings used for genomic DNA extraction.

3. Young leaves were minced into little pieces and administered orally at a dose of about 150 mg. It was given 600µl of extraction buffer. The ground samples were placed in a 1.5 ml eppendorf tube, vortex for 20 seconds in a vortex mixture, and then incubated for 20 minutes at 65°C in a hot water bath.

4. The tube was filled with an equal volume (600µl) of phenol, chloroform, and isoamyl alcohol (25: 24: 1). After that, it underwent a 20-second vortex.

5. After that, the solution was centrifuged at 13000 rpm for 10 minutes. Without touching the lower part, the supernatant was removed with a tiny pipette tip and put into a fresh eppendorf tube. Thereafter, an equal amount of chloroform: isoamyl alcohol (24: 1) was added to the 400–450 µl that had been taken. For ten seconds, the solution was vortexed.

6. The solution was centrifuged once more for 10 minutes at 13000 rpm.

7. The lower layer was discarded and the supernatant was obtained in a different eppendorf tube.

8. The solution's volume was multiplied by 0.6 before being mixed with the same volume of isopropanol (0.6 volume of liquid).

9. A finger was then used to tap it for 20 to 30 seconds (The genomic DNA was visible as cotton like structure).

10. The material was once more centrifuged at 13000 rpm for 15 minutes after tapping. The liquid was totally discarded, and the DNA solution was then precipitated again using 500µl of absolute (100%) cold ethanol and 20 µl of 0.3 M Sodium acetate.

11. It was lightly rattled. The pellet was separated by tapping. The sample was centrifuged for 15 minutes at 13000 rpm. Complete liquid removal allowed for the isolation of precipitated DNA.

12. After being introduced, 500µl of 70% ethanol was vortexed for 10 seconds. It was then centrifuged at 10,000 rpm for 10 minutes. The clear DNA precipitate was then left in the tube after the top solution was withdrawn.

13. After that, the DNA pellet was air dried for 2 to 3 hours while the tube was wrapped in tissue paper. The RNA was then removed by dissolving it in the proper volume (30 to 40µl) of TE buffer and treating it for 15-20 minutes at 37°C with 3 µl of RNAse. It was then spin for four to five seconds.

14. The extracted DNA samples were then placed in a freezer and kept at -20°C.

3.6 Confirmation of extracted genomic DNA

To confirm the extracted DNA sample 1% Agarose gel, working sample of each genomic DNA, 2x loading dye and de-ionized H_2O was needed.

3.6.1 Agarose gel preparation (1%)

Reagents

- Agarose powder
- 1x TBE buffer (pH 8.3)

- Ethidium Bromide

Procedure for making gel

A 500 ml Erlenmeyer flask with 70 ml of 1x TBE buffer was added to 0.7g of agarose powder. To stop excessive evaporation, aluminium foil paper was used to wrap the flask. To create a homogeneous, crystal-clear suspension, the flask was heated in a microwave for about two minutes, sometimes whirling it to create uniform suspension. To make the DNA visible under an ultraviolet light box, 0.5 µl of ethidium bromide (DNA stain) was added and thoroughly mixed by moderate shaking after the agarose solution had been cooled to a temperature of 45 to 50° C. (Trans-illuminator). Onto a fresh gel bed (l5×15×2 cm^3 size), the molten gel was immediately poured. This was set on a flat surface, and an adequate comb was inserted with the bottom of the teeth sitting about 2 mm above the plate. The comb was carefully removed after the gel had softly hardened and had cooled to room temperature after 25 minutes. The DNA samples could then be loaded onto the gel.

3.6.2 DNA sample preparation for electrophoresis

All of the samples were in a buffer at the same concentration. An eppendorf tube was filled with 3 µl of de-ionized water, 2 µl of 2x loading dye (0.25% xylene ethanol, 0.25% bromophenol blue, 30% glycerol, and 1 mM EDTA), and 3.0 µl of sample DNA for each sample. In order to keep the sample in the well, loading dye was employed to monitor loading, the development of the electrophoresis, and to raise the density of the sample. Lastly, 8.0 µl of the anticipated DNA was introduced and thoroughly mixed. The sample was then put into the gel's well, where it was left to sink to the bottom. The gel was introduced. The gel was positioned in the electrophoresis chamber, kept horizontal, and covered with a layer of 1x TBE buffer (running buffer). To transport DNA from a negative to a positive electrode (black to red), the gel tank was covered, and the electrophoresis power source was turned on. A 50-minute electrophoresis procedure was performed at 75 volts.

Table 2. DNA confirmation tools and their quantities

Components	Quantity (µl)
Deionized water	3.0
Working DNA sample	3.0
2x loading dye	2.0
Total	8.0

Plate 3. Confirmation of genomic DNA of rice (representative) genotypes.

Lane: 1= BRRI dhan58; 2= BR18; 3 = Dholi Aman; 4= BR16; 5= BRRI dhan36; 6= BRRI dhan97; 7= BR8; 8= IRBB5; 9= Manik Gira; 10= BRRI dhan29; 11= Laxmi Digha; 12= Anguli Aman; 13= Dudhsor; 14= Dula Bech; 15= Kartik Sail; 16= Kartik Jhul; 17= Dulai Aman; 18= BRRI dhan55; 19= Atash; 20= Katari; 21= Mota Kartik Sail; 22= Ijule Khoma; 23= Lali Khoma; 24= Kata Mukul; 25= Hira; 26= IRBB24; 27= IRBB60; 28= IRBB64; 29= IRBB65; 30= IRBB2277; 31= BR7; 32= BR19

3.6.3 Records for the DNA samples

After removing the gel from the chamber, it was examined with an ultraviolet light box (UV trans-illuminator) and shot with a Gel-Cam camera. DNA samples with better-quality bands were then collected for quantification and working solution preparation for the subsequent phase.

3.7 SSR and gene specific marker synthesis

For molecular diversity analysis in fifteen disseminated deep water rice five (Matin *et. al*., 2012) well-known SSR primers RM1, RM85, RM252, RM13, RM206 (Table 3) and five (Yap *et al*., 2016) gene specific primers Xa4F/4R, RM604F/604R, Xa7F/7-1R/7-2R, Xa13F/13R, Xa21F/21R (Table 4) were chosen to identify the bacterial blight resistance genes. This is a list of the primers that were utilized.

3.7.1 Preparation of primer working solution from stock solution

To make 100 µl working primer solution, 10 µl stock primer was taken in a 1500 µl eppendorf tube. After that 90 µl ddH_2O was added to make total volume 100 µl**.**

Table 3. List of SSR markers used for genetic diversity analysis of 15 deep water Aman rice

Sl. No.	Marker Name	Chromosome No.	Product Size(bp)	Forward primer (5′–3′)	Reverse primer (3`-5`)	Annealing temp.
1	RM1	1	113	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC	55°C
2	RM85	3	107	CCAAAGATGAAACCTGGATTG	GCACAAGGTGAGCAGTCC	
3	RM252	4	216	TTCGCTGACGTGATAGGTTG	ATGACTTGATCCCGAGAACG	
4	RM13	5	141	TCCAACATGGCAAGAGAGAG	GGTGGCATTCGATTCCAG	
5	RM206	11	147	CCCATGCGTTTAACTATTCT	CGTTCCATCGATCCGTATGG	

Table 4. List of five functional markers used for identifying bacterial leaf blight disease resistant rice germplasm

Sl. No.	Resistant gene	Chr. no.	Marker name	Forward primer(5′–3′)	Reverse primer(3`-5`)	Annealing temp. (°C)
1	*Xa4*	11	Xa4F/4R	GCAGCACCATCTCCATCGTTTC	CTGCTATAAAAGGCATTCGGGTCTC	57
2	*xa5*	5	RM604F/604R	AGGTGACCATCCTCTTTCTT	TTAACGAACGAACGCGAG	
3	*Xa7*	6	Xa7F/7-1R/7-2R	GGTCGGAAGGTGAGAAAGAGGAGG	GCATGTCTGTGTCGATTCGTCCGTACGA	
4	*xa13*	8	Xa13F/13R	TACCTCCTGATATGTGAGGTAGTGAGAG	AGAGAGAGGTAACTTGAAGAAAGGGAT	
5	*Xa21*	11	Xa21F/21R	GCTATTTCCTGATCCAGCATATCTGATC	GATCGGTATAACAGCAAAACTATTTCCG	

3.7.2 Working solution of DNA sample preparation

DNA concentration were adjusted to 25 ng/µl for doing PCR using the following formula:

$V1 \times S1 = V2 \times S2$

Where, V1 = Initial volume of DNA solution (µl)

S1 = Initial DNA concentration (ng/µl)

V2 = Final volume of DNA solution (µl)

S2 = Final DNA concentration (ng/µl)

3.8 Amplification of SSR markers by PCR

To amplify definite gene segment in the rice germplasm polymerase chain reaction was done with primer and then amplification was observed.

3.8.1 The SSR marker's amplifying principle

Tandem repeats of 1-6 nucleotides are known as microsatellites or SSR. Consider the following examples: (A)n, (AT)n, (ATG)n, (GATT)n, (CTACG)n, (TACGAC)n etc. all organisms genetics have abandoned them. To create primers and perform PCR to amplify SSR-containing sequences, it is possible to use the sequence of the SSR's distinctive flanking regions. SSRs were initially employed as markers in human genomic mapping (Litt and Luty, 1989).

3.8.2 Amplification by the polymerase chain reaction

Using a GeneAtlas G (Astec, Japan) 96-well thermal cycler, PCR analysis was carried out in a reaction sample of 10 µl comprising 3 ng of DNA template, 4.5 µl of Go Taq G2 Green Master Mix (Canada), 1.5 µl of Nuclease-Free Water, and 0.5 µl of each 10 M forward and reverse primer (Table 5). The DNA was transferred from the dilution plate to the PCR plate using a 12-channel pipette. To stop the mixture from evaporating, 10 µl of mineral oil were added on top. Adhesive film was used to cover the PCR plate. The following are the components of the PCR reaction for SSR markers:

Table 5. PCR cocktail ingredients and preparation (master mix)

Reagent	Amount (µl)
Master mix	2
DNA	3.0
Primer(F)	0.5
Primer(R)	0.5
ddH_2O	4
Total	10

After initial denaturation for 5 minutes at 94°C, each cycle comprised 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min extension with a final extension for 5 min at 72°C at the end of 35 cycles for genetic diversity analysis (Table 6) and bacterial leaf blight gene specific marker were used for initial

denaturation at 95°C for 15 minutes, each cycle comprised 30 sec denaturation at 94°C, 2 min annealing at 57°C, and 2 min extension at 72°C with a final extension for 30 min at 60°C at the end of 30 cycles (Table 7).

Table 6. Temperature profile of PCR for diversity analysis (Easy shortcut new method)

Step	Temperature	Time	No. of Cycle
Initial Denaturation	94	5 min	
Denaturation	94	1 min	35
Annealing	55	1 min	
Extension	72	1 min	
Final extension	72	5 min	
Hold at	4°C		99:99 (overnight)

Table 7. Ideal PCR condition for bacterial leaf Blight resistance

Step	Temperature	Time duration	No. of Cycle
Initial Denaturation	95	15 min	
Denaturation	94	30 sec	30
Annealing	57	2 min	
Extension	72	2 min	
Final extension	60	30 min	
Hold at	4°C		99:99 (overnight)

3.9 Agarose Gel Electrophoresis

To verify DNA quantity and PCR amplification, three microliters of PCR products containing SSR markers were electrophoresed on agarose gel at 75 volt at various time settings. However, the complete methodology is provided below:

3.9.1 Glass plate assembly

1. Using laboratory detergent (based on bleaching powder), two glass plates, two spacers, and one comb were adequately cleaned before being rinsed with water. A 0.5 M NaOH solution was also used to wash glass plates. The inside surfaces of some glass plates were selected and sprayed with 100% ethanol before being air dried and wiped with lint-free tissue.

2. Starting from one side of the short plate (round-bottom), the rubber gasket was fastened. The corners of the gasket's notches were lined up. The inner surface of the plate was exposed by the gasket's circular part.

3. The interior side of the short plate was towards the table. The spacers were then placed along the gasket's inner edges.

4. The second plate was positioned on top of the first one.

5. The plate assembly was positioned flat on the table after the clamps were tightened on both sides of the plates.

3.9.2 Agarose gel preparation

To manufacture 1% PAGE gel, the following substances and their quantities were employed. Electric oven was used to melt the agarose powder and gel solution in a beaker.

Table 8. Agarose gel preparation and composition

Reagents	**Final cont.**	**1% gel**
10X TBE buffer	1X	70 ml
Agarose gel	1%	0.7 g
Ethidium bromide	0.1%	0.5 µl
Total		70.0 ml

After adding agarose, the beaker with TBE buffer was put in electric oven for 2 minutes. After that ethidium bromide was added with caution. The gel solution was then smoothly and continuously poured into the glass plate assembly, starting from one corner, to prevent air bubbles. The gel was allowed to polymerize for 30 minutes.

3.9.3 Agarose gel electrophoresis

The gasket was removed from the plate assembly starting from one corner after the gel had polymerized. The tank's base was filled with approximately 500 ml of 0.5X TBE buffer, and the top of the tank with approximately 300 ml of 0.5X TBE buffer before the comb was gently removed.

1. Each well containing 10 µl of PCR product received 2 µl of 2X loading dye, and the plates were centrifuged at 3000 rpm for 30 sec in a high-speed refrigerator-based centrifuge. Using a 2-2.5 µl pipette, approximately 2 µl of the mixer was added to the PAGE gel wells. A 50 bp DNA size marker DNA ladders had been loaded for size determination. Thermo Scientific Gene Ruler 1Kb Plus DNA Ladder was used from Thermo Scientific company (Canada).

2. After connecting the electrodes to the power source and covering the tank, the gel was operated at 75 volts for around 50 to 60 minutes. Running time was shown to be influenced by PCR fragment size.

3.9.4 The Gel's staining and visualization

1. The plates were taken out of the tank and the power supply unit was turned off. Carefully removing the agarose gel with DNA sample.

2. The stained gels were placed in the gel documentation system's exposure cabinet (Molecular Imager Gel Doc XR System, BIO-RAD, Korea). By first exposing the gel to white light, it was possible to view it on the computer monitor. The gel inside the exposure box was moved to make the appropriate modifications. UV light was used to photograph the gel after which the gel image was saved as a JPEG file.

3.10 Analysis of SSR data

Using POWERMAKER version 3.25 (Liu and Muse, 2005), a genetic marker data analysis program, the summary statistics, including the number of alleles per locus, major allele frequency, gene diversity, and Polymorphism Information Content (PIC) values, were calculated. The software AlphaEaseFC (Alpha Innotech Corporation), version 4.0 was used to estimate the molecular weights of each microsatellite product in base pairs. The following formula (Anderson *et al*., 1993) was used to calculate polymorphic information content (PIC) values:

$PIC_i = 1 - \sum^{n}_{j=1} p^2 ij$

Where, n is the number of marker alleles for marker i and P_{ij} is the frequency of the j^{th} allele for marker i

Shannon diversity index was quantified using following formula

$I = -\sum^{n}_{j=1} pi_{ln} pi$, where pi is the frequency of the j^{th} allele of a particular locus containing a total of n alleles.

Each fragment was given an allele designation for the relevant microsatellite loci. Moreover, genetic diversity was evaluated, and phylogenetic trees based on Nei's (1983) genetic distance were created using MEGA 6.0 (Tamura *et al*., 2013; Liu et al., 2021).

Some Pictorial View of lab work

Plate 4. Leaf grinding for DNA extraction

Plate 5. Centrifugation for identifying DNA containing layer

Plate 6. Discarding upper layer leaving DNA pellet at the tube

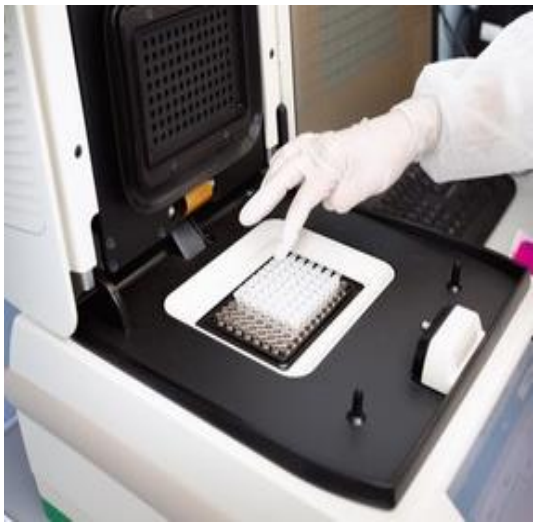

Plate 7. Sample arrangement for PCR reaction in PCR machine

Plate 8. Gel preparation for DNA sample

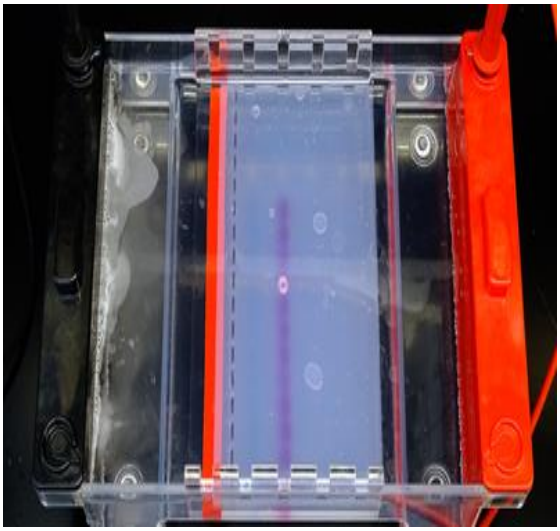

Plate 9. Gel analysis after running with TBE 1x buffer

CHAPTER IV

RESULTS AND DISCUSSION

CHAPTER IV

RESULTS AND DISCUSSION

The present study focused on the molecular diversity study of deep water aman rice using simple sequence repeats (SSR) highly repeatable and polymorphic PCR based marker to assess polymorphism and similarity among local genotypes and gene specific marker to assess bacterial leaf blight resistance genes in rice germplasm. Results identified from the research have been presented below under the following title.

4.1 Amplification of DNA by PCR and polymorphic banding pattern

Amplifying with 41 (15 deep water Aman rice) rice genotypes, RM1, RM85, RM252, RM13, RM206 five SSR primers and Xa4F/4R, RM604F/604R, Xa7F/7-1R/7-2R, Xa13F/13R, Xa21F/21R five gene specific markers exhibited various banding patterns. By maintaining proper PCR condition this method helps to amplify DNA content. Mondini (2009) states that PCR, or polymerase chain reaction method involves amplifying a number of distinct DNA products that originate from DNA regions that are bordered by high-homology primer regions. Agarose Gel helps to investigate desirable banding pattern of rice genotype with definite marker. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to profile total seed proteins in order to examine the genetic variety of rice types (Noreen *et al.*, 2020)**.** Idrees and Irshad (2014) used 1.5- 2.0% agarose gels and stained with ethidium bromide (EtBr) to get PCR products. Some *L*. *perenne* accessions displayed variance upon basic agarose gel electrophoresis of amplification products of marker TeaCpSSR27 (Diekmann, 2012). Table 9 and plates 1 to 11 show the results of the amplification of each SSR and the gene-specific primers.

Sixteen fragment of DNA amplification were noticed by SSR primer RM1. The size of the amplification ranged from 76bp to 300bp. These bands indicate a highly polymorphic band and total number of allele was 47. Kartik Jhul and Dholi Khoma produced a band at 76bp and 98bp respectively. DNA fragment 100 bp was obtained from Anguli Aman, Dula Bech, Dulai Aman, Katari. On the other hand, Dudhsor, Kartik Jhul, Ijule Khoma, Lali Khoma, Kata Mukul gave their amplification at 105 bp. Fragment 110 bp was produced by Laxmi Digha and Mota Kartik Sail. The 115 bp fragment was produced by Manik Gira, Anguli Aman, Dula Bech, Dulai Aman, Katari. 125bp band was observed in Laxmi Digha, Anguli Aman, Dulai Aman, Katari and Mota Kartik Sail.

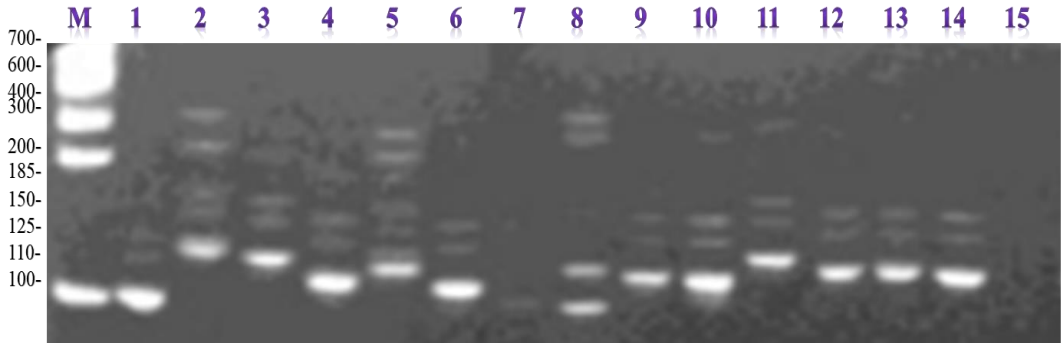

Figure 1. DNA profile of 15 deep water rice using SSR primer RM 1

M = Molecular Marker (1 kb plus DNA Ladder, Bio Basic, Canada); Lane: 1 = Dholi Khoma; 2 = Manik Gira; 3 = Laxmi Digha; 4 = Anguli Aman; 5 = Dudhsor; 6 = Dula Bech; 7 = Kartik Sail; 8 = Kartik Jhul; 9 = Dulai Aman; 10 = Katari; 11 = Mota Kartik Sail; 12 = Ijule Khoma; 13 = Lali Khoma; 14 = Kata Mukul; 15 = Hira.

Again, Dula Bech, Ijule Khoma, Lali Khoma, Kata Mukul amplified at 135bp. Rice germplasms Manik Gira, Dudhsor, Dulai Aman, Katari, Mota Kartik Sail produced band at 140bp. Furthermore, Laxmi Digha, Dudhsor, Ijule Khoma, Lali Khoma, Kata Mukul gave their amplification at 150bp. Laxmi Digha and Katari formed band at 155bp. Laxmi Digha and Dudhsor amplified at 200bp. Manik Gira amplified at 220bp. At 250bp Dudhsor, Kartik Jhul, Katari amplified. Only Mota Kartik Sail showed band at 280bp. Manik Gira and Kartik Jhul produced band at 300bp (Figure 1). Allhgholipour *et al*. (2014) identified RM14 and RM1 on chromosome 1 and RM276 and RM5642 on chromosome 6 showed highly polymorphic band having more than 9 observed allele.

PCR was done for 15 deep water rice germplasm using RM85 SSR marker that was able to amplify fourteen fragments of DNA. A total number of 42 alleles were observed. The size of amplification was 91 to 800bp. Dholi Khoma, Manik Gira and Laxmi Digha amaplified at 91 and 95bp respectively. Anguli Aman, Dudhsor and Dula Bech, Kartik Sail produced band at 97 and 100bp respectively. Kartik Jhul, Dulai Aman, Katari, Mota Kartik sail displayed band at 105bp. Ijule Khoma, Lali Khoma and Kata Mukul, Hira amplified band at 110 and 115bp. At 250bp and 265bp Dudhsor, Kartik Jhul, Mota Kartik Sail, Lali Khoma and Laxmi Digha, Anguli Aman, Katari displayed band respectively.

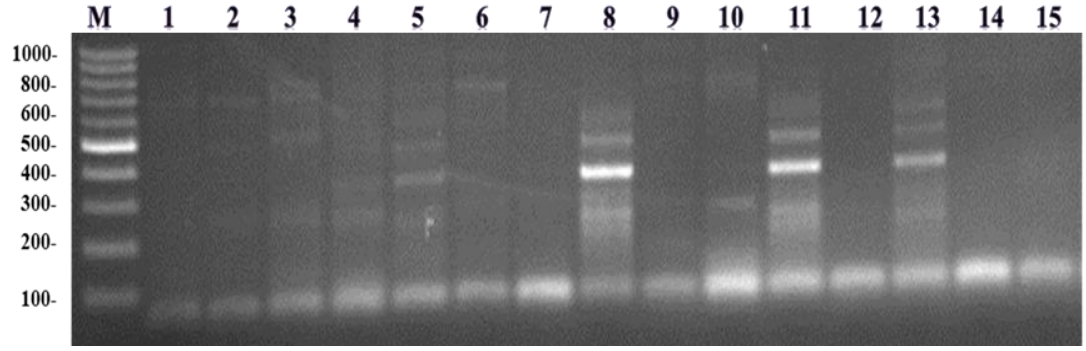

Figure 2. DNA profile of 15 deep water rice using SSR primer RM 85

M = Molecular Marker (1 kb plus DNA Ladder , Bio Basic, Canada); Lane : 1 = Dholi Khoma; 2 = Manik Gira; 3 = Laxmi Digha; 4 = Anguli Aman; 5 = Dudhsor; 6 = Dula Bech; 7 = Kartik Sail; 8 = Kartik Jhul; 9 = Dulai Aman; 10 = Katari; 11 = Mota Kartik Sail; 12 = Ijule Khoma; 13 = Lali Khoma; 14 = Kata Mukul; 15 = Hira.

Again, Anguli Aman, Dudhsor, Kartik Jhul, Mota Kartik Sail, Lali Khoma produced band at 350bp. At 500bp and 600bp Dudhsor, Kartik Jhul, Mota Kartik Sail, Lali Khoma amplified. Dholi Khoma, Manik Gira, Laxmi Digha showed banding pattern at 700bp. Finally, Laxmi Digha, Dula Bech and Katari formed band at 800bp. Manik Gira, Anguli Aman, Katari shows three allele and Dholi Khoma, Dula Bech shows two polymorphic band. KartiSail, Ijule Khoma, Kata Mukul, Hira shows monomorphic band (Figure 2). Manjunatha *et al*. (2021) observed four amplicons in case of RM85 SSR marker. SSR markers showed polymorphism by producing multiple allele ranging from 2 alleles (RM510, RM244, and RM277) to 6 alleles (RM 163), with an average of 3.33 alleles across 9 loci (Sajib *et al*., 2012). Primers RM85 showed 100% polymorphism detecting 8 alleles that is the lowest number of amplicons (Wang *et al*., 2022).

SSR primer RM252 detected eleven amplification-related DNA fragments. The size of the amplification ranged from 195bp to 295bp. All the germplasms forms polymorphic banding pattern and total number of allele was recorded 36. Ijule Khoma forms band at 195bp. Laxmi Digha, Anguli Aman, Mota Kartik Sail and Lali Khoma gave their amplification at 200bp. Again, 205bp was observed in Dholi Khoma, Manik Gira, Dudhsor, Kartik Jhul, Dulai Aman and Katari. Whereas, Kartik Sail amplified band at 210bp.

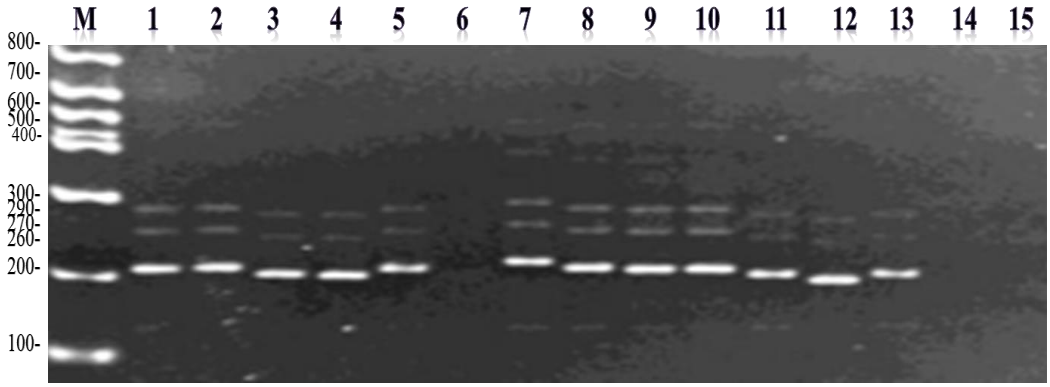

Figure 3. DNA profile of 15 deep water rice using SSR primer RM 252

M = Molecular Marker (1 kb plus DNA Ladder , Bio Basic, Canada); Lane : 1 = Dholi Khoma; 2 = Manik Gira; 3 = Laxmi Digha; 4 = Anguli Aman; 5 = Dudhsor; 6 = Dula Bech; 7 = Kartik Sail; 8 = Kartik Jhul; 9 = Dulai Aman; 10 = Katari; 11 = Mota Kartik Sail; 12 = Ijule Khoma; 13 = Lali Khoma; 14 = Kata Mukul; 15 = Hira.

Laxmi Digha, Anguli Aman, Mota Karatik Sail, Lali Khoma amplified at both 250 and 280bp. At 260bp Ijule Khoma amplified. Dholi Khoma, Manik Gira, Dudhsor, Kartik Jhul, Dulai Aman, Katari formed band at both 265 and 290bp. Kartik Jhul produced band at both 270 and 295bp (Figure 3). Chauhan *et al*. (2015) investigated 48 soybean germplasms with 21 SSR primers displayed 84 alleles that were polymorphic in nature.

Figure 4. states that, SSR primer RM13 was able to amplify eleven different fragments of DNA with a highly polymorphic banding pattern. The size of amplification ranged from 90bp to 395bp. Total number of allele was 59. Dholi Khoma, Manik Gira, Laxmi Digha, Anguli Aman, Dudhsor, Kartik Sail, Kartik Jhul, Dulai Aman, Katari, Mota Kartik Sail, Ijule Khoma, Lali Khoma, Kata Mukul displayed band at 90bp. Dholi Khoma, Manik Gira, Laxmi Digha, Anguli Aman,

Lali Khoma, Kata Mukul formed band at 100bp. Laxmi Digha, Dulai Aman, Katari, Ijule Khoma showed band at 120bp. Again, Dholi Khoma, Kartik Jhul and Mota Kartik Sail obtained band at 140bp. Dholi Khoma, Manik Gira, Laxmi Digha, Anguli Aman, Dulai Aman, Mota Kartik Sail, Lali Khoma, Kata Mukul formed band at 150bp. Dudhsor, Kartik Jhul, Katari, Ijule Khoma displayed band at 155bp. Anguli Aman, Dudhsor, Katari, Kata Mukul showed amplification at 170bp. Laxmi Digha, Katari formed band at 180bp.

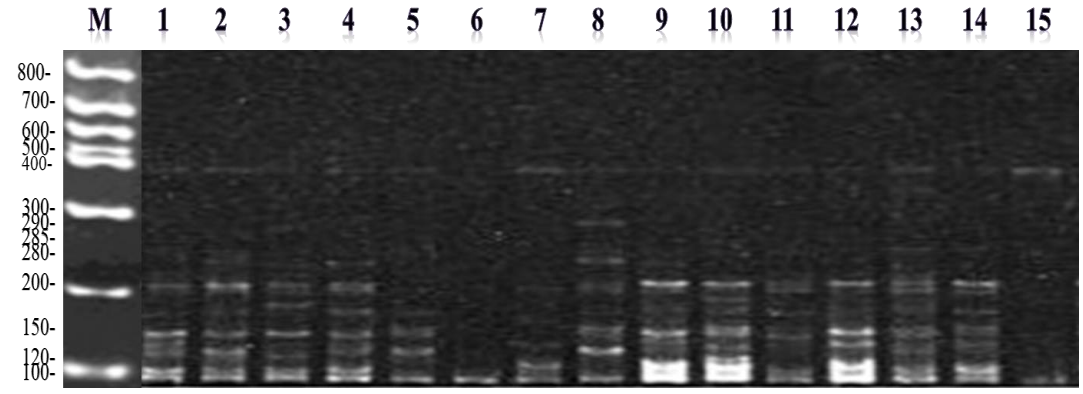

Figure 4. DNA profile of 15 deep water rice using SSR primer RM 13

M = Molecular Marker (1 kb plus DNA Ladder , Bio Basic, Canada); Lane : 1 = Dholi Khoma; 2 = Manik Gira; 3 = Laxmi Digha; 4 = Anguli Aman; 5 = Dudhsor; 6 = Dula Bech; 7 = Kartik Sail; 8 = Kartik Jhul; 9 = Dulai Aman; 10 = Katari; 11 = Mota Kartik Sail; 12 = Ijule Khoma; 13 = Lali Khoma; 14 = Kata Mukul; 15 = Hira.

Most of the genotypes formed band at 210 bp that are polymorphic in nature. Dholi Khoma, Manik Gira, Laxmi Digha, Anguli Aman, Kartik Jhul, Dulai Aman, Katari, Mota Kartik Sail, Ijule Khoma, Lali Khoma, Kata Mukul amplified at 210bp. Anguli Aman, Kartik Jhul, Lali Khoma produced band at 240bp. At 290bp Kartik Jhul amplified. The amplification of primer RM13 is presented in Figure 4. Shiran *et al*. (2007) selected 18 (out of 26) SSR primers for testing almond reproducibility and they revealed highly polymorphic result. SSR markers revealed the number of identified alleles ranged from 3 to 10 alleles per locus with a mean value of 6.64 alleles per locus.

SSR primer RM206 detected seven fragments of DNA amplification. Amplifications are polymorphic and sizes ranged from 100bp to 220bp bases. Seven types of band indicate highly polymorphic band. Total 55 allele was observed. Dholi Khoma, Laxmi Digha, Anguli Aman, Dudhsor, Dula Bech, Kartik Sail, Kartik Jhul, Dulai Aman, Katari, Mota Kartik Sail, Ijule Khoma, Lali Khoma, Kata Mukul, Hira amplified at 100bp. When Dholi Khoma, Laxmi Digha, Anguli Aman, Dudhsor, Dula Bech, Kartik Sail, Kartik Jhul, Dulai Aman, Mota Kartik Sail, Ijule Khoma, Lali Khoma, Kata Mukul, Hira amplified at 120bp. Anguli Aman and Dulai Aman obtained band at 137 bp. Dudhsor, Dula Bech, Kartik Sail and Kartik Jhul rice genotypes yielded a DNA fragment of 145 bp.

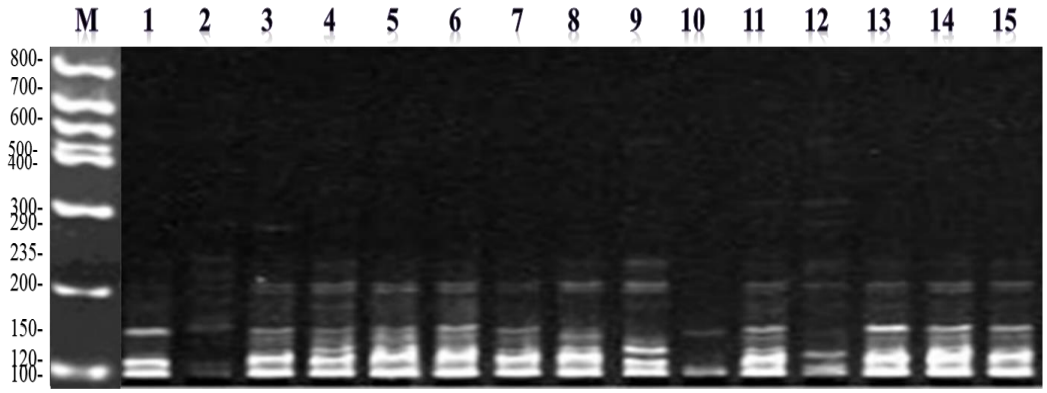

Figure 5. DNA profile of 15 deep water rice using SSR primer RM 206

M = Molecular Marker (1 kb plus DNA Ladder , Bio Basic, Canada); Lane : 1 = Dholi Khoma; 2 = Manik Gira; 3 = Laxmi Digha; 4 = Anguli Aman; 5 = Dudhsor; 6 = Dula Bech; 7 = Kartik Sail; 8 = Kartik Jhul; 9 = Dulai Aman; 10 = Katari; 11 = Mota Kartik Sail; 12 = Ijule Khoma; 13 = Lali Khoma; 14 = Kata Mukul; 15 = Hira**.**

The amplification of the genotypes Laxmi Digha, Anguli Aman, Dudhsor, Dula Bech, Kartik Sail, Mota Kartik Sail, Lali Khoma, Kata Mukul and Hira occurred at 150 bp. Laxmi Digha, Anguli Aman, Dudhsor, Dula Bech, Kartik Sail, Kartik Jhul, Dulai Aman, Mota Kartik Sail, Ijule Khoma, Lali Khoma, Kata Mukul, Hira generated fragment at 210bp. At 220bp Dulai Aman amplified (Figure 5**).** In chinese sugarcane (*Sacchuram* spp.) 117 SSR alleles were detected by PAGE. Primer pairs SMC336BS, SMC31CUQ, and SMC597CS amplified more than eight alleles detectable by PAGE. (Ali *et al*., 2017)

4.2 Polymorphism information content (PIC) in different deep water Aman rice genotypes

Number of alleles, range of DNA fragment, number of polymorphic alleles, allele frequency, gene diversity and polymorphism information content (PIC) found among experimental germplasms for 5 SSR markers are presented in Table 9. Five polymorphic SSR loci yielded a total of 59 alleles, with an average of 11.8 alleles per locus and a range of 7 to 16 alleles (Table 6). The number of alleles per locus ranged from 4 to 21, and there were a total of 337 alleles found with an average of 11 alleles per locus (Masuduzzaman *et al*., 2016). Singh *et al*. (2016) selected 36 HvSSR markers that amplified 112 alleles with an average of 3.11 per locus for 729 rice genotypes. It was discovered that the experimental genotypes had a total of 47 polymorphic alleles. All of the RMs (RM1, RM85, RM252, RM13, RM206, and RM413) found distinct alleles. The DNA fragments range in size from 76 base pairs (bp) to 800 base pairs (bp). While primer RM85 amplified a DNA fragment of 800bp, primer RM1 only amplified a 76bp fragment. The major allele's frequency ranged from 0.556 (RM252) to 0.945 (RM206). The average Polymorphic Information Content (PIC) score for the five SSR markers was 0.317, with values ranging from 0.103 (RM206) to 0.494 (RM252). Genotyping of 114 rice genotypes using 65 SSR markers detected 147 alleles with the average polymorphic information content (PIC) value of 0.51 (Verma et al., 2019). In various sets of rice varieties, Shah *et al*. (2013) and Pachauri *et al*. (2013) reported mean PIC values of 0.37 and 0.38, respectively, which were closer to our finding. The PIC value for RM252 (0.494) was the highest, followed by RM1 (0.418), RM85 (0.337), RM13 (0.234), and RM206 (0.103) in that order. Salgotra *et al*. (2015) have reported mean PIC value 0.40 in a basmati collection of the north-western Himalaya, which was somewhat higher than our result. The primer RM206 displayed the lowest PIC value of 0.103. According to the PIC value, it is evident that RM252 is the top marker for 15 rice genotypes, followed by RM1, RM85, RM13, and RM206. Once more, RM206's lowest PIC value makes it the least effective marker. The findings show a high degree of genetic similarity among the 15 genotypes of Aman rice. They once more show a significant level of heterozygosity. They also demonstrate significant genetic differentiation, polymorphism, and intra-varietal group variety exist to a degree that is clearly discernible.

Table 9. Range of DNA fragment, number of alleles, major allele, major allele frequency and Polymorphism information content (PIC) found among 15 deep water Aman rice genotypes for 5 microsatellite markers

Sl. No.	Primer name	Position	Chromosome no.	Amplicon size range	Allele no.	Major allele	Major allele frequency	PIC value
1	RM1	4.63	1	76-300	16	33	0.702	0.418
2	RM85	66.76	3	91-800	14	33	0.786	0.337
3	RM252	45.21	4	195-295	11	20	0.556	0.494
4	RM13	8.27	5	90-395	11	51	0.864	0.234
5	RM206	21.97	11	100-220	7	52	0.945	0.103
Total					59	154	3.8533	1.5864
Mean					11.8	30.8	0.771	0.317

4.3 Diversity revealed by different markers

The polymorphism information content (PIC) value is a reflection of allelic diversity and frequency among the varieties. The used five markers have different level of diversity on the basis of PIC value. Among them, RM13 and RM206 showed low genetic diversity. Again, RM1, RM85, RM13, RM252 showed moderate diversity. Kartik Sail, Manik Gira and Katari produced unique alleles. The results revealed that the varieties produced unique alleles that could be used for molecular characterization and DNA fingerprinting of these Aman rice genotypes. Because, these unique alleles may have some special characteristics which may be helpful in breeding as well as other research program concerning rice development. The result revealed to broaden the genetic base for wider adaptability.

Table 10. Diversity level showed by different markers based on PIC value

Sl. No.	PIC value	Diversity level	Marker
1.	≤ 0.25	Low	RM13, RM206
2.	0.25-0.50	Moderate	RM1, RM85, RM252

4.4 Nei’s genetic distance among 15 deep water rice genotypes

On the basis of genetic similarity, the genetic distance between 15 rice genotypes was calculated using Nei's (1983) method of pair-wise comparison. The similarities ranged from 0.3729 to 0.9375, with an average similarity of 0.7048 (Table 11). Pair wise Nei’s genetic distance ranged from 0.07 to 0.95 between basmati rice genotypes (Ashfaq, M. and Khan, A. S., 2012). Lowest genetic distance value was indicated by the highest genetic similarity score (0.9375). Dula Bech and Kartik Sail showed the least genetic distance and had the highest genetic similarity (0.9375), indicating that they were most closely connected to one another. The biggest genetic distance value was described by a lower genetic similarity value (0.3729). The lowest Nei’s genetic similarity (0.3729) was observed in Laxmi Digha vs. Kartik Jhul. Minimum genetic similarity value revealed that they were distantly related with highest genetic distance. The highest genetic distance between them indicated that genetically they are dissimilar and also highly diverse. The difference between the highest and lowest genetic identity indicates the presence of variability among 15 genotypes of Aman rice. Those (genotypes) which have higher genetic dissimilarity is more dissimilar than those which have lower value. The average genetic similarity among the 15 genotypes was observed as 0.7048 (Table 11). Using both ILP and SSR markers, Nei's genetic distance, a measure of genetic diversity was calculated though the result obtained by SSR markers were significantly higher (Huang *et al*., 2010). Wang *et al*. (2014) calculated Nei's gene diversity and Shannon's information index and observed that, the mean genetic distance was higher between groups (0.006) than the mean genetic distance within groups (0.0048) of *Villosiclava virens* isolates. The geographical factor played a more important role in the selection of *V. virens* isolates than rice cultivars as greater genetic similarity among isolates was originated from individual fields but different rice cultivars.

Table 11: The degree of genetic similarity pair (below diagonal) values among discussed fifteen deep water rice genotypes

Genotypes	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15
G1	1.0000														
G2	0.8475	1.0000													
G3	0.6441	0.5932	1.0000												
G4	0.6441	0.6271	0.7288	1.0000											
G5	0.6102	0.5932	0.4915	0.5593	1.0000										
G6	0.6667	0.6458	0.6042	0.6875	0.6458	1.0000									
G7	0.6744	0.6744	0.6512	0.6512	0.7442	0.9375	1.0000								
G8	0.6610	0.5763	0.3729	0.4746	0.7119	0.5417	0.5581	1.0000							
G9	0.7458	0.7288	0.6271	0.7288	0.5593	0.6875	0.7209	0.5763	1.0000						
G10	0.6271	0.6441	0.6441	0.6441	0.5763	0.6250	0.6512	0.5593	0.8136	1.0000					
G11	0.6441	0.5932	0.6949	0.7288	0.6271	0.6042	0.6744	0.6102	0.6949	0.5763	1.0000				
G12	0.6949	0.6102	0.6441	0.6102	0.6441	0.7292	0.7674	0.5932	0.7119	0.6610	0.6102	1.0000			
G13	0.6441	0.5932	0.6949	0.7627	0.6610	0.6458	0.6512	0.6102	0.5932	0.4746	0.7966	0.7458	1.0000		
G14	0.7708	0.7083	0.7083	0.7500	0.6667	0.7292	0.7813	0.5625	0.6667	0.5625	0.6667	0.8333	0.8333	1.0000	
G15	0.7619	0.7143	0.7619	0.7619	0.6190	0.8095	0.8571	0.5714	0.7143	0.6667	0.7143	0.8571	0.7143	1.0000	1.0000

Legend: GI (Dholi Khoma), G2 (Manik Gira), G3 (Laxmi Digha), G4 (Anguli Aman), G5 (Dudhsor), G6 (Dula Bech), G7 (Kartik Sail), G8 (Kartik Jhul), G9 (Dulai Aman), G10 (Katari), G11 9 (Mota Kartik Sail), G12 (Ijule Khoma), G13 (Lali Khoma), G14 (Kata Mukul), G15 (Hira).

4.5 Fifteen deep water Aman rice genotypes cluster analysis

A dendrogram was created using the Nei's genetic distance estimate of 15 Aman rice germplasm. According to the Unweighted Pair Group Method of Arithmetic Mean (UPGMA), the 15 Aman rice genotypes may be divided into four major clusters: Cluster I was made up of the deep water aman rice genotypes G1 (Dholi Aman), G2 (Manik Gira), G6 (Dula Bech), G7 (Kartik Sail), G12 (Ijule Khoma), G14 (Kata Mukul), and G15 (Hira). Cluster II had the genotypes G9 (Dulai Aman) and G10 (Katari). The Aman rice genotypes named G3 (Laxmi Digha), G4 (Anguli Aman), G11 (Mota Kartik Sail), and G13 (Lali khoma) were classified as cluster III, while genotypes G5 (Dudhsor) and G8 (Kartik Jhul) were classified as cluster IV (Figure

6). Deep water Aman rice genotypes that share a higher degree of genetic similarity are grouped together in this dendrogram. The dendrogram showed that genotypes that were derivatives of the same genetic type grouped together. The genotypes are classified into one cluster because of their lower genetic distance, whereas those with more genetic dissimilarity are grouped into a different cluster because of their greater genetic distance. Cluster-I, Cluster-II, Cluster-III, and Cluster-IV genotypes are more dissimilar from one another than other genotypes. Mazid *et al*. (2013) grouped 41 rice genotypes and 13 morphological features into six main categories. Cluster I was the largest group, with 27 genotypes, while cluster III, IV, and V had three, three, and six genotypes.

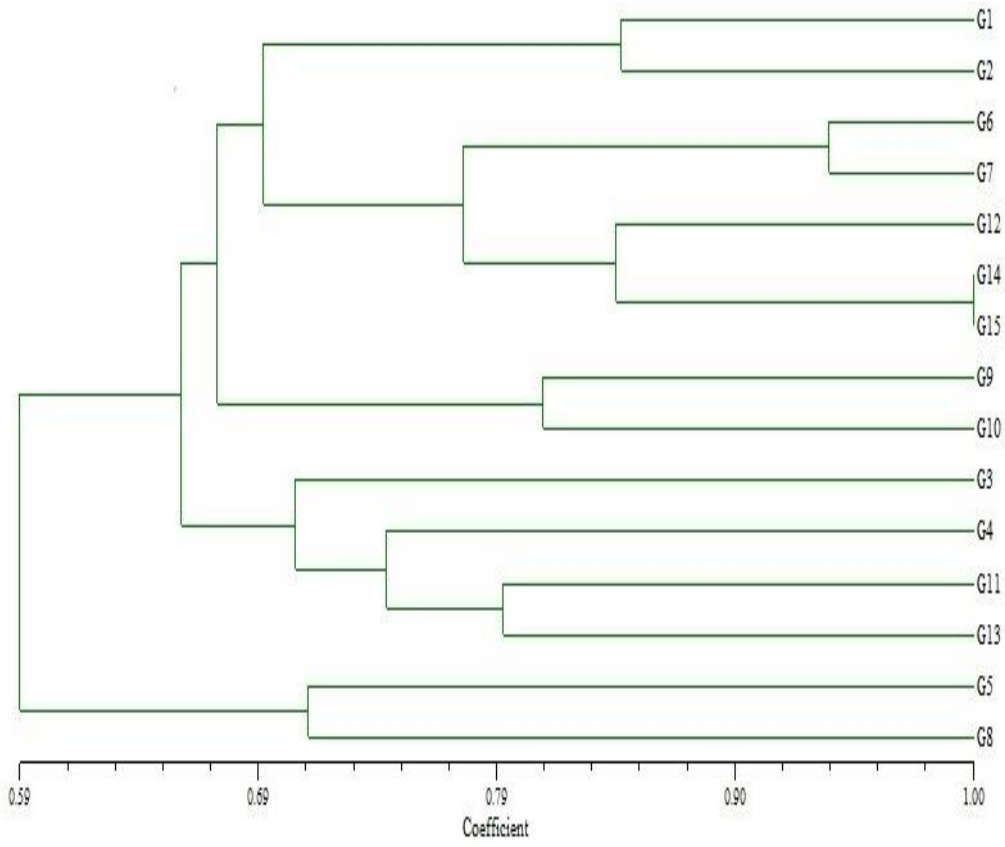

Figure 6. A UPGMA cluster dendrogram illustrating the genetic links of 15 deep water Aman rice germplasms of Bangladesh

Table 12. List of 15 deep water rice genotypes with their cluster based on UPGMA dendrogram

Cluster	Genotype
I	G1 (Dholi Khoma), G2 (Manik Gira), G6 (Dula Bech), G7 (Kartik Sail), G12 (Ijule Khoma), G14 (Kata Mukul), and G15 (Hira)
II	G9 (Dulai Aman) and G10 (Katari)
III	G3 (Laxmi Digha), G4 (Anguli Aman), G11 (Mota Kartik Sail), and G13 (Lali khoma)
IV	G5 (Dudhsor) and G8 (Kartik Jhul)

4.6 PCR genotyping for identifying bacterial leaf blight resistant gene

In order to analyze 41 rice germplasms named BRRI dhan58, BR18, Dholi Khoma, BR16, BRRI dhan36, BRRI dhan97, BR8, IRBB5, Manik Gira, BRRI dhan29, Laxmi Digha, Anguli Aman, Dudhsor, Dula Bech, Kartik Sail, Kartik Jhul, Dulai Aman, BRRI dhan55, Atash, Katari, Mota Kartik Sail, Ijule Khoma, Lali Khoma, Kata Mukul, Hira, IRBB24, IRBB60, IRBB64, IRBB65, IRBB2277, BR7, BR19, BRRI dhan28, BRRI dhan35, BRRI dhan45, BRRI dhan47, BRRI dhan50, BRRI dhan81, BRRI dhan84, BRRI dhan99, BRRI dhan100, the five gene specific markers created for the BLB resistance genes *Xa4, Xa5, Xa7, Xa13, and Xa21* were employed. Using primers designed for the NBS and LRR sections of the *Rpg1b* genes, the Indian variety of soybean was tested for the existence of the *Rpg1b* gene (Singh, 2021).

The Xa4F/4R primer pair was used to amplify a 217-bp segment of the *Xa4* gene in 11 types of rice genotypes, including BR16, BRRI dhan36, BRRI dhan97, BR8, IRBB5, Manik Gira, BRRI dhan29, Laxmi Digha, Anguli Aman, Dudhsor, and Dula Bech. These genotypes showed the presence of genes that are resistant to Bacterial Blight. Breeding programs can utilize these genotypes as donor parents to develop even better varieties of rice with increased resistance to Bacterial Blight. On the other hand, three rice genotypes, namely BRRI dhan58, Dholi Aman, and Kartik Sail, showed a susceptible allele to Bacterial Blight disease, as evidenced by 198bp amplification (Figure 7).

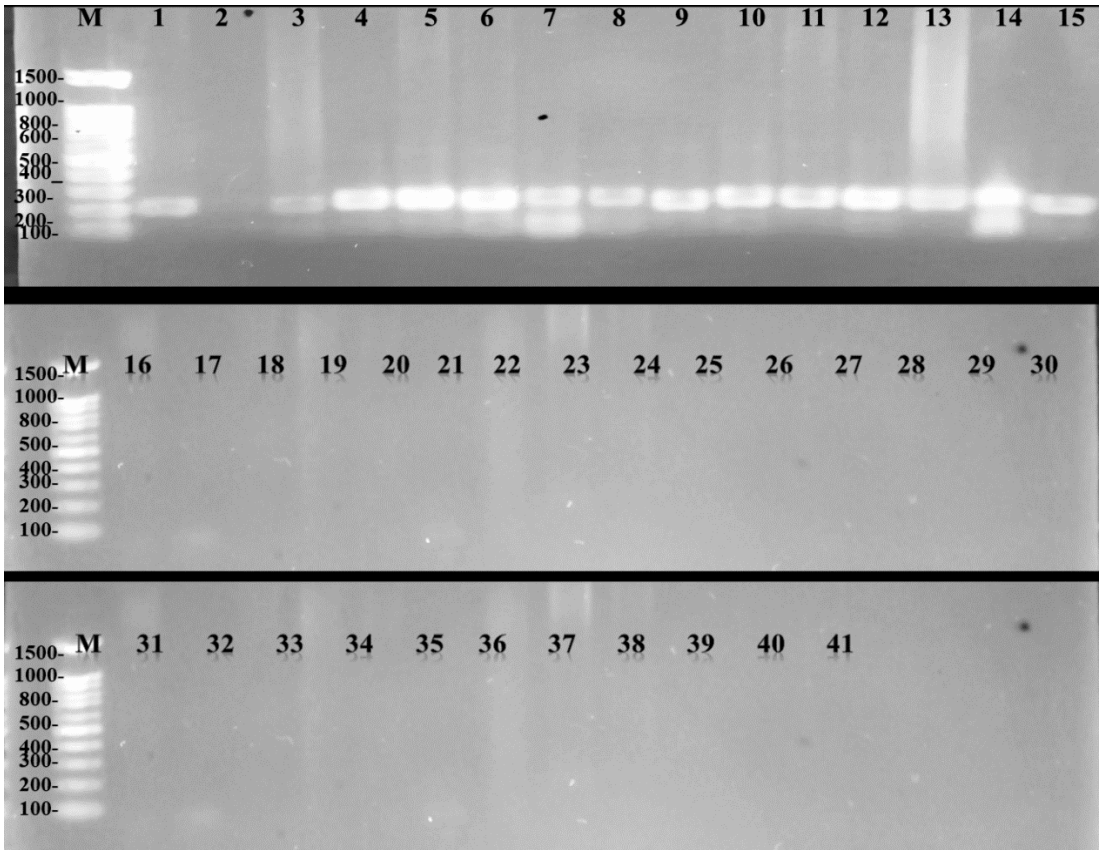

Figure 7. Identification of bacterial leaf blight disease resistance rice genotypes using functional marker Xa4F/4R

M= Molecular Marker (1 kb plus DNA Ladder, Bio-Basic, Canada); Lane: 1= BRRI dhan58; 2= BR18; 3= Dholi Khoma; 4= BR16; 5= BRRI dhan36; 6= BRRI dhan97; 7= BR8; 8= IRBB5; 9= Manik Gira; 10= BRRI dhan29; 11= Laxmi Digha; 12= Anguli Aman; 13= Dudhsor; 14= Dula Bech; 15= Kartik Sail; 16= Kartik Jhul; 17= Dulai Aman; 18= BRRI dhan55; 19= Atash; 20= Katari; 21= Mota Kartik Sail; 22= Ijule Khoma; 23= Lali Khoma; 24= Kata Mukul; 25= Hira; 26= IRBB24; 27= IRBB60; 28= IRBB64; 29= IRBB65; 30= IRBB2277; 31= BR7; 32= BR19; 33= BRRI dhan28; 34= BRRI dhan35; 35= BRRI dhan45; 36= BRRI dhan47; 37= BRRI dhan50; 38= BRRI dhan81; 39= BRRI dhan84; 40= BRRI dhan99; 41= BRRI dhan100.

Four rice genotypes called BRRI dhan58, BR18, BR16, and Anguli Aman express band when amplified with primer pair RM604F/604R for a 103-bp *Xa5* fragment in the hopes that these would contain genes for bacterial leaf blight resistance. Molecular and conventional approaches were used to identify Pakistani rice germplasm for the presence of *xa5*, a bacterial blight resistance gene with the help of Polymerase chain reaction (PCR) with primers specific for *xa5* resistances gene (Abbasi *et al.*, 2011). These can be used as donor parents in breeding to create better Bacterial Blight resistant varieties. 107-bp amplification was found in four rice genotypes- IRBB5, Manik Gira, BRRI dhan29, and Laxmi Digha-shows that each of these has an allele that makes it susceptible to the bacterial leaf blight disease (Figure 8)

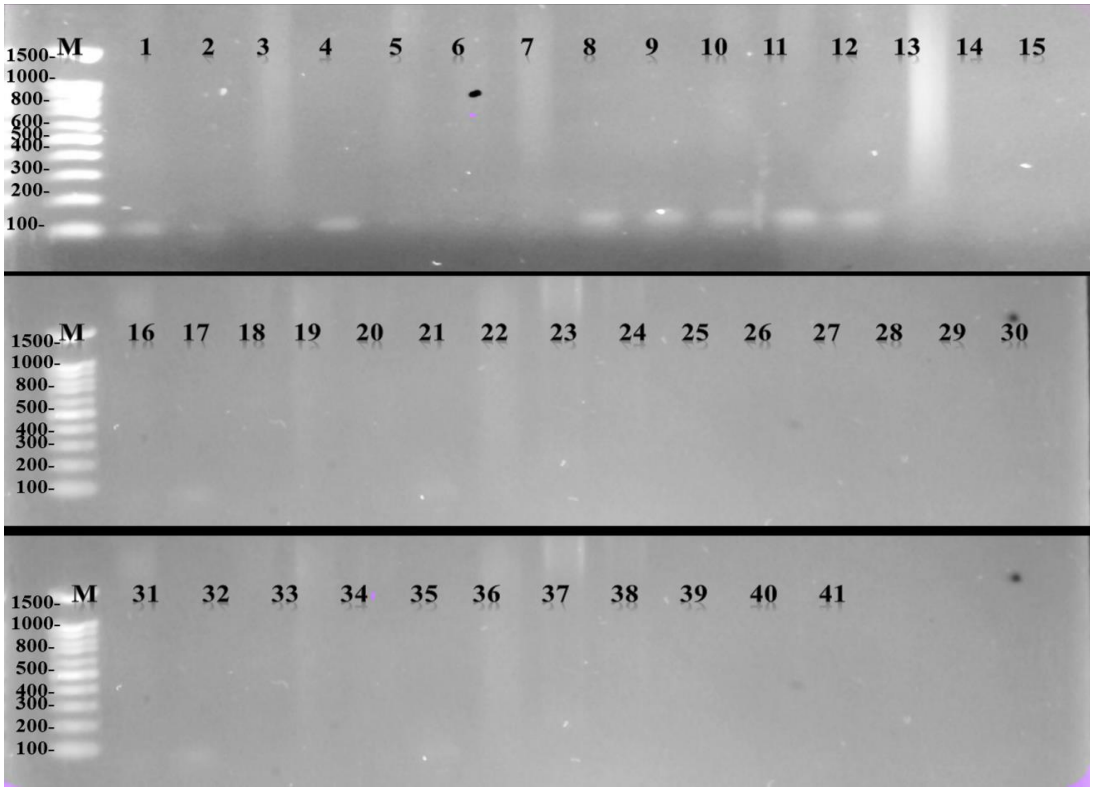

Figure 8. Identification of bacterial leaf blight disease resistance rice genotypes using functional marker RM604F/604R

M= Molecular Marker (1 kb plus DNA Ladder, Bio-Basic, Canada); Lane: 1= BRRI dhan58; 2= BR18; 3 = Dholi Khoma; 4= BR16; 5= BRRI dhan36; 6= BRRI dhan97; 7= BR8; 8= IRBB5; 9= Manik Gira; 10= BRRI dhan29; 11= Laxmi Digha; 12= Anguli Aman; 13= Dudhsor; 14= Dula Bech; 15= Kartik Sail; 16= Kartik Jhul; 17= Dulai Aman; 18= BRRI dhan55; 19= Atash; 20= Katari; 21= Mota Kartik Sail; 22= Ijule Khoma; 23= Lali Khoma; 24= Kata Mukul; 25= Hira; 26= IRBB24; 27= IRBB60; 28= IRBB64; 29= IRBB65; 30= IRBB2277; 31= BR7; 32= BR19; 33= BRRI dhan28; 34= BRRI dhan35; 35= BRRI dhan45; 36= BRRI dhan47; 37= BRRI dhan50; 38= BRRI dhan81; 39= BRRI dhan84; 40= BRRI dhan99; 41= BRRI dhan100

The Xa7F/7-1R/7-2R primer pair was used to amplify a 179-bp region of the *Xa7* gene in four different rice genotypes, Dulai Aman, Katari, Kata Mukul, and Hira, revealing the existence of genes resistant to bacterial leaf blight. Fatimah et al., (2018) shown the sequence alignment of Xa7LD37 in two resistant and three susceptible cultivars. Breeding programs can employ these genotypes as donor parents to develop more effective rice varieties with increased resistance to Bacterial Blight. Conversely, nine other rice genotypes namely BR7, BR19, BRRI dhan35, BRRI dhan45, BRRI dhan47, BRRI dhan81, BRRI dhan84, BRRI dhan99, and BRRI dhan100, displayed a susceptible allele to bacterial leaf blight disease, as verified by an amplification of 87bp (Figure 9)

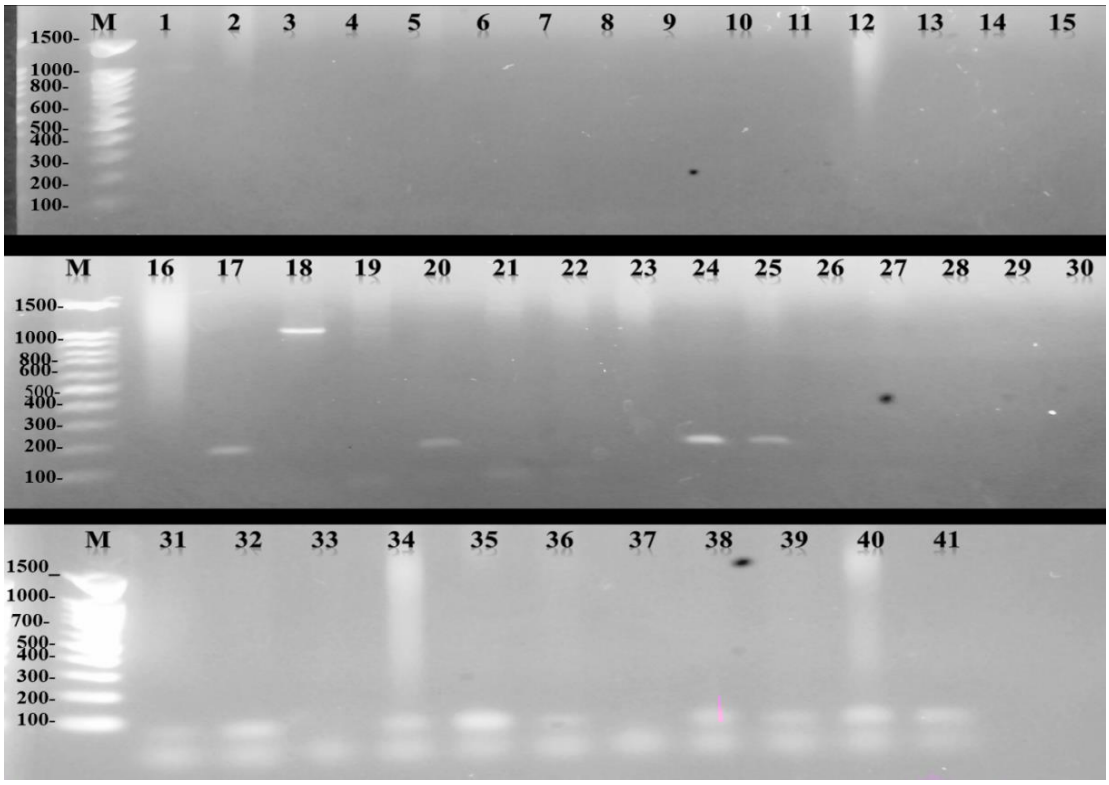

Figure 9. Identification of bacterial leaf blight disease resistance rice genotypes using functional marker Xa7F/7-1R/7-2R

M= Molecular Marker (1 kb plus DNA Ladder, Bio-Basic, Canada); Lane: 1= BRRI dhan58; 2= BR18; 3= Dholi Khoma; 4= BR16; 5= BRRI dhan36; 6= BRRI dhan97; 7= BR8; 8= IRBB5; 9= Manik Gira; 10= BRRI dhan29; 11= Laxmi Digha; 12= Anguli Aman; 13= Dudhsor; 14= Dula Bech; 15= Kartik Sail; 16= Kartik Jhul; 17= Dulai Aman; 18= BRRI dhan55; 19= Atash; 20= Katari; 21= Mota Kartik Sail; 22= Ijule Khoma; 23= Lali Khoma; 24= Kata Mukul; 25= Hira; 26= IRBB24; 27= IRBB60; 28= IRBB64; 29= IRBB65; 30= IRBB2277; 31= BR7; 32= BR19; 33= BRRI dhan28; 34= BRRI dhan35; 35= BRRI dhan45; 36= BRRI dhan47; 37= BRRI dhan50; 38= BRRI dhan81; 39= BRRI dhan84; 40= BRRI dhan99; 41= BRRI dhan100

A 381-bp fragment of *Xa13* was amplified in 12 rice genotypes, including BRRI dhan58, BR18, BRRI dhan29, Dudhsor, Dula Bech, Kartik Sail, Dulai Aman, BRRI dhan55, Kata Mukul, BR7, BR19, and BRRI dhan100, indicating that these may include bacterial leaf blight resistance genes. According to Bharani et al. (2009), BLB resistance gene Xa-3 and Xa-4 were found in 52 accessions, Xa-5 in 25 accessions, Xa-7 in 23 accessions, Xa-8 in 30 accessions of rice, Xa-10 in 5 accessions, and Xa-13 in 6 accessions. To develop further improved bacterial leaf blight resistant variety these can be used as donor parent for breeding purpose. Dholi Khoma, BRRI dhan36, BR8, IRBB5, Manik Gira, Laxmi Digha, Kartik Jhul, Atash, Katari, Mota Kartik Sail, Ijule Khoma, Lali Khoma, Hira, IRBB24, IRBB60, IRBB65, IRBB2277, BR7, BRRI dhan35, BRRI dhan45, BRRI dhan99, BRRI dhan100 twenty two genotypes of rice included a 391-bp amplification that express susceptibility to bacterial leaf blight disease (Figure 10).

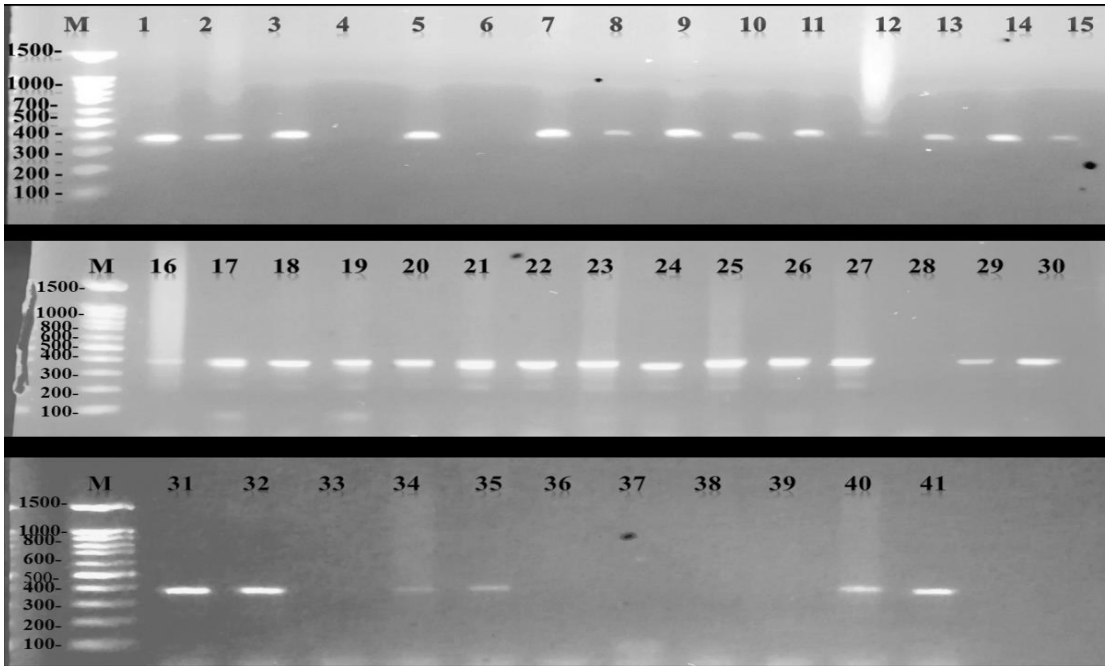

Figure 10. Identification of bacterial leaf blight disease resistance rice genotypes using functional marker Xa13F/13R

M= Molecular Marker (1 kb plus DNA Ladder, Bio-Basic, Canada); Lane: 1= BRRI dhan58; 2= BR18; 3= Dholi Khoma; 4= BR16; 5= BRRI dhan36; 6= BRRI dhan97; 7= BR8; 8= IRBB5; 9= Manik Gira; 10= BRRI dhan29; 11= Laxmi Digha; 12= Anguli Aman; 13= Dudhsor; 14= Dula Bech; 15= Kartik Sail; 16= Kartik Jhul; 17= Dulai Aman; 18= BRRI dhan55; 19= Atash; 20= Katari; 21= Mota Kartik Sail; 22= Ijule Khoma; 23= Lali Khoma; 24= Kata Mukul; 25= Hira; 26= IRBB24; 27= IRBB60; 28= IRBB64; 29= IRBB65; 30= IRBB2277; 31= BR7; 32= BR19; 33= BRRI dhan28; 34= BRRI dhan35; 35= BRRI dhan45; 36= BRRI dhan47; 37= BRRI dhan50; 38= BRRI dhan81; 39= BRRI dhan84; 40= BRRI dhan99; 41= BRRI dhan100.

By using the primer pair Xa21F/21R, a 595-bp segment of the *xa21* gene was amplified in three rice genotypes, specifically Katari, Mota Kartik Sail, and Ijule Khoma. This suggests that these plants may possess genes that are resistant to bacterial leaf blight. Bacterial leaf blight genes *Xa4*, *Xa5*, and *Xa21* were identified using Super Basmati as the recipient and IRBB60 as the donor parent and there were developed numerous lines with the BLB resistance gene combinations *Xa4*, *Xa5* and *Xa4*, *Xa5*, *Xa21* (Sabar et al., 2019). These can be utilized as donor parents for breeding in order to create new, improved Bacterial Blight resistant varieties. 29 varieties of rice, specifically BRRI dhan58, BR18, Dholi Khoma, BRRI dhan36, BR8, BRRI dhan29, BRRI dhan55, Atash, Kata Mukul, Hira, IRBB24, IRBB60, IRBB65, IRBB2277, BR7, BR19, BRRI dhan35, BRRI dhan45, BRRI dhan47, BRRI dhan81, BRRI dhan84, and BRRI dhan99, were tested for susceptibility to bacterial leaf blight disease. The result showed that all of them have a vulnerable gene to the disease, as evidenced by the 467-bp amplification (Figure 11).

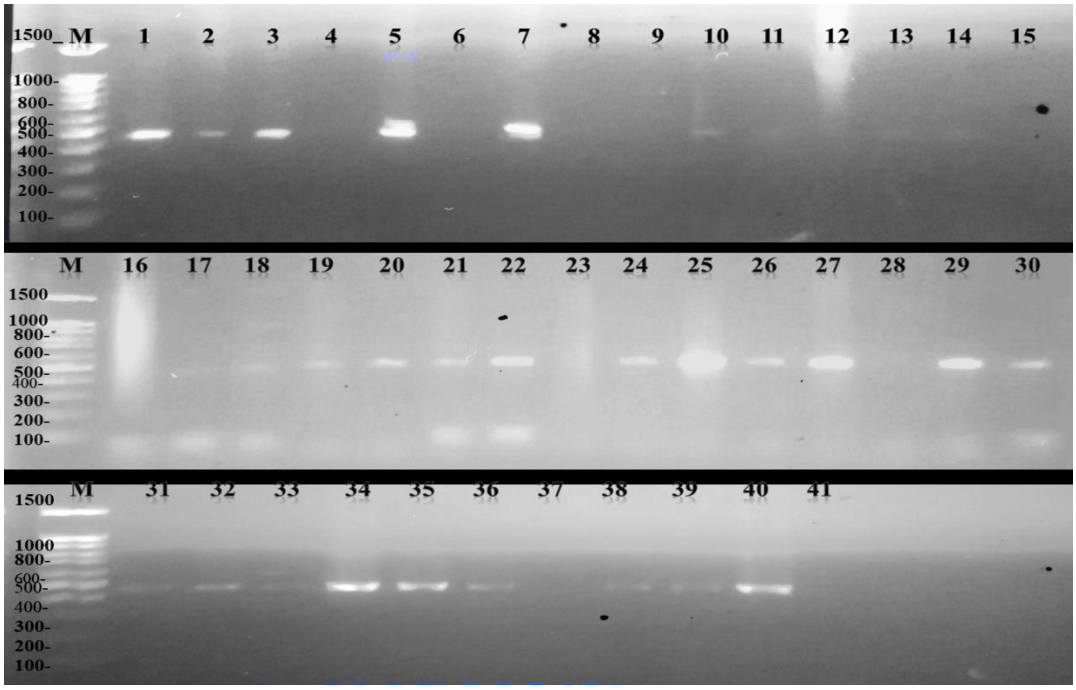

Figure 11. Identification of bacterial leaf blight disease resistance rice genotypes using functional marker Xa21F/21R

M= Molecular Marker (1 kb plus DNA Ladder, Bio-Basic, Canada); Lane: 1= BRRI dhan58; 2= BR18; 3= Dholi Khoma; 4= BR16; 5= BRRI dhan36; 6= BRRI dhan97; 7= BR8; 8= IRBB5; 9= Manik Gira; 10= BRRI dhan29; 11= Laxmi Digha; 12= Anguli Aman; 13= Dudhsor; 14= Dula Bech; 15= Kartik Sail; 16= Kartik Jhul; 17= Dulai Aman; 18= BRRI dhan55; 19= Atash; 20= Katari; 21= Mota Kartik Sail; 22= Ijule Khoma; 23= Lali Khoma; 24= Kata Mukul; 25= Hira; 26= IRBB24; 27= IRBB60; 28= IRBB64; 29= IRBB65; 30= IRBB2277; 31= BR7; 32= BR19; 33= BRRI dhan28; 34= BRRI dhan35; 35= BRRI dhan45; 36= BRRI dhan47; 37= BRRI dhan50; 38= BRRI dhan81; 39= BRRI dhan84; 40= BRRI dhan99; 41= BRRI dhan100.

Ten germplasm samples had a maximum of two different gene combinations, with Dudhsor and Dula Bech carrying the *Xa4*, *Xa13* gene and Dulai Aman and Kata Mukul carrying the *Xa7, Xa13* gene. Once more, the genes for *Xa4*, *Xa5* and *Xa7*, *Xa21* were found in the genotypes of Anguli Aman and Katari, respectively. The genes *Xa4*, *Xa5*, and *Xa5, Xa13* were discovered in BR16 and BR18, respectively. The *Xa4*, *Xa13*, and *Xa5, Xa13* genes are present in the BRRI dhan29 and dhan58, respectively. IRBB5, Manik Gira, Laxmi Digha, BRRI dhan36, and BRRI dhan97 all have the *Xa4* gene and BR7, BR19 having the *Xa13* gene. The *Xa7* and *Xa21* genes are present in Hira and Mota Kartik Sail, respectively. Kartik Sail, BRRI dhan55, and BRRI dhan100 all carry the *Xa13* gene.

Table 13. List of rice genotypes that carries different bacterial leaf blight resistant gene

Resistant gene	**Rice genotypes**
Xa4	10 genotypes (Dudhsor, Dula Bech, Anguli Aman, BR16, BRRI dhan29, IRBB5, Manik Gira, Laxmi Digha, BRRI dhan36, and BRRI dhan97)
xa5	4 genotypes (Anguli Aman, BR16, BR18, BRRI dhan58)
Xa7	4 genotypes (Dulai Aman, Kata Mukul, Katari, Hira)
xa13	12 genotypes (Dudhsor, Dula Bech, Dulai Aman, Kata Mukul, BR18, BRRI dhan29, BBRI dhan58, BR7, BR19, Kartik Sail, BRRI dhan55, and BRRI dhan100)
Xa21	2 genotypes (Katari, Mota Kartik Sail)

4.7 Phylogenetic Tree of 41 Rice Genotypes

Among 41 rice germplasm, a phylogenetic tree (Figure 12) was calculated using Molecular Evolutionary Genetics Analysis (MEGA6.0 version). Phylogenetic tree indicated the segregation of 41 rice germplasms into three main clusters: Dholi Khoma, Manik Gira, Dula Bech, Kartik Sail, Lali Khoma, Kartik Jhul, Anguli Aman, Dulai Aman, Katari, Laxmi Digha, Hira, Mota Kartik Sail, Dudhsor, Ijule Khoma, Kata Mukul, BRRI dhan58, BRRI dhan55, IRBB5, IRBB65, IRBB24, IRBB65, IRBB60, BR7, IRBB2277 were grouped in cluster I and BRRI dhan28, BR19, BR18, BRRI dhan35, Atash, BRRI dhan 100, BRRI dhan47, BRRI dhan81 BR16. BRRI dhan50, BRRI dhan99 were grouped in cluster II. Here, "G" indicated the Aman rice germplasms.

In cluster I, Dholi Khoma, Manik Gira, Dula Bech, Lali Khoma, Kartik Jhul, Anguli Aman, Dulai Aman, Katari, Laxmi Digha, Hira, Mota Kartik Sail, Dudhsor, Ijule Khoma, Kata Mukul grouped together in sub cluster 1: here, BRRI dhan58, BRRI dhan55, IRBB5, IRBB65, IRBB24, IRBB65, IRBB60, BR7, IRBB2277 formed sub cluster 2 of cluster I. Again, BRRI dhan28, BR19 formed sub cluster 1 and BR18, BRRI dhan35, Atash, BRRI dhan 100, BRRI dhan47, BRRI dhan81, BR16, BRRI dhan50, BRRI dhan99 formed sub cluster 2 of cluster II.

In this phylogenetic tree, rice genotypes of more genetic similarity are placed in same sub cluster. The genotypes grouped in the same cluster due to lower genetic distance and the other genotypes having more genetic dissimilarity grouped in another cluster due to higher genetic distance. It is clear that IRBB5, IRBB65, IRBB24, IRBB65, IRBB60 are more different from most of the genotypes.

In the context of 41 rice germplasm, phylogenetic trees can illustrate how different rice varieties are related to one another in terms of their genetic ancestry. As significant sources of genetic variety, ancestral or wild rice lineages can be located using phylogenetic trees. Breeding efforts to incorporate beneficial features from wild rice into cultivated varieties can be guided by knowledge of the genetic links between relatives of cultivated rice and wild rice. In summary, phylogenetic trees are a effective tool for visualizing and analyzing the genetic diversity of rice germplasm. They provide insights into the evolutionary relationships among rice varieties, help

identify ancestral lines, aid in the selection of breeding parents, and support the conservation and utilization of rice genetic resources for improved crop varieties.

Table 14. List of 41 rice germplasm with their cluster based on MEGA 6.0 version of phylogenetic tree

Cluster	Sub cluster	Genotype
I	1	Dholi Khoma, Manik Gira, Dula Bech, Kartik Sail. Lali Khoma, Kartik Jhul, , Anguli Aman, Dulai Aman, Katari, Laxmi Digha, Hira, Mota Kartik Sail,, Dudhsor, Ijule Khoma , Kata Mukul
	2	BRRI dhan58, BRRI dhan55, IRBB5, IRBB65, IRBB24, IRBB65, IRBB60, BR7, IRBB2277
II	1	BRRI dhan28, BR19
	2	BR18, BRRI dhan35, Atash, BRRI dhan 100, BRRI dhan47, BRRI dhan81, BR16, BRRI dhan50, BRRI dhan99

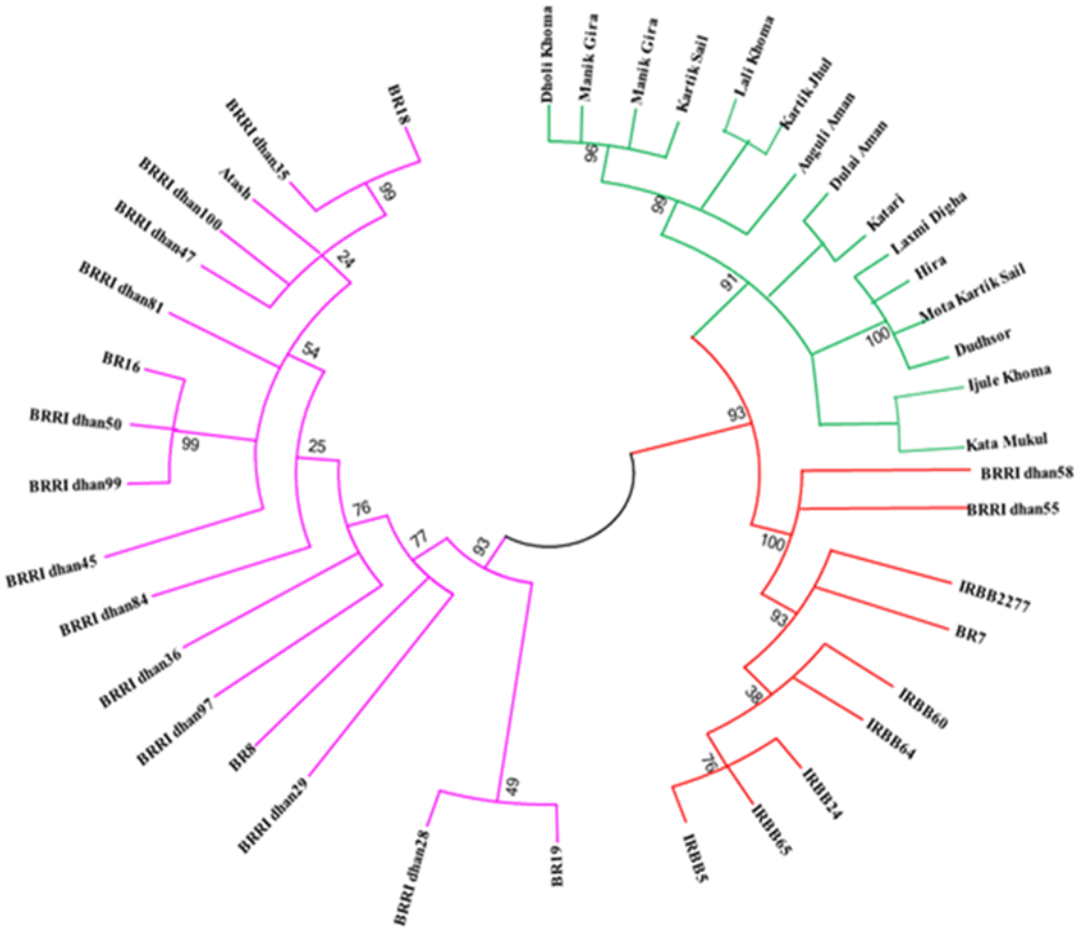

Figure 12. Phylogenetic tree of 41 rice germplasm

CHAPTER V

SUMMARY AND CONCLUSION

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In Bangladesh, rice is dominant crop to agriculture and the national economy. It has been persistently contributing to higher rice production in last successive years. The experiment was conducted to assess the genetic variation , genetic relatedness among 15 deep water Aman rice genotypes and identifying bacterial leaf blight resistant genes within 41 rice genotypes by using five highly polymorphic SSR markers and functional gene specific marker respectively.

Five SSR primers RM1, RM85, RM252, RM13, RM206 showed distinct polymorphic amplification. There were 59 alleles created by the primers, ranging from 7 to 16 allelic fragments per locus, and 11.8 on average were observed. Additionally, 47 polymorphic alleles were found. The main allele's frequency ranged from 0.556 (RM252) to 0.945 (RM206). The average Polymorphic Information Content (PIC) score for the five SSR markers was 0.317, with values ranging from 0.103 (RM206) to 0.494 (RM252). Primer RM252 demonstrated the greatest gene diversity, and primer RM206 the least. According to PIC value, RM252 was found to be the most effective marker for 15 rice genotypes, followed by other markers, while RM206 was found to be the least effective marker for those genotypes.

Using Xa4F/4R, RM604F/604R, Xa7F/7-1R/7-2R, Xa13F/13R, Xa21F/21R gene specific markers five bacterial leaf blight resistance genes *Xa4*, *xa5*, *Xa7*, *xa13* and *Xa21* were found in ten, four, four, twelve and two genotypes respectively. Among 41 rice genotypes, ten genotypes contained two different gene combination and twelve genotypes contained single gene that is dissimilar to one another.

As SSR markers exhibit a high level of polymorphism, they are useful tools for determining the genetic diversity of rice populations. In rice breeding programs, they can be used for marker-assisted selection (MAS) to increase yield, disease resistance, and other desirable qualities. A molecular screening of bacterial blight resistant genes in rice using gene-specific markers can provide valuable information about the presence of *Xa4*,*xa5*, *Xa7*, *xa13*, *Xa21* bacterial leaf blight resistant genes in rice genotypes. All these information can play a major role for further breeding programme developing variety with high qualitative and quantitative characters.

RECOMMENDATIONS

The findings of this study offered some helpful ramifications for establishing the sovereignty of the Bangladeshi rice gene pool. According to this study, genetic diversity of rice germplasm through SSR markers were useful in identifying polymorphism and gene-specific markers were helpful to identify the presence or absence of specific genes associated with resistance to bacterial leaf blight. Overall, molecular screening techniques can be a powerful tool for identifying desirable traits in rice germplasm, including resistance to deep water conditions and bacterial leaf blight disease. The current study is generally regarded as appropriate in all rice germplasm-related concerns. It can serve as a model for future scholars interested in rice experimentation. For further research, following points could be considered for developing rice genotypes with desirable characteristics:

- To produce more diversified results, a lot of germplasm could be used for study.

- High throughput molecular markers, such as Single Nucleotide Polymorphism (SNP), could be employed to cover the entire genome of rice germplasm in order to obtain more precise results.

- The cultivar which carries resistant genes could be grown in field for their phenotypic characteristics analysis. After confirmation, the selected cultivar could be used as donor parent for breeding purpose developing bacterial leaf blight resistant variety.

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APPENDICES

Appendix I: Preparation of chemicals needed for DNA extraction and PCR work

I (a): Composition and preparation of the DNA extraction buffer

Reagent	Amount (500ml)
Tris HCL (1M)	50 ml
EDTA(0.5M, pH= 8.0)	20 ml
NaCl (5M, pH= 8.0)	50 ml
SDS	30 ml
DD H_2O	350 ml

I (b): Composition and preparation of the phenol: chloroform: isoamyl alcohol (25:24:1)

Reagent	Amount (200 ml)
Phenol	100 ml
Chloroform	96 ml
Isoamyl alcohol	4 ml

I (c): Composition and preparation of the 10X TBE buffer

Reagent	Amount (1 L)
Tris HCl	108 ml
Boric Acid	55 ml
EDTA	9.3 ml
dd H_2O	Up to 1 L

I (d): Composition and preparation of the 1X TBE buffer

Reagent	Amount (1 L)
10 X TBE	100 ml
De-ionized water	900 ml

I (e): Composition and preparation of the TE buffer

Reagent	Amount (100 ml)
Tris HCL (1M, pH= 8.0)	1 ml
EDTA 0.5 M	200 µl
dd H_2O	98.8 ml

I (f): Primer dilution

Primer was centrifuged at 13000 rpm for 5 min.

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TE buffer was added (10 times more) according to the concentration labelled on the bottle

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Kept overnight at 4°C

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Next day, it was centrifuged at 5000 rpm for 15 seconds

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Stored at -20°C freeze

I (g): PCR cocktail for 56 samples

Reagent	**Amount (µl)**
Master mix	112
Primer(F)	28
Primer (R)	28
DD H_2O	224
Sample DNA	168

1 (h): Composition and preparation of Gel

Reagent	**Amount**
1X TBE Buffer	70 ml
Agarose	0.7 g
Ethydium Bromide	0.5 µl