BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF SOME ADVANCED MUTANT RICE (*Oryza sativa*) LINES OF BINA

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This is to certify that the thesis entitled, "**BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF SOME ADVANCED MUTANT RICE *(Oryza sativa)* LINES OF BINA**" Submitted to the Department of Biochemistry, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE IN BIOCHEMISTRY** embodies the result of a piece of bona fide research work carried out by **MD. KAWSAR ALAM NADIM, Registration No. 14-05993** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma in any other institutes.

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

Dated: December, 2020
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Dedicated to
My Beloved
Parents

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ABSTRACT

Rice (*Oryza sativa*) is one of the oldest cereal grains in the world and the main staple food of Bangladesh. In rice, amylose and protein are the main biochemical factors in selection and breeding of good eating quality varieties. A total of 12 rice genotypes [10 advanced mutant lines, 1 released mega variety (BRRI dhan49) and 1 landrace (Luxmidigha)] was incorporated in this study to assess their amylose and protein content as well as their genetic diversity relationship. Among the genotypes, Magic-86 was highest in amylose content (25.6%). The second and third highest amylose content were RM-40(C)-4-2-8 (25.1%) and Magic-10 (24.4%), respectively. Amylose of other genotypes ranged from 23.9 to 20.3%. SSB-3 was found to be the highest in protein content (8.86%) whereas Magic-86 (8.78%) and Luxmidigha (8.73%) were the second and third highest, respectively. Protein content of other genotypes were ranged from 8.50 to 5.04%. The highest gene diversity (0.81) was observed in RM519 and the lowest (0.28) was in RM111. The PIC values ranged from 0.24 to 0.78. The highest Nei's genetic distance value 0.87 was found in RM-16(N)-10 vs RM-40(C)-4-2-8 and the lowest value 0.18 was found in LD-200-1-3-3-8 vs LD-200-1-3-2-4. A dendogram was constructed based on Nei's similarity coefficient using UPGMA method to group the selected genotypes and observe relationship among them. Six major clusters were built at 0.7 cut off similarity coefficient. The highest number of genotypes was observed in cluster-6 (4 genotypes) and the lowest in cluster-1, cluster-2 and cluster-4 (each contained 1 genotype). Cluster-3 and cluster-5 contained 3 and 2 genotypes, respectively. The genotypes showing diverse ranges of amylose and protein content tended to cluster together in the dendogram with some exceptions. These findings may play potential role for the selection of parents or lines in future breeding program to develop better quality rice varieties for Bangladesh.

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ABBREVIATIONS

AC	: Amylose Content
APS	: Ammonium Per Sulfate
BAU	: Bangladesh Agricultural University
BINA	: Bangladesh Institute of Nuclear Agriculture
BRRI	: Bangladesh Rice Research Institute
BBS	: Bangladesh Bureau of Statistics
bp	: Base pair
Contd.	: Continued
CF	: Conversion Factor
DNA	: Deoxyribo Nucleic Acid
^0C	: Degree Celsius
et al.	: and others
EDTA	: Ethelene Diamine Tetra Acetic Acid
FAO	: Food and Agriculture Organization
g	: Gram
g/L	: Gram per liter
%	: Percentage
HCl	: Hydrochloric Acid
$HClO_4$	: Perchloric Acid
H_2SO_4	: Sulphuric Acid
mg/L	: Milligram Per Litre
ml	: Millilitre
1N	: 1 Normal
nm	: Nanometer
NaCl	: Sodium Chloride
NaOH	: Sodium Hydroxide
N:P	: Nitrogen:Protein

µl	: Microliter
Ng	: Nano gram
rpm	: Revolutions Per Minute
PAGE	: Poly Acrylamide Gel Electrophoresis
PC	: Protein Content
PCR	: Polymerase Chain Reaction
PIC	: Polymorphic Information Content
psi	: Pounds per Square Inch
QTL	: Quantitative Trait Locus
SAU	: Sher-e-Bangla Agricultural University
SDS	: Safety Data Sheet
SSR	: Simple Sequence repeats
TEMED	: N,N,N,N-tetramethyl diamine
UPGMA	: Unweighted Pair Group Method of Arithmetic Means

CHAPTER 1

INTRODUCTION

Rice, popularly known as 'global grain' is a major cereal crop grown as a staple food exclusively for over half of the world's population. It is a semi-aquatic grass plant belongs to the genus *Oryza* in the Gramineae (Poaceae) family that includes all cereal crops. The genus *Oryza* includes approximately 24 species among which only 2 species *Oryza sativa* and *Oryza glaberrima* are cultivated for human consumption (Jena, 2010). *Oryza sativa* evolved from *O. rufipogon* around 9000 years ago at a site close to the Yangtze valley of China and then split into *Indica* and *Japonica* groups around 4000 years ago (New York University, 2011). The other cultivated species of rice, *O. glaberrima*, evolved in the Niger river basin from *O. barthii* 2000-3000 years ago and remained confined to West Africa (Linares, 2002). South Asia has been described as the "food basket" and "food bowl" of Asia because of being one of the major centers for rice domestication. Overall rice production in South Asia has reached 16.9 crore tons on milled basis (USDA,2021).

In Bangladesh, rice (usually cultivated *Oryza sativa*) is commonly known as "dhan". With a production of 3.6 crore tons, Bangladesh stands in third position after China and India that produce 14.6 crore tons and 11.8 crore tons, respectively (USDA, 2021). Being the major crop, rice engages more than 70% of the rural population and the national economic system of Bangladesh (Anonymous, 2002). In this country less than 75% of total cultivable land is used for rice production but the population is growing approximately 1.20% per year (BBS, 2018). If this rate of increasing population continues, the total population will be approximately 238 million by 2050 (Shelly *et al.,* 2016). Hence rice production need to increase in order to meet the demand of food for the growing population. Additionally, quality of the grain is of much importance with respect to the rice scientists, producers and consumers.

The quality of rice is significantly affected by the two attributes amylose contents and protein contents in the rice (Champagne et al., 1998). The amylose content(AC) is strictly associated by the sensual possessions of the freshly cooked rice (Champagne et al., 2004); although, the protein content (PC) dictates to the consistency (texture) of

the cooked rice by hindering absorption of the water and starch puffiness during cooking (Xie et al., 2008).

Rice is consumed primarily as a full grain and the texture of the whole grain is a matter of prime significance. Cultivars of waxy and non-waxy rice are generally categorized conferring to their amylose content, grain sizes, amylograph reliability, gelatinization possessions of the take out starches and of the texture (hardness and sensory dimensions) of the cooked rice (Juliano, 1985). Texture is a significant characteristic of food acceptance by the consumers and also a dire step in the assessment of quality of rice. Texture is proposed as it is the sensory appearance of the food arrangement and the style in which that arrangement responds to the applied force; thus, amylose content in rice variety affects the rice texture (Szczesniak, 1968). The greatest essential aspect inducing the cooking and processing appearances of rice is amylose content which is considered to be one of them. It is normally used as an objective index for the texture of cooked rice (Webb,1991). Low amylose contents are linked with cohesive, tender, and glossy rice on the cooking. On the contrary, high levels of amylose content cause rice to absorb extra water and accordingly expand more throughout the cooking; also, the cooked grains tend to be dry, fluffy and detached (Juliano, 1971). Rice breeders are consistently anxious regarding having new rice lines with suitable amylose content and protein contents. Protein is an essential component required for growth, antibodies production and immunity in human beings. Rice is a vital source of protein, delivering additional 50% of the entire protein consumed in the more or less countries. Even a modest rise in protein contents levels in rice would provide an important nutritional enhancement to the hundreds of millions of people who rely on it. In the selection of each variety and market value, determination of rice quality is very much important in many countries (Fitzgerald et al., 2008; Champagne et al., 1999). Milled rice is the staple food of tropical Asia and it contributes 40 to 80 percent of the calories and 40 per cent of the protein requirement (Shobarani *et al.,* 2006). The average protein content of milled rice is relatively low (about 7% at 14% moisture) whereas brown rice contains about 8% protein. Increasing the protein content of rice would mean an increased supply of protein in rice based diets.

Generally, rice genotypes are recognized and identified based on morpho-biochemical traits. Majority of the traits are quantitative in nature and it misguides the plant scientist to recognize a particular genotype. Therefore, molecular characterization

along with morphological traits would be the best solution. Exploring the diversity at the molecular level is one of the prerequisites in modern plant breeding to address the world food security challenges. Molecular markers can reveal abundant difference among genotypes at the DNA level. They provide a more direct, reliable and efficient tool for germplasm characterization, screening and evaluation. A powerful technique for DNA fingerprinting is the Polymerase Chain Reaction (PCR) amplification of tandem repeat sequences. This has long been known to be polymorphic and widespread in plant genomes and is referred to as Simple Sequence Repeats (SSR) or Micro satellite polymorphism (Cregan, 1992; Morgante and Olivieri, 1993). It has been demonstrated that SSRs as primers are highly informative, locus specific markers in many species of plants (Akkaya *et al.*, 1992; Legarcrantz *et al.*, 1993; Wu and Tanksley, 1993). SSRs are increasingly useful for integrating the genetic, physical, and sequence-based maps of rice, and they simultaneously provide breeders with an efficient tool to link phenotypic and genotypic variation.

In rice, micro-satellites are abundant and well distributed throughout the genome (Wuand Tanksley, 1993; Akagi *et al.*, 1996; McCouch*et al.*, 1997). They are valuable as genetic markers because they are co-dominant in nature, detect high levels of allelic diversity and are assayed efficiently by the PCR technique (McCouch*et al.*, 2002). The level of average genome wide coverage provided by micro-satellites in rice, one marker every 6 centimorgans (Temnykh *et al.*, 2000), was sufficient to be useful for assessment of hybrid seed purity and for genotype identification. However, these markers can detect simple sequence length polymorphism (SSLP) and are quickly relocating restriction fragment length polymorphisms (RFLPs) for many kinds of genetic approaches, largely because of their easy technique They just need small amount of starting DNA, rapid turn-around time, the comparatively low cost for the users and high power of genetic resolution.

Nutrient quality evaluation and molecular characterization as well as diversity analysis of germplasm will provide valuable information to plant breeders for helping them in varietal improvement program. Therefore, the overall aim of this study was to analyze protein and amylose content as well as to characterize and evaluate the genetic diversity of 10 mutant rice lines of Bangladesh Institute of Nuclear Agriculture (BINA) and two checks using molecular markers.

The specific objectives of this study were to -

1. compare amylose and protein content of the selected 10 advanced rice genotypes, a landrace and a released variety for their qualitative analysis;
2. assess genetic diversity of the selected 12 rice genotypes using SSR markers;
3. make DNA fingerprint and to know genetic relationship among the selected advanced rice genotypes; and
4. characterize the selected rice genotypes for their better utilization in breeding program.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Experiment 1: Estimation of protein and amylose content

L-J Zhu *et al.* (2011) investigated digestibility of starches in four rice samples with amylose content (AC) from 1.7 to 55.4%, including a newly developed high-amylose rice in China. An *in vitro* enzymatic starch digestion method and an AOAC method were applied to correlate rapidly digestible starch (RDS), slowly digestible starch (SDS), resistant starch (RS), and total dietary fiber (TDF) content with the AC in the samples. Results revealed that, amylose content was positively correlated with RS and TDF content in rice flours, and starches ranged from 1.7 to 55% amylose content. However, no correlation between granule size and digestibility of starch or amylose content was found in the rice starches.

S. Aiyswaraya *et al.* (2017) conducted a research on screening and evaluation of protein content in 150 rice germplasm accessions was undertaken to identify protein rich germplasm. The results of the study revealed that total soluble protein content ranged from 7.54 g/100g to 14.54 g/100g of sample. Among 150 accessions, eight lines had recorded significantly higher protein content (>10.50 g/100g), 48 lines had registered moderate content (9.01 to 10.50 g/100g) and 94 lines had registered low protein content (< 9.00 g/100g). Cluster analysis based on available protein content revealed that, 150 accessions were grouped into two major clusters at the similarity coefficient of 2.24. Cluster I is comprised of three genotypes *viz.,* RG1 (Mapillaisamba), RG7 (Kudaivazhai) and RG110 (Norungan) which recorded higher protein content (14.54 to 12.38g/100g) and these are landraces of Tamil Nadu. Cluster II comprised of 147 accessions that branched into three sub clusters. Sub-cluster 1 had genotypes with high protein content in the range of 10.70 to 10.96 g/100g, while sub cluster II consisted of 48 genotypes with moderate protein content in the range of 9.16 to 10.25g/100g. Finally, 94 genotypes with low protein content (7.54 to 8.71g/100g) fell in sub cluster 3.

S. Yu *et al.* (2010) evaluated the starches and flours from four different rice cultivars for composition, crystallinity characteristics, blue value, turbidity, swelling power, solubility, pasting properties, and textural and retro-gradation properties. The amylose content of starches and flours from different rice cultivars differed significantly. The results showed that the physicochemical properties of rice starch and rice flour were correlated to amylose content. The crystallinity degree of rice starch and flour depended on amylose content. The blue value, turbidity value, and gel hardness were positively correlated to amylose content; however, the swelling power, solubility, and gel adhesiveness were negatively correlated to amylose content. Furthermore, the rice starch structure and molecule, proteins, and lipids significantly influenced the physicochemical properties of rice flours.

M, Riaz *et al.* (2018) tested Protein, amylose and moisture contents of the rice samples of nine (9) fine lines from five different locations of Punjab, Pakistan during the year 2014. Different environment tested entries were evaluated and found that all the values have highly significant effect of environment and genotypes. The environment and genotype ranking in the Additive Main effect and Multiplicative Interaction (AMMI) model were studied and PK8680-13-3-1 and check variety Basmati 515 were found to be most stable lines in most micro environment with respect to amylose contents and moisture contents. Protein contents were studied in PK8892-4-2-1-1 and PK3810-30-1 and are best suited in all the environments. The results indicated that grain analyzer may be used for amylose and protein contents along with effect of different locations on these traits in early breeding generations for quality control in the food industry.

A.M Magomya et al. (2014) determined Protein contents of the plant samples via the kjeldahl method using the conventional nitrogen to protein (N:P) conversion factor 6.25 (i.e. total nitrogen $\times$ 6.25) and by summation of amino acid residues (considered more accurate and taken there as the actual protein content). From data of total amino acid and total nitrogen, specific N:P conversion factors were calculated for each sample. The N:P factors ranged from 3.24 to 5.39, with an overall average of 4.64. Protein contents were also calculated using this new factor. Comparison of the calculated protein contents showed that the traditional conversion factor of 6.25 overestimated the actual protein content of the samples. The degree of overestimation

ranged from 16%-93%. Protein contents calculated with their adjusted factor (4.64) gave results that were in good agreement with the actual protein content. The results indicated that calculation of protein content by N × 6.25 was highly unsuitable for plant samples.

Q. Liu et al. (2020) determined the influence of the ranges in amylose and protein variation on rice eating quality and explored the characteristics of amylose and protein in high eating-quality rice varieties. A total of 105 *japonica* rice varieties (lines) from the middle and lower reaches of Yangtze River were studied. The results showed that the relationships among AC, PC, and eating quality were affected by the ranges of AC and PC variation. Rice varieties of the low amylose and low protein (LALP) combination should be the best choice for the high eating quality variety in the middle and lower reaches of Yangtze River.

G-K Ge *et al.* (2008) conducted an experiment in which the genetic relationships between amylose content (AC) and appearance quality traits of *indica* rice (*Oryza sativa L.*) were investigated using conditional analysis and unconditional analysis. Comparing the results from the unconditional and conditional analysis, it appeared that, for the same brown rice weight, it was possible to improve the amylose content and simultaneously noticeably reduce the brown rice length and brown rice thickness. Furthermore, if breeders improve the rice amylose content while maintaining the same protein content, the brown rice length could be reduced while simultaneously increasing the brown rice width and the brown rice thickness.

P. Eze (2020) conducted a research in which some NERICA varieties were studied to assess their proximate composition and amylose content. These varieties include; FARO 44, FARO 52, FARO 57, FARO 60, and FARO 61. Crude protein was conducted and the recorded results ranged from 7.58 to 10.29%. It was observed that NERICA contains much protein due to their high crude protein content. Results of the carbohydrate content ranged from 79.11 to 81.34%, and high carbohydrate indicated that products made from NERICA flour would provide high quality energy sources. The recorded starch results of the NERICA varieties ranged from 76.55 to 82.48%. Result indicated that NERICA recorded less degree of amylose content and this attributes to its swelling capability due to the frail support of the internal work by amylose molecules. In summary, the study on the proximate composition and amylose

content of NERICA flour varieties has revealed some vital information of the new rice varieties.

F. Shamim *et al.* (2017) conducted a research in which the cooking quality and physico-chemical characteristics of 14 newly developed lines and two check varieties widely grown in Punjab, Pakistan were investigated. Significant variation ($P<0.05$) was detected among the 15 rice varieties for all the traits evaluated. The results showed that most of the physico-chemical characteristics such as amylose, protein and gelatinization temperature were significantly correlated (positively or negatively) with some of the cooking quality traits i.e., elongation ratio, CGL indicating that efforts aimed at selecting rice varieties with improved cooking quality traits would warrant a consideration of the physico-chemical attributes of the rice grain. The overall cooking quality and physico-chemical attributes of some of new lines were even relatively better than the Check (Super Basmati).

P.T.T. Ha et al. (2016) evaluated total 44 lines/varieties obtained from Cuu Long Rice Research Institute gene bank, and 30 F_1 generation combinations. Three varieties IR75499-73-1-B, OM6162, and OM4900 were found to have good amylose content, gel consistency, protein content, gelatinization temperature, and aroma. A very notable finding was that the cross between OM6162/SwarnaSub$_1$ that low amylose content (20.2%), high gel consistency (78.2 mm), high protein content (8.1%), appropriate gelatinization temperature (scale 5), low chalkiness (level 0), high heritability (0.9) for grain yield trait/cluster, and (0.84) for the number of panicles/cluster.

2.2 Experiment 2: Molecular characterization of advanced rice mutants using Simple Sequence Repeat markers

2.2.1 Molecular marker

A gene or unique DNA sequence with a known position on a chromosome is a molecular marker or DNA marker which is readily identified and whose inheritance can be easily monitored. Molecular markers in DNA sequences are based on naturally occurring polymorphisms (i.e., base pair deletions, substitutions, additions or patterns) (Mishra et al., 2014). There are various types of molecular markers such as,

i. Simple Sequence Repeat (SSR) (Levinson et al., 1987),

ii. Single Nucleotide Polymorphism (SNP) (Vieux et al., 2002),

iii. Random Amplification of Polymorphic DNA (RAPD) (Willium et al., 1990; Tingey et al., 1993),

iv. Restriction Fragment Length Polymorphism (RFLP) (Becker et al., 1995; Paran et al., 1993),

v. Amplified Fragment Length polymorphism (AFLP), (Mackil et al., 1996; Zhu et al., 1998).

Simple Sequence Repeats are also known as microsatellites or variable numbers of tandem repeat markers that are readily available in any genome area. SSR markers have been successfully used in rice in the study of genetic diversity and relatedness, QTL mapping, marker assisted selection, rice domestication and many others.

2.2.2 Features of ideal molecular markers

An ideal molecular marker must have certain desirable properties, according to Joshi *et al.* (2011) such as,

- Highly polymorphic in nature.
- Easy access (availability): Identification should be easy, quick and inexpensive.
- High replicability.
- Frequent genome incidence: A marker should be distributed uniformly and frequently throughout the genome.
- Co-dominant inheritance: the determination of diploid organisms' homozygous and heterozygous states.
- Easy and rapid assay.
- Easy data exchange between laboratories.
- Selective neutral behaviors: any organism's DNA sequences are neutral to circumstances or management activities in the environment.
- Molecular markers are one of the most powerful tools for genome analysis and enable heritable traits to be correlated with underlying genomic variation.

2.2.3 Concept and implementation of SSR marker

Microsatellites or Simple Sequence Repeat (SSR) markers are widely used for diversity analysis among the numerous molecular markers available (Gao et al., 2005; Zhang et al., 2007; Thomson et al., 2009). Litt et al. (1989) coined the term microsatellite. SSR markers are short DNA stretches of 1-6 base pairs called motif tandem repeat units that are abundantly present both in coding and non-coding regions of DNA in the eukaryote genome.

Typically, they are 1-100 bp long and embedded with unique sequences in DNA. As they are co-dominant, efficient and cost effective, stable, reproducible, locus-specific in nature, highly polymorphic and hyper variable, SSR markers are the most common and versatile genetic markers (Gupta et al., 2000). Microsatellites are more effective tools than RAPD markers that can detect finger variation rates among closely related breeding lines (Olufowote et al., 1997).

These markers may detect a substantially higher degree of rice polymorphism (Ni et al., 2002; Okoshi et al., 2004) that is suitable for genetic diversity and intensive studies of genetic mapping (Cho et al., 2000). In the coastal saline zone of Bangladesh, where rice cultivation is largely affected by the identification of salt-tolerant landraces using modern biotechnological methods, a large cultivable area lies. Rice genetic diversity assessment is an effective method for rice (Oryza sativa L.) breeding and an integral factor in the characterization and conservation of germplasm.

The significance of rice genotype diversity analysis is,

- Establishment of genetic relationship among varieties released and lines of mutants.
- To raise the probability of genetic mitigation or genetic drift.
- For better utilization through molecular breeding of mutant rice genotypes.

2.2.4 SSR markers in the study of genetic diversity

Siddique *et al*. (2016) state that assessment of genetic diversity and molecular characterization among rice landraces of Bangladesh is important for their

identification. Genetic diversity of 96 Aman (rainfed, partially irrigated) rice landraces of Bangladesh were evaluated using eight SSR markers to characterize the landraces and also to establish the sovereignty of Bangladeshi rice gene pool. A total of 159 alleles were detected. The number of alleles per locus ranged from 13 (RM60, RM237) to 34 (RM163), with an average of 19.88. The polymorphism information content (PIC) which ranged from 0.86 (RM237) to 0.95 (RM163) with an average of 0.90 revealed much variation among the studied landraces. RM163 was the best marker for identifcation and diversity estimation of Aman rice landraces as revealed by PIC values. The allele frequency ranged from 8.33% (RM163) to 22.92% (RM60, RM125) with an average of 15.89. The UPGMA dendrogram based on Nei's genetic distance revealed seven distinct clusters with a similarity coefficient of 0.09. The two-dimensional graphical view of Principal Coordinate Analysis (PCoA) revealed that the landraces Pankaij, Lotha, Chinigura, Patjag, Chinikanai, Badshabogh, Panati, Jirasail, Joria, Dudhmoni, Jhingasail were found far away and distributed around the centroid of the cluster. The findings of that study were useful for landraces identification to assist plant breeders in selecting suitable genetically diverse parents for breeding programs.

T. Halder *et al*. (2016) carried out an investigation to analyze the genetic diversity of 12 Bangladeshi local Boro rice (*Oryza sativa* L.) germplasm using morphological traits and molecular markers. A set of eight SSR primer pairs was used for molecular characterization resulting 49 alleles, where average of allele number was 6.13. The polymorphic information content (PIC) values ranged from 0.67 (RM1) to 0.86 (RM314) with an average of 0.76. The highest PIC value (0.86) was obtained for RM314 which also gave maximum alleles. The PIC value revealed that RM314 was the best marker for 12 genotypes tested. The cluster analysis based on UPGMA system grouped 12 genotypes into four clusters.

Joachim (2015) carried out a study to evaluate the genetic diversity of rice germplasms collected from Eastern and Southern Africa countries (Burundi, Kenya, Malawi, Tanzania including Zanzibar and Rwanda) based on morphological, molecular and quality traits for utilization in breeding programs. A total of 191 rice germplasms were characterized in this study. Twenty four qualitative and quantitative morphological traits, eight grain quality traits and 18 Simple Sequence Repeat (SSR) markers were used for analysis. Genetic variation analysis of morphological and grain

quality traits resulted in grouping of the germplasms into seven clusters. Principal component analysis showed that 75.37% of the variability was contributed by the first six principal components. A total of 18 SSR's markers were used and 16 found to be polymorphic. A total of 121 alleles were obtained on polymorphic SSR with an average of 7.56 allele per marker and the number of alleles ranged from 2 to 20. The Polymorphism Information Content (PIC) values ranged from 0.01 to 0.89 with an average value of 0.49. The genetic diversity of each SSR locus appeared to be associated with number of allele detected per locus. The cluster analysis based on similarity index of simple matching grouped the studied rice genotypes into six clusters.

Shaheen *et al*. (2017) performed an experiment in which seedlings of different rice genotypes were screened for drought tolerance using traditional methods and molecular characterization of selected rice genotypes using microsatellite markers for drought tolerance. The research used 21 rice genotypes obtained from the Plant Breeding Division, RARS, Pattambi. Seedlings of these genotypes were initially subjected to conventional PEG-6000 drought screening (6 MPa and 8 MPa). Just 14 genotypes were selected from the 21 rice genotypes based on the seedling vigor index and used for molecular characterization. Sample DNA extraction was carried out using the CTAB method followed by PCR amplification with two specific SSR markers, namely RM103 and RM212. With RM103, monomorphic bands were observed in all fourteen genotypes; thus, it cannot be used as a drought tolerance marker in these genotypes. In the studied genotypes for the marker RM212, polymorphic bands were observed. However, there was no significant linkage between the RM212 marker and the seedling vigor index under drought stress.

Mawuli *et al*. (2020) conducted a research titled by molecular characterization of popular rice (*Oryza sativa* L.). Phenotyping showed 37 leaf blast resistant lines among 103 rice germplasm samples. Following that, using gene-specific markers, genotyping of the germplasm for 5 essential blast resistance genes in India, namely Pitp, Pi1, Pi2, Pi9, and Pi54, revealed that Pitp was more prevalent than Pi2 in the tested lines. RM234 had the highest PIC value of 0.77 in an initial genetic diversity analysis, suggesting its high polymorphic behavior. Analysis of molecular variance showed a difference between the populations of 19 percent, while 81 percent within the populations. Using 101 SSR markers, the association study reported ten new marker-

trait associations using the General Linear Model approach and two new marker-trait associations using the Mixed Linear Model approach. They suggested that, these markers may be used in marker-assisted selection after being validated in a large collection of germplasm.

Samal *et al*. (2019) conducted a study in which 98 unique rice landraces have characterized. The landraces were collected from coastal regions of India, affected by submergence and salinity, based on *Sub1* and *Saltol* quantitative trait loci (QTL) linked microsatellite markers. A high level of genetic diversity of H_e = 0.349 and 0.529 at *Sub1* and *Saltol* QTL region was detected by QTL-linked microsatellite markers, respectively. At *Sub1* region one genotype, AC34902, was detected with maximum allelic similarity with FR13A, a known submergence tolerant variety. Besides, five genotypes (IC211188-1, IC536604-1, IC536604, IC536558 and IC536559) showed comparatively close genetic relationship with the salt tolerant variety FL478 for *Saltol* QTL and were clustered together in the neighbor joining dendrogram. Considering the haplotype structure, five genotypes (IC203801, IC203778, IC324584, IC413608 and IC413638) were identified which did not contain any common allele similar to FR13A but were still tolerant to submergence.

Supari *et al*. characterized 21 Malaysian rice cultivars using primers of 27 microsatellite loci to investigate the molecular diversity. To amplify the possible microsatellite loci, extracted DNA and PCR-based methods have been used. 61 alleles were amplified, including 27 rare and 15 unique alleles, and 88.89 % of all microsatellite loci analyzed were polymorphic, according to the findings. In addition, di-nucleotide repeat motifs (49.28 %) were shown to be more frequent than tri-nucleotide repeat motifs (48.28 %). Polymorphism information content (PIC) ranged from 0.090 (RM411) to 0.940 (RM105) with a mean of 0.531. In addition, all varieties were grouped into three major clusters by the UPGMA derived dendrogram. The relatively high degree of genetic diversity among the cultivars studied could explain the high proportion of polymorphism (88.89%). Gene mapping, marker-assisted selection (MAS) amplification, and rice breeding programs in Malaysian rice cultivars depend heavily on chromosome 4 (0.917) and chromosome 7 (0.607).

CHAPTER 3

MATERIALS AND METHODS

The study was conducted for the estimation of amylose and protein content as well as genetic diversity among twelve genotypes of rice on the basis of qualitative analysis and molecular characterization.

3.1 Experiment 1: Estimation of amylose and protein content

3.1.1 Location and time

Amylose content was carried out at Biochemistry and Molecular Biology (BMB) laboratory, Bangladesh Agricultural University (BAU), Mymensingh, and Biotechnology laboratory, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. Protein content was carried out at the laboratory of Soil Science Division, BINA, Mymensingh. The study was conducted during the period of September to December, 2020. The place is geographically situated at about $24^{o}75'$ North Latitude and $90^{o}50'$ East Longitude.

3.1.2 Materials

A total of 10 rice mutant genotypes, a landrace and a commercially available check variety was included in the study. The check variety was collected from Bangladesh Rice Research Institute (BRRI), Gazipur and other eleven genotypes from Plant Breeding Division, BINA, Mymensingh (Table 1).

Table 1. List of the genotypes of rice

Sl. No.	Name of the genotypes	Source
1	RM-16 (N)-10	BINA
2	RM- Kas-80(C)-1	BINA
3	LD-200-1-3-3-5	BINA
4	LD-200-1-3-3-8	BINA
5	LD-200-1-3-2-4	BINA
6	RM-40(C)-4-2-8	BINA

Table 1 (cont'd)

7	SSB-3	BINA
8	Magic-10	BINA
9	Magic-72	BINA
10	Magic-86	BINA
11	BRRI dhan49	BRRI
12	Luxmidigha	BINA

BRRI dhan49

BRRI dhan49 is a mega variety released in 2008. It is cultivated all around Bangladesh during Aman season. Life cycle of this variety is around 135 days and plant height is about 100 cm. Average yield is 5.0 tons/ha. The variety is popular for its good quality grain and constant yield.

Luxmidigha

Luxmidigha is a landrace of deep water rice generally grown in Barishal and Gopalganj region in Late Boro season. It has excellent stem elongation capacity (up to 5 cm/day) under submergence condition. Life cycle and average yield of luxmidigha ranges from 148-165 days and 0.9-1.3 tons/ha, respectively.

3.1.3 Sample preparation

At first, 100 g seeds of each rice genotype was peeled by hand. Then the seeds were crushed well by grinder. After that the rice powder of each genotype was sieved separately by 120 mesh sieve. Finally, the powder of each genotypes was taken in separate zipper bags and stored in a desiccator.

3.1.4 Analysis of Amylose Content (AC)

3.1.4.1 Reagents and Equipment required

Table 2. Reagents and equipment required for analysis of amylose content

Reagents	Equipment
1N Oxalic Acid	Hot water bath
1N NaOH	Spectrophotometer
1N Acetic acid	Desiccator
Ethanol	Micropipette
Potassium iodide	Weighing Balance
Iodine	Microwave Oven
Phenolphthalein powder	Autoclave

3.1.4.2 Reagents preparation

i. 1N Oxalic acid (500 ml)

Exactly 31.5 g dry oxalic acid powder was dissolved in 300 ml distilled water. Then the volume was made up to 500 ml by adding distilled water.

ii. 1N NaOH (1000ml)

Exactly 40 g NaOH pellet was dissolved in 500 ml distilled water. Then the volume was made up to 1000 ml by adding distilled water.

iii. 1N Acetic acid (1000 ml)

Carefully 60 ml acetic acid was poured to 500 ml distilled water in a 1000 ml volumetric flask. Then the volume was made up to 1000 ml by adding distilled water.

iv. Iodine solution (100 ml)

Appropriately 0.1079 g KI was dissolved in 5 ml distilled water in a 100 ml volumetric flask. Then 0.0315 g I_2 was added and the flask was shaken well. Finally, the volume was made up to 100 ml. The solution was stored in a dark bottle at 4°C until used.

v. Standard stock solution (100 ml)

Firstly 0.04 g amylose standard of rice was weighed and poured in a 100 ml volumetric flask. Secondly, 1 ml 95% ethanol and 9 ml 1N NaOH was added to that. Thirdly, the mixture was boiled in hot water bath for 15 minutes. Finally cooling the mixture at room temperature, the volume was made up to 100 ml by adding distilled water.

3.1.4.3 Standardization process by titration

i. Standardization of 1N NaOH by 1N Oxalic acid

At first, a drop of indicator (1% phenolphthalein) was added in 5 ml 1N NaOH solution in a beaker. The color of the solution turned into colorless to purple. Then 5 ml 1N Oxalic acid was added drop by drop through a 5 ml pipette. The color was not changed which means 1N NaOH was stronger than 1N Oxalic acid.

Exactly 2 ml distilled water was added in 1N NaOH solution and the process was repeated. Then the purple color of 1N NaOH solution with indicator mixture was turned into colorless after adding exactly 5 ml 1N oxalic acid. Thus 1N NaOH was standardized.

ii. Standardization of 1N Acetic acid by standard 1N NaOH

Firstly, a drop of indicator (1% phenolphthalein) was added in 5 ml 1N Acetic acid solution in a beaker. The solution remained colorless. Then 5 ml 1N NaOH was added drop by drop through a 5 ml pipette. The colorless state of the solution was not changed which means 1N Acetic acid was stronger than 1N NaOH.

Exactly 2.5 ml distilled water was added in 1N NaOH solution and the process was repeated. Then the purple color was appeared in the solution mixture after adding exactly 5 ml standard 1N NaOH solution. Thus 1N Acetic acid was standardized.

3.1.4.4 Preparation of standard amylose curve

According to Table 3, specific amount of standard stock solution, 1N acetic acid, iodine solution was poured in 5 separate 100 ml volumetric flasks as well as a blank to set zero. Then the 6 volumetric flasks were volume up to 100 ml.

Table 3. Reaction mixtures for standard amylose curve

Sl. No.	1N Acetic acid (ml)	Iodine sol^n (ml)	Standard stock sol^n (ml)	Amylose Equivalence (%)
1	-	2	-	0
2	0.4	2	1	8
3	0.8	2	2	16
4	1.2	2	3	24
5	1.6	2	4	32
6	2.0	2	5	40

The mixtures then measured at 620 nm in a spectrophotometer. A standard amylose curve was prepared by using the absorbance reading with different amylose equivalence.

3.1.4.5 Determination of amylose content in rice samples

At first, 0.1 g of each rice samples were weighed and put in 100 ml volumetric flask. One ml 95% ethanol and nine ml 1N NaOH was added and shaken well. The mixture was boiled in a hot water bath for 15 minutes. After cooling at room temperature the solution was volume up to 100 ml.

Then 5 ml from 100 ml solution was transferred to another 100 ml volumetric flask for each sample. Fifty ml distilled water, 1 ml 1N acetic acid and 2 ml iodine solution was added to that and volume up to 100 ml. Each sample solution was measured at 620 nm to get absorbance reading. Finally, amylose content of each samples was determined by calculating with the standard amylose curve.

3.1.5 Analysis of Protein Content by Kjeldahl Method

The Kjeldahl method can conveniently be divided into three steps: digestion, distillation and titration. In this study, digestion process was done manually and a machine “UKD 159 Automatic Distillation & Titration System” was used that could perform distillation and titration automatically.

3.1.5.1 Reagents and Equipment required

Table 4. Reagents and Equipment required for analysis of protein

Reagents	Equipment
H_2SO_4	Fume hood
$HClO_4$	Hot plate
Boric acid	Automatic Distillation & Titration System
NaOH	Micropipette
Bromocresol green	Weighing Balance
Methyl red	Microwave Oven

3.1.5.2 Reagents preparation (Stock solution)

i. Indicator mixture (17 ml)

At first, 0.1 g bromocresol green was dissolved in 100 ml ethanol. Then 0.1 g methyl red was dissolved in 100 ml ethanol. Finally, 10 ml bromocresol green from 100 ml solution and 7 ml methyl red from 100 ml solution were mixed in a 100 ml beaker. Thus 17 ml indicator mixture was prepared.

ii. Diacid mixture (500 ml)

Exactly 333 g H_2SO_4 was mixed with 167 g $HClO_4$ in a 500 ml conical flask (H_2SO_4: $HClO_4$ = 2:1) and stored.

iii. 4% Boric acid solution (1000 ml)

Forty gram of boric acid powder and 500 ml distilled water were poured in a 1.0 L beaker. Then dissolved the solution with heat and volume that up to 1000 ml. Finally, 17 ml indicator mix added to the mixture.

iv. 32% NaOH solution (1000 ml)

Appropriately 320 g NaOH tablet was placed in a 1.0 L beaker with distilled water and dissolved that with heat on a hot plate. Then the solution was volume up to 1000 ml.

3.1.5.3 Procedure of protein analysis

- At first 0.5 g of each sample was added with 10 ml Diacid mixture in 100 ml conical flasks.
- The mixture containing conical flasks were placed in a fume hood and kept there overnight.
- Then the mixture containing conical flasks were placed on a hot plate in another fume hood at 350℃ and heat 3 hours for complete digestion.
- After complete digestion, the conical flasks were kept at room temperature overnight to become cool.
- Then the mixtures were volume up to 50 ml with distilled water.
- The solution for each sample then poured in the UKD 159 Automatic Distillation & Titration System.
- Finally, total N% and Protein % in each sample were found from the reading of UKD 159 Automatic Distillation & Titration System. N:P conversion factor 5.95 for rice was used in this system (Greenfield and Southgate, 1992).

3.2 Experiment 2: Molecular characterization of selected Rice genotypes

The molecular characterization of the selected rice genotypes was performed during September to December, 2020 at the laboratory of Biotechnology Division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh.

3.2.1 Collection of leaf sample for DNA extraction

Initially, a healthy portion of the young, vigorous leaves from 21 days old seedling was cut apart with sterilized scissors and washed in 70% ethanol and distilled water. The leaf samples were then placed in polythene bags and labelled. To prevent any damage to the leaf tissues, the bags were placed in an ice box and transferred to the laboratory. Finally, the samples were stored at - 20°C freezer.

3.2.2 Extraction of genomic DNA

The Cetyl Trimethyl Ammonium Bromide (CTAB) mini-prep method was used to extract DNA from the leaves of each genotype. The IRRI-developed simplified mini scale procedure for DNA isolation in PCR analysis was used.

3.2.3 Reagents required

1. Extraction buffer (pH=8.0)

- 1M tris-HCl
- 0.5 M EDTA (Ethylene diamine tetra acetic acid)
- 5 M NaCl

2. 20% SDS (Sodium Dodecyl Sulphate)

3.Chloroform: Isoamyl alcohol= 24:1 (v/v); equilibrated to pH near 8.0 with TE buffer.

4.TE (Tris-EDTA) buffer; pH= 8.0

- 10mM Tris= HCl
- 1mM EDTA

5. Sodium acetate (3 M), pH= 5.2

6. Absolute (100%) ethanol

7. Isopropanol

8. 70% Ethanol

9. CTAB

3.2.4 Equipment required

- Weighing Balance
- Mortar and pestle
- Refrigerator
- pH Meter
- Hot water Bath
- Water Bath with Shaker
- UV Spectrophotometer
- Centrifuge machine
- Nano drop spectrophotometer
- Gel Electrophoresis Unit

- Gel Documentation
- Eppendorf tube
- PCR machine
- Magnetic stirrer
- Microwave Oven
- Micropipettes and tips

3.2.5 Reagents used for DNA Isolation

a. Extraction Buffer (pH=8.00)

- 2M Tris-HCl (pH=8.00)
- 5M NaCl
- 0.5M EDTA (pH=8.00)
- 4g CTAB
- β-marcapto ethanol (3%)

b. 20% SDS

c. 70% Ethanol

d. 100% Ethanol

e. Phenol: Chloroform: Isomyl alcohol (25:24:1)

f. 100% Isopropanol

g. 1X TE Buffer (pH=8.00)

h. 3M sodium Acetate (pH=5.2)

3.2.6 Reagents preparation for DNA extraction (stock solution)

a. Extraction Buffer (100 ml)

- Four gram CTAB was taken in a measuring cylinder.
- Five ml 2M tris-HCl was added to the solution.
- Then 75 ml 5M NaCl was added in measuring cylinder.

- Then 4 ml of 0.5M EDTA was added.
- Finally sterilized distilled H_2O was added to make the volume up to 100 ml.
- After that, the mixture was autoclaved at 121℃ for 20 minutes.
- Three ml β-marcapto ethanol (3%) was added just before use.

b. 20% SDS (100 ml)

- Twenty gram SDS was added into 100 ml sterile ddH_2O.
- The mixture was not autoclaved, because SDS is hazardous.

c. 5M NaCl (100 ml)

- Exactly 29.22 g NaCl was weight in an electric balance
- Then 100 ml ddH_2O was added to make the solution
- Then it was autoclaved at 121℃ for 20 minutes.

d. 2M Tris-HCl (100 ml)

- Appropriately 31.52 g Tris base was taken in a volumetric flask.
- Exactly 75 ml ddH_2O was added.
- pH was just adjusted to 8.0.
- The volume was made up to 100 ml and autoclaved at 121℃ for 20 minutes.

e. 0.5 M EDTA (100ml)

- Exactly 18.612 g EDTA was added to 75 ml ddH_2O.
- pH was adjusted to 8.0 with NaOH.
- The volume was made 100 ml and autoclaved at 121℃ for 20 minutes.

f. 70% Ethanol (50 ml)

- Fifteen ml ddH_2O was added to 35 ml absolute Ethanol.

g. Phenol: Chloroform: Isoamyl alcohol (25:24:1) 100 ml

- Fifty ml phenol was added with 48 ml Chloroform and then 2 ml isoamyl alcohol was added to make volume 100 ml.

h. 3M Na-acetate (100 ml)

- Exactly 24.609 g Na-acetate was added in 100 ml ddH_2O.
- pH was just adjusted to 5.2 with Glacial acetic acid.

i. 1X TE Buffer

- One ml 1M Tris-HCl (pH 8.0) was taken in a conical flask.
- Then 0.2 ml 0.5M EDTA was added and the volume was made up to 100 ml with ddH_20.
- Autoclaved at 121°C for 20 minutes.

j. 100% Ethanol

k. 100% Isopropanol

- It was done simply by keeping the Isopropanol in -20°C refrigerator.

l. 10X TAE buffer, (1000 ml)

- At first 48.4 g Tris-base dissolved in 800 ml ddH_20.
- Then 11.4 ml glacial acetic acid was added.
- Then 3.7 g EDTA was added.
- Finally, the volume was adjusted to 1000 ml.

m. 1X TAE (1000 ml)

- It was made by adding 100 ml 10×TAE in 900 ml ddH_2O.

n. Ethidium bromide preparation (10 mg/ml)

- One gram of ethidium bromide was added in 100 ml of ultra-pure water. Then the mixture was stirred vigorously for several hours to ensure that the dye was dissolved.
- The container (dark bottle) was then wrapped in aluminum foil.
- Stored at room temperature.

3.2.7 Extraction procedure of genomic DNA

- Using a pestle and mortar, the leaf samples were cut into 2-3 cm pieces and ground.

- An extraction buffer of 800 µl was added to the paste and ground until it was fully dissolved.
- Each dissolved sample was put in a 2 ml eppendorf tube.
- Samples were vortexed thoroughly and incubated for 10 minutes at 65°C in a hot water tank.
- The samples were vortexed once more and incubated in a 65°C hot water bath for another 10 minutes.
- Each sample tube was filled with 800 µl of chloroform mix (Chloroform: isoamylalcohol = 24:1).
- The chloroform solution was thoroughly mixed with the sample by continuously inverting for 3 minutes.
- The samples were then centrifuged for 8 minutes at 11000 rpm.
- The 500 µl supernatant was then transferred to a new clean1.5 ml eppendorf tube.
- Then 1 ml of ice cold absolute ethanol was added.
- To get a better pellet, the mixture was inverted and held at -20° C for 2-2.5 hours.
- After centrifuging the samples for 12 minutes at 13,200 rpm, a small pellet was visible at the bottom of the tube.
- The aqueous solution was carefully poured out to keep the pellet from falling off.
- Then 1 ml of ice cold 70% ethanol was added.
- The samples were centrifuged for 3 minutes at 13,200 rpm.
- The ethanol was removed, and the pellets were allowed to air dry for 1.5–2 hours.
- To re-suspend the pellets, 1X TE buffer (50 µl) was added.
- The pellets were dissolved by gently tilting the samples and immersing them in a 65°C hot water bath for 30-45 minutes.
- After that, the sample was held at -20°C.

Precautions

To prevent contamination, all glassware, micropipette tips, centrifuge tubes, glass pipettes, distilled water, and buffer solutions were properly autoclaved. Scissors and forceps were sterilized using absolute ethanol.

3.2.8 Confirmation of DNA using gel electrophoresis

Using Agarose gel electrophoresis, the DNA samples were examined qualitatively. Here is the process for sample preparation, 0.8% gel preparation, and electrophoresis.

a. Preparation of 0.8% Agarose gel (250 ml)

- A flask was loaded with 100 ml of 0.5X TBE (electrophoresis buffer) and 0.8 g of agarose.
- To dissolve the mixture, it was put in a micro-oven and cooked for 3 minutes.
- The gel was allowed to cool down to a tolerable temperature at room temperature for 20-30 minutes.
- The gel was then carefully poured into the gel mold.
- In the meantime, combs were laid out on the gel.
- The gel was solidified within 30 minutes.
- In the gel tank, the gel was immersed in 0.5X TBE buffer.
- The combs were taken out of the gel.
- The gel was then ready for loading the DNA samples.

b. DNA sample preparation and electrophoresis

Reagents required

- Loading dye (0.25% xylene cyanol, 0.25% bromophenol blue, 30% glycerol and 1mM EDTA)
- DNA marker (DNA ladder)

Procedure

- Using a micropipette, loading dye (4 µl) was placed on a piece of para-film paper.
- Two µl extracted DNA sample was added to it, which was thoroughly mixed before being placed into the gel's slot.
- The first lane of the gel was loaded with a recognized DNA marker (DNA ladder).
- The electrophoresis apparatus was connected to the power supply unit and the gel tank was submerged in 0.5X TBE.
- The DNA was transferred from the negative to the positive electrode after the switch was turned on.
- After a few minutes, two colors (dye) appeared.
- The separation was detected by the migration of the dye in the gel.
- The power supply was turned off when the first dye (bromophenol blue) had reached two-thirds of the gel volume.
- Electrophoresis was conducted for 60-90 minutes at 120 volts.

c. Documentation of the DNA samples

- After electrophoresis, the gel was carefully removed from the gel chamber and stained with a previously prepared ethidium bromide solution.
- After 30 minutes of staining, the image was put on the UV trans-illuminator in the Image Documentation System's dark chamber.
- The system's UV light was turned on. The picture was visualized on the monitor by using Alpha Easefc 4.0 software and the image was saved in the Gel Doc computer.

3.3 Quantification of DNA concentration

To estimate the quantity and quality (in terms of protein and RNA contamination) of isolated genomic DNA of the samples, Nano-drop spectrophotometry (thermo-scientific model nano-drop 1) was used to quantify DNA in per micro litter.

The concentration of DNA (ng/µl) and absorbance at 260/280 nm were measured (Table 5).

Table 5. DNA absorbance reading and DNA concentration

Name of the genotypes	Absorbance reading (at 260/280 nm)	DNA concentration (ng/µl)
RM-16 (N)-10	1.55	966.26
RM- Kas-80(C)-1	1.72	917.78
LD-200-1-3-3-5	2.13	552.05
LD-200-1-3-3-8	2.06	455.24
LD-200-1-3-2-4	1.92	457.45
RM-40(C)-4-2-8	1.74	875.01
SSB-3	2.00	362.35
Magic-10	2.03	734.13
Magic-72	1.62	945.62
Magic-86	1.94	760.33
BRRI dhan49	1.88	844.30
Luxmidigha	1.88	888.02

3.4 Preparation of working solution of DNA sample

Before running the samples through the PCR system, the DNA concentrations were diluted to 50 ng/µl using the following formula:

$$S_1 \times V_1 = S_2 \times V_2$$

Where,

S_1= Initial concentration of DNA (ng/µl)

S_2= Final concentration of DNA (ng/µl)

V_1= Initial volume of DNA solution (µl)

V_2= Final volume of DNA solution (µl)

$$V_1 = (V_2 \times S_2)/S_1$$

3.5 Amplification of SSR markers by polymerase chain reaction (PCR)

The SSR analysis was carried out at the Laboratory of Biotechnology Division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh.

3.6 Selection of Primers

The strength of bands, consistency between individuals, presence of smearing, and potential for population discrimination were all considered when assessing primers. 16 random SSR markers were screened for twelve rice genotypes in this experiment to see if they were optimal for amplifying DNA sequences that could be properly scored (Table 6).

Table 6. Name of the screened SSR markers

SL. NO.	Markers name	Chromosome No.
1.	RM 217	6
2.	RM 42	8
3.	RM 237	1
4.	RM 431	1
5.	RM 307	4
6.	RM 105	9
7.	RM 171	10
8.	RM 228	10
9.	RM 206	11
10.	RM 536	11
11.	RM 519	12
12.	RM 286	12
13.	RM 111	6
14.	RM 475	2
15.	RM 168	3
16.	RM 11	7

Primers were used to select the heterozygosity of alleles (Table 7).

Table 7. Sequence of SSR markers, DNA band size and Annealing temperature

Markers Name	Product Size (bp)	Sequences	Annealing temp.(°C)
RM 217	133	Forward: ATCGCAGCAATGCCTCGT Reverse: GGGTGTGAACAAAGACAC	55
RM 42	166	Forward: ATCCTACCGCTGACCATGAG Reverse: TTTGGTCTACGTGGCGTACA	55
RM 237	130	Forward: CAAATCCCGACTGCTGTCC Reverse: TGGGAAGAGAGCACTACAGC	55
RM 431	251	Forward: TCCTGCGAACTGAAGAGTTG Reverse: AGAGCAAAACCCTGGTTCAC	55
RM 307	174	Forward: GTACTACCGACCTACCGTTCAC Reverse: CTGCTATGCATGAACTGCTC	55
RM 105	134	Forward: GTCGTCGACCCATCGGAGCCAC Reverse: TGGTCGAGGTGGGGATCGGGTC	55
RM 171	328	Forward: AACGCGAGGACACGTACTTAC Reverse: ACGAGATACGTACGCCTTTG	55
RM 228	154	Forward: CTGGCCATTAGTCCTTGG Reverse: GCTTGCGGCTCTGCTTAC	55
RM 206	147	Forward: CCCATGCGTTTAACTATTCT Reverse: CGTTCCATCGATCCGTATGG	55
RM 536	243	Forward: TCTCTCCTCTTGTTTGGCTC Reverse: ACACACCAACACGACCACAC	55
RM 519	122	Forward: AGAGAGCCCCTAAATTTCCG Reverse: AGGTACGCTCACCTGTGGAC	55
RM 286	110	Forward: GGCTTCATCTTTGGCGAC Reverse: CCGGATTCACGAGATAAACTC	55

Table 7 (cont'd)

RM 111	124	Forward: CACAACCTTTGAGCACCGGGTC Reverse: ACGCCTGCAGCTTGATCACCGG	55
RM 475	235	Forward: CCTCACGATTTTCCTCCAAC Reverse: ACGGTGGGATTAGACTGTGC	55
RM 168	116	Forward: TGCTGCTTGCCTGCTTCCTTT Reverse: GAAACGAATCAATCCACGGC	55
RM 11	140	Forward: TCTCCTCTTCCCCCGATC Reverse: ATAGCGGGCGAGGCTTAG	55

3.7 Polymerase Chain Reaction (PCR)

The PCR cocktail was placed in the PCR tubes and operated in the DNA thermal cycler, with a total volume of 10 µl/reaction mixture containing 1 µl of DNA based on the IRRI rice protocol of SSR analysis.

3.7.1 Components of PCR Cocktail

Table 8. PCR Cocktail Components for SSR

Sl. No.	Components	Quantity for single reaction (µl)
1	Go Taq Green master mix	5.0
2	Nuclease free water (ddH$_2$O)	3.0
3	Primer Forward	0.5
4	Primer Reverse	0.5
5	Diluted DNA	1.0
	Total	10.0

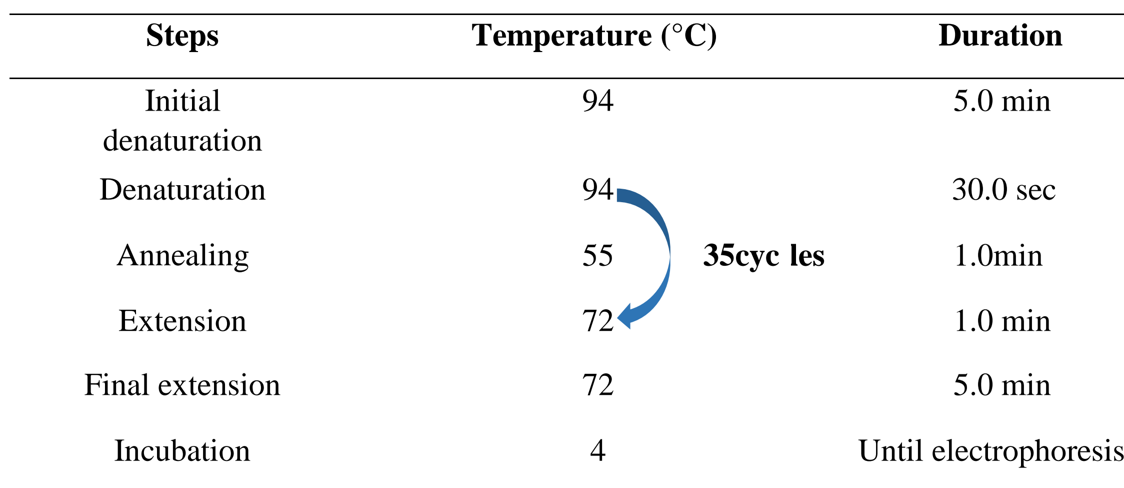
3.7.2 Procedure for PCR Cocktail Preparation (for each sample)

- A 1.5 ml PCR tube was filled with 3 µl of sterilized ddH2O and 5 µl of Go Taq Green master mix.
- Then 0.5 µl of forward primer and 0.5 µl of reverse primer were added together.
- After that, the mixture was vortexed.
- Finally, 1 µl of diluted DNA sample was pipetted into the PCR tubes' wells, and the tube was centrifuged for 30-40 seconds to ensure proper mixing.
- Thus a total of 10 µl of PCR sample was prepared.
- The PCR tubes were numbered precisely. The tubes were kept on ice during the process, and all chemicals were kept on ice as well.

3.7.3 Profile of PCR amplification

The PCR tubes were placed in the thermo-cycler plate's wells. The system was then run according to the instructions in the manual (Table 9).

Table 9. Thermal profile of PCR cycle



Steps	Temperature (°C)		Duration
Initial denaturation	94		5.0 min
Denaturation	94		30.0 sec
Annealing	55	**35cyc les**	1.0min
Extension	72		1.0 min
Final extension	72		5.0 min
Incubation	4		Until electrophoresis

3.8 Polyacrylamide Gel Electrophoresis (PAGE)

Compared to agarose gel electrophoresis, polyacrylamide gel electrophoresis (PAGE) has several advantages. The key advantage of acrylamide over agarose is that it has smaller pores, which makes it ideally suited for separating smaller DNA molecules that an agarose gel would be unable to separate.

3.8.1 Reagents required

- 40% Acrylamide
- 10% APS
- 10X TBE Buffer
- TEMED (N,N,N',N'- tetramethylethane-1,2-diamine)

3.8.2 Glass plate assembly

- The glass plates were washed by 70% alcohol and rinsed with distilled water. Then the plates were air-dried and cleaned using lint-free tissue.
- Starting from one sight of the plate, the rubber gasket was tightened with a short plate (round bottom). The gasket's notches were aligned on the corners, and the gasket's circular part was exposed on the plate's inner hand.
- The tubing side of the short plate was set up on the table. The spacers were placed along the gasket's inside edges.
- On top of the short plate, the other plate was placed.
- The plates were clamped on both sides and the plate assembly was laid flat on the surface. The table's surface was even.

3.8.3 Preparation of polyacrylamide gel

3.8.3.1 Reagent composition for PAGE

I. Ten percent Ammonium per sulfate (APS), 10 ml

- 1 g APS
- 9 ml RNase free water

II. 10X TBE buffer (1000 ml)

- 108 g Tris-base
- 50 gram Boric acid
- Exactly 9.3 g EDTA, pH 8.0

III. 40% Acrylamide (1000 ml)

- Exactly 380 g Acrylamide powder
- Twenty gram Bisacrylamide powder
- Six hundred ml distilled water

IV. Premix preparation (1000 ml)

- Two hundred ml of 40% Acrylamide
- One hundred ml of 10X TBE Buffer
- Exactly 690 ml of dH_2O

The gel solution was prepared in a beaker placing on a magnetic stirrer. The reagents were added in the following order (Table 10):

Table 10. Polyacrylamide Gel Components

SL. NO.	Reagents	Volume
1	Premix	50ml
2	10% APS	650µl
3	TEMED (N,N,N',N'- tetramethylethane-1,2-diamine)	72µl
	Total	50.722 ml

3.8.4 Gel casting

Starting from one corner, the gel was poured smoothly and steadily until it reached the top portion of the short plate. The comb was slowly inserted into the gel until half of it was completely inserted. For 15 minutes, the gel was allowed to polymerize.

3.8.5 Electrophoresis

- The gasket from the plate assembly was removed before beginning electrophoresis, and the tank was loaded with 10X TBE buffer. To prevent a short circuit, the plate assembly was put in the tank so that the short glass plate faced the inner side and there were no bubbles at the base. On top of the tank, 10X TBE buffer was added, and the comb was carefully removed.

- Each well was loaded with 2.5 µl of each PCR substance. As an indicator, DNA ladders of different sizes (20 bp, 100 bp, and 1 kb) were used.
- Then, the electrodes were connected to the power supply, and the tank was powered at 70 volts for around 1.5 hours (running time may vary depending on the size of the fragments).

3.8.6 Staining of ethidium bromide

After electrophoresis was done, the gel was soaked in ethidium bromide (10 mg/ml) solution for 20-30 minutes.

10 mg/ml Ethidium bromide preparation:

- In 10 ml ddH2O, 0.1 g Ethidium bromide was added.
- After that, the mixture was vigorously stirred for several hours.
- The container was then covered in aluminum foil and placed at room temperature.

Precautions

- Hand gloves were used when handling Ethidium bromide because it is a potent mutagen and carcinogen.
- UV radiation with a wavelength of 254 nm is emitted by a trans-illuminator. This wavelength has the potential to be harmful to the eyes (short term: burns, long term: cataracts and skin cancers). So, before turning on UV radiation, the gel doc's door was closed.

3.8.7 DNA sample documentation

The gel was carefully removed from the staining tray and placed on the gel doc's high-performance ultraviolet light box (UV trans-illuminator) to check the DNA bands. Using Alpha Easefc 4.0 software, the DNA was identified as a band and the records were saved.

3.9 Data analysis

The experiments were conducted through CRD design. The recorded data for amylose and protein content were analyzed using MSTAT-C package program (Gomez and Gomez, 1984). POWER MARKER version 3.23 (Liu and Muse, 2005), a genetic analysis software was used to calculate statistical results such as the number of alleles per locus, major allele frequency, gene diversity, and polymorphism information Content (PIC) values. The Alpha Ease 4C software was used to calculate the molecular weights of microsatellite products in base pairs. The alleles of the relevant microsatellite loci were assigned to the individual fragments. The genetic distance or phylogeny reconstruction based on neighbor-joining method (Saitou and Nei, 1983) as implemented in POWER MARKER with the tree viewed using TREEVIEW was also determined using allele molecular weight data.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Experiment 1: Estimation of Amylose content (AC) and protein content %

4.1.1 Amylose content

The amylose content of selected 12 rice genotypes was determined with reference of the standard amylose curve. Standard amylose curve was obtained from amylose equivalence and the absorbance of the standard mixtures at 620 nm in a spectrophotometer.

Table 11. Absorbance of the standard mixtures at 620 nm for amylose equivalence

Sl. No.	Amylose Equivalence (%)	Absorbance
1	8	0.015 e
2	16	0.031 d
3	24	0.044 c
4	32	0.057 b
5	40	0.069 a
	CV (%)	1.78

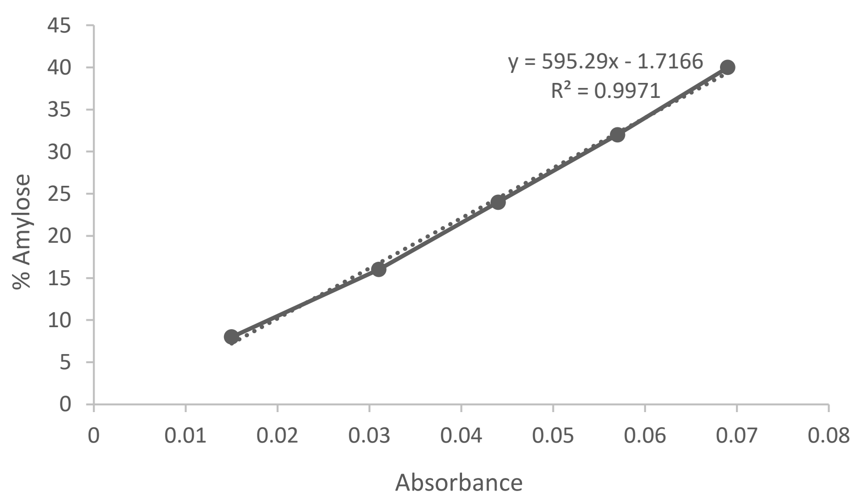


Fig 1. Standard amylose curve

The amylose content of 12 rice genotypes varied from 20.3% to 25.6 %. The highest amylose content (25.6%) was found in Magic-86; followed by RM-40(C)-4-2-8 (25.1%), Magic-10 (24.4%), BRRI dhan49 (23.9%), Magic-72 (23.9%), LD-200-1-3-3-8 (23.3%), SSB-3 (22.7%), RM-Kas-80(C)-1 (22.1%), LD-200-1-3-2-4 (22.1%), LD-200-1-3-3-5 (21.5%), Luxmidigha (20.9%) and RM-16(N)-10 (20.3%). There were 10 groups (a, b, etc.) in which the means were not significantly different from one another at 5% level of significance. Critical Value for Comparison was 0.1685. These values were similar to those reported by Paul (2020).

Table 12. Amylose contents of 12 rice genotypes

Sl. No.	Name of the genotypes	Amylose content (%)
1	RM-16 (N)-10	20.3 j
2	RM- Kas-80(C)-1	22.1 g
3	LD-200-1-3-3-5	21.5 h
4	LD-200-1-3-3-8	23.3 e
5	LD-200-1-3-2-4	22.1 g
6	RM-40(C)-4-2-8	25.1 b
7	SSB-3	22.7 f
8	Magic-10	24.4 c
9	Magic-72	23.9 d
10	Magic-86	25.6 a
11	BRRI dhan49	23.9 d
12	Luxmidigha	20.9 i
	CV (%)	0.44

4.1.2 Protein content

The protein content of 12 rice genotypes was varied widely. The highest protein content (8.86%) was found in SSB-3; followed by Magic-86 (8.78%), Luxmidigha (8.73%), Magic-72 (8.50%), LD-200-1-3-3-8 (8.41%), LD-200-1-3-3-5 (8.32%), LD-200-1-3-2-4 (8.28%), BRRI dhan49 (7.49%), Magic-10 (7.47%), RM-16(N)-10 (6.12%), RM-Kas-80(C)-1 (5.79%) and RM-40(C)-4-2-8 (5.04%). There were 9 groups (a, b, etc.) in which the means were not significantly different from one another

at 5% level of significance. These protein contents were similar to those reported by Magomya *et al*. (2014).

Table 13. Protein contents of 12 rice genotypes

Sl. No.	Name of the genotypes	Protein %
1	RM-16 (N)-10	6.12 g
2	RM- Kas-80(C)-1	5.79 h
3	LD-200-1-3-3-5	8.32 e
4	LD-200-1-3-3-8	8.41 d
5	LD-200-1-3-2-4	8.28 e
6	RM-40(C)-4-2-8	5.04 i
7	SSB-3	8.86 a
8	Magic-10	7.47 f
9	Magic-72	8.50 c
10	Magic-86	8.78 ab
11	BRRI dhan49	7.49 f
12	Luxmidigha	8.73 b
	CV (%)	0.59

4.2 Experiment 2: Molecular characterization of Rice using SSR markers

The analysis of genetic variation is a very important factor for the development of rice lines, which can be achieved through DNA profiling techniques that displays high amount of loci for large variability. The sample of rice cultivars collected from various origins were analyzed by using SSR markers which is basically a PCR based technique.

4.1.1 Allelic and loci variation within 12 rice genotypes

In this SSR marker based DNA fingerprinting technique, 12 rice genotypes were analyzed using 16 loci. Amplified microsatellite loci were analyzed to find out diversity. All 16 microsatellite loci had 6 alleles (mean). The bands obtained were compared to the band of BRRI dhan49 and Luxmidigha (Figure 2-11).

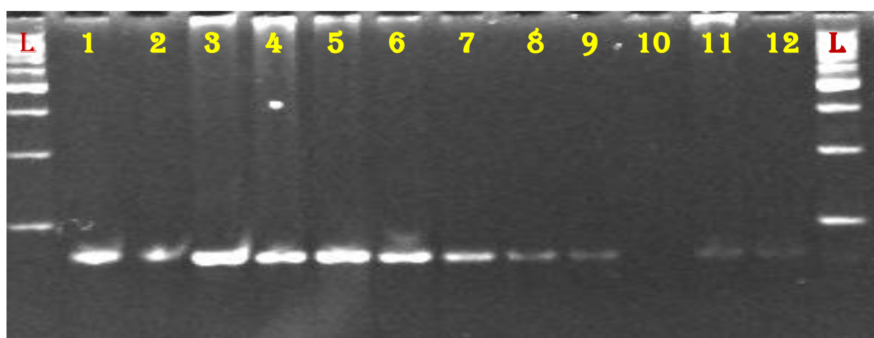


Figure 2. Microsatellite profiles of 12 rice genotypes at locus RM217

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

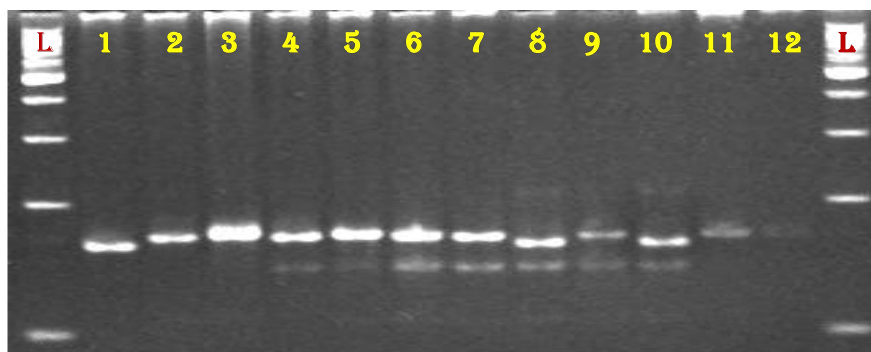


Figure 3. Microsatellite profiles of 12 rice genotypes at locus RM42

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

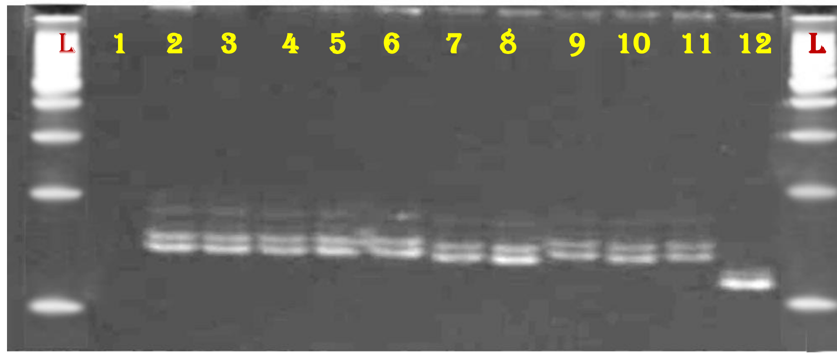


Figure 4. Microsatellite profiles of 12 rice genotypes at locus RM237

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

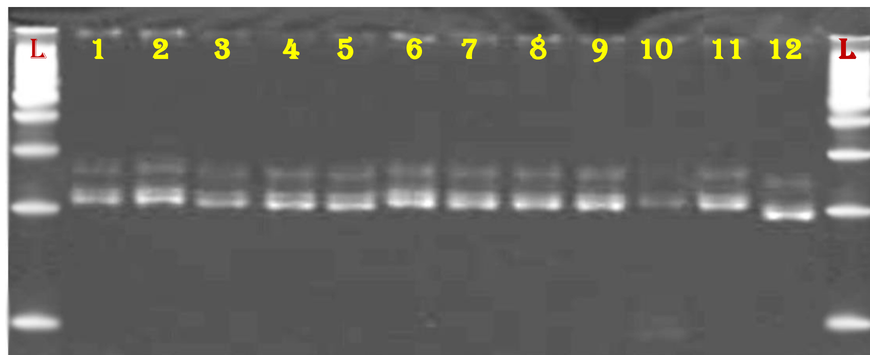


Figure 5. Microsatellite profiles of 12 rice genotypes at locus RM431

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

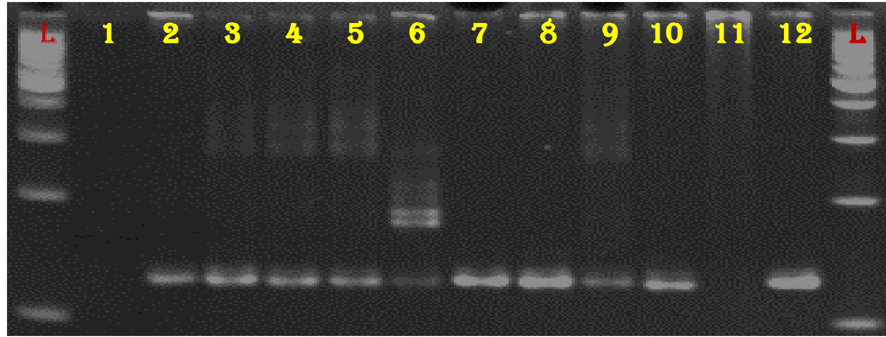


Figure 6. Microsatellite profiles of 12 rice genotypes at locus RM307

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

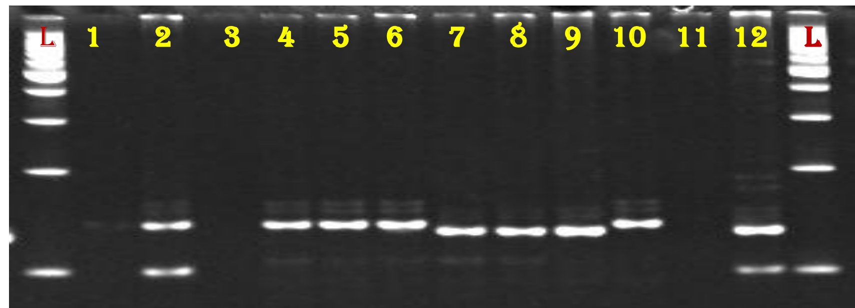


Figure 7. Microsatellite profiles of 12 rice genotypes at locus RM105

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

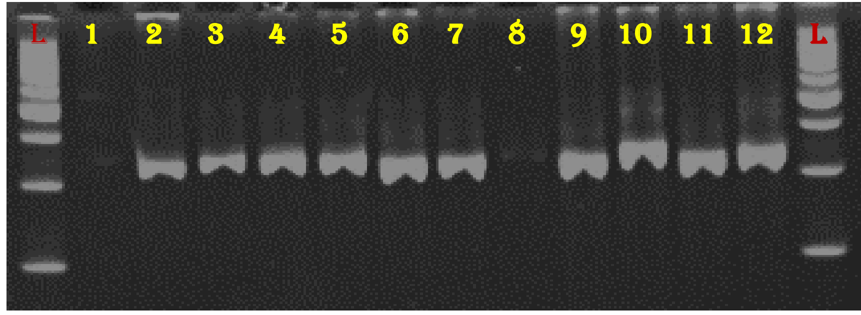


Figure 8. Microsatellite profiles of 12 rice genotypes at locus RM171

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

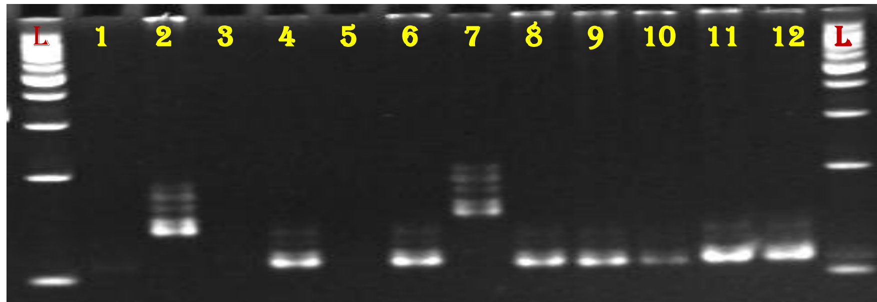


Figure 9. Microsatellite profiles of 12 rice genotypes at locus RM228

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

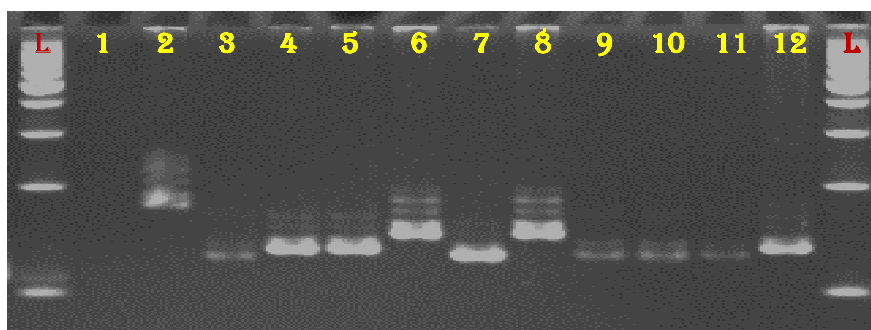


Figure 10. Microsatellite profiles of 12 rice genotypes at locus RM206

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

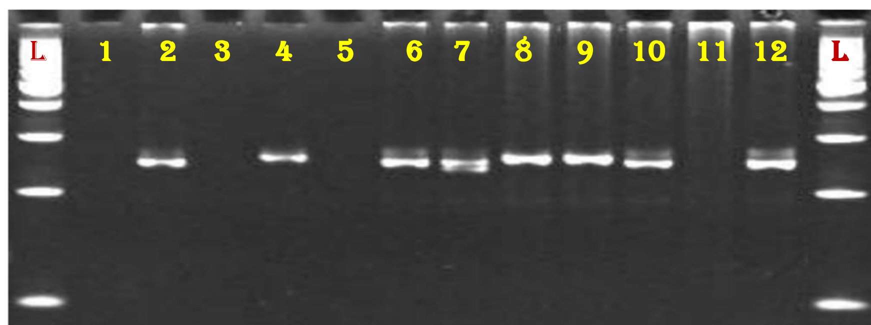


Figure 11. Microsatellite profiles of 12 rice genotypes at locus RM536

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

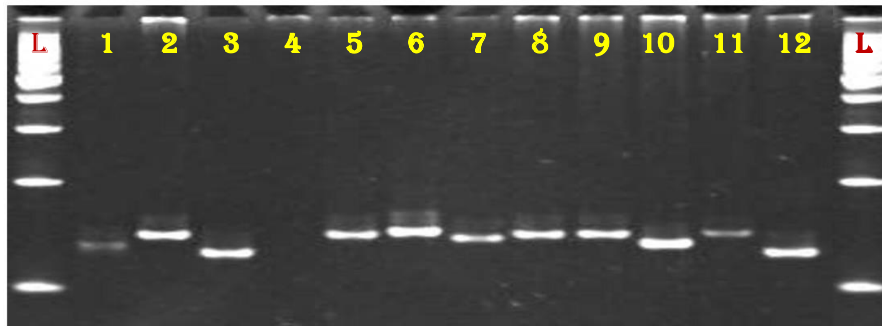


Figure 12. Microsatellite profiles of 12 rice genotypes at locus RM519

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

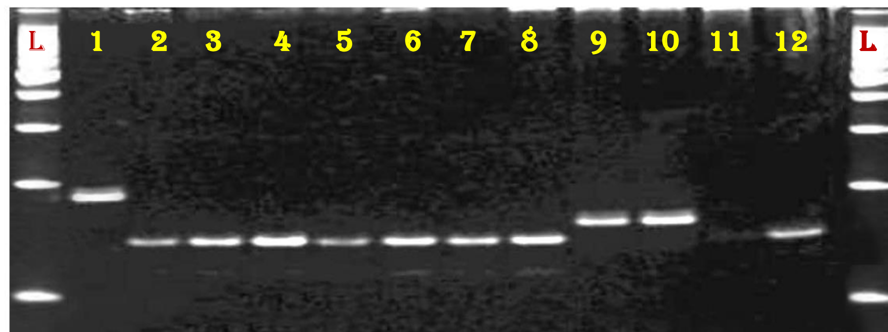


Figure 13. Microsatellite profiles of 12 rice genotypes at locus RM286

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

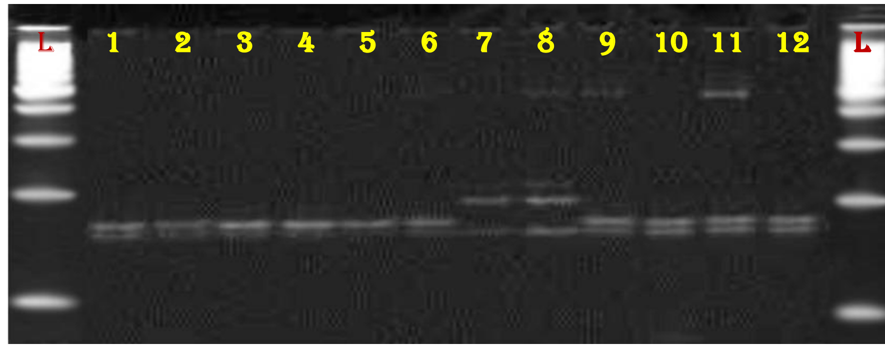


Figure 14. Microsatellite profiles of 12 rice genotypes at locus RM111

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

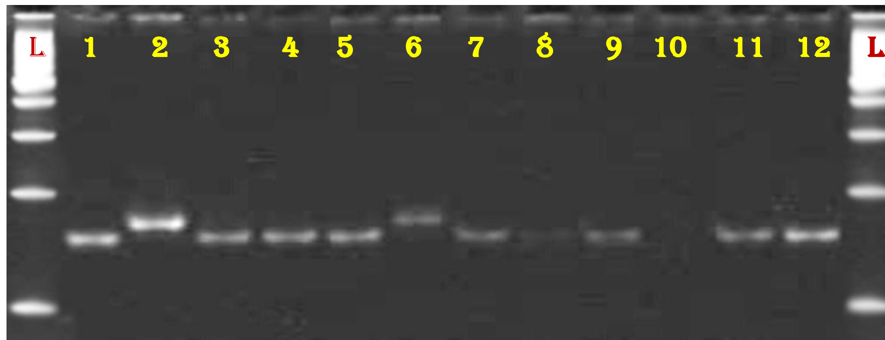


Figure 15. Microsatellite profiles of 12 rice genotypes at locus RM475

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

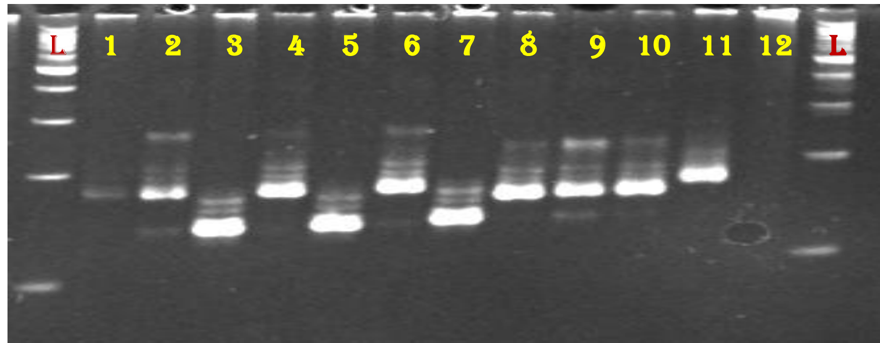


Figure 16. Microsatellite profiles of 12 rice genotypes at locus RM168

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

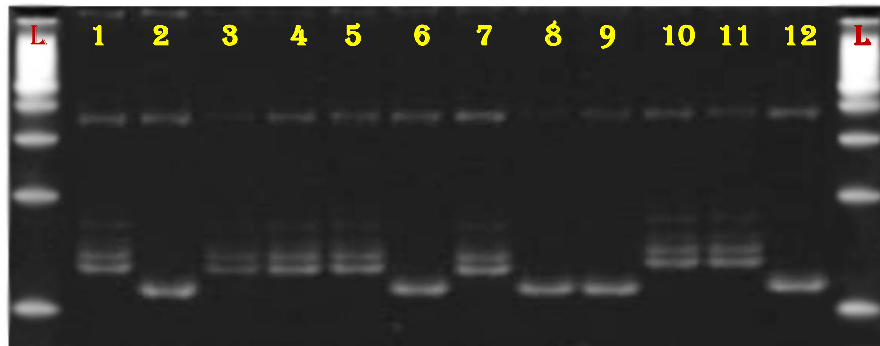


Figure 17. Microsatellite profiles of 12 rice genotypes at locus RM11

Legend: 1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha, **L**=25 bp ladder.

4.1.2 Size and frequency of alleles

In case of RM217, allele size was 145bp; RM42, RM237, RM431, RM307, RM105, RM171, RM228, RM206, RM536, RM519, RM286, RM111, RM475, RM168 and RM11 displayed the range 157-167bp, 120-130 bp,234-241bp, 170-175bp, 135-140bp, 320-330bp, 142-160bp, 147-154bp, 243-246bp, 118-127bp, 115-119bp, 124-128bp, 235-240bp, 117-120bp and 140-143bp, respectively. Almost similar result has been reported by Siddique et al. (2017).

4.1.3 Major allele

The allele with the highest frequency is termed as major allele or most common allele at each locus, which can be defined as major allele. The size of various major alleles at different loci ranges from 115bp (RM286) to 322bp (RM171). Among all the genotypes, on an average, 54% of them shared a common major allele ranging from 25% (RM105 and RM519) to 83% (RM307 and RM111) at each locus (Table 14). The result was more or less similar to the result reported by Siddique *et al*. (2016).

4.1.4 Gene diversity

The highest gene diversity (0.81) was observed in RM519 and the lowest (0.28) was in RM111, having an average diversity of 0.55. It was found that marker detecting the higher number of alleles showed higher gene diversity, on the other hand lower number of alleles expressed lower gene diversity (Table 14). Heenan *et al*. (2000) also found similar result of gene diversity.

4.1.5 PIC Value

Polymorphic information content (PIC) value is a reflection of allele diversity and frequency among the genotypes. PIC value of each marker can be determined on the basis of its allele. PIC varied significantly for all the studied SSR loci. In this study, the level of polymorphism among 12 rice genotypes was evaluated by calculating PIC values for each of the 16 SSR loci. The PIC values ranged from 0.24 to 0.78, having an average of 0.51 per locus. According to Jiang *et al*. (2010), a PIC value of greater than 0.5 is reflective of a good marker. The highest PIC value was 0.78 for RM519 and the lowest was 0.24 for RM111 in this study (Table 14).

Table 14. Data of major alleles, gene diversity and PIC value found among 12 rice genotypes for 16 SSR markers

Marker	Major Allele		Allele No.	Gene Diversity	PIC
	Size (bp)	Frequency (%)			
RM 42	163	41	5	0.65	0.58
RM 237	130	67	8	0.51	0.48
RM 431	238	67	8	0.49	0.42
RM 307	170	83	10	0.29	0.27
RM 105	137	25	3	0.78	0.74
RM 171	322	33	4	0.72	0.67
RM 228	142	58	7	0.58	0.53
RM 206	152	33	4	0.74	0.69
RM 536	246	41	5	0.65	0.58
RM 519	125	25	3	**0.81**	**0.78**
RM 286	115	75	9	0.40	0.36
RM 111	124	83	10	**0.28**	**0.24**
RM 475	235	75	9	0.40	0.36
RM 168	118	58	7	0.58	0.53
RM 11	143	58	7	0.49	0.37
Mean		**54**	**6**	**0.55**	**0.51**

4.1.6 Nei's (1973) genetic distance

The pair wise comparison values of Nei's (1973) genetic distance among 12 rice genotypes were calculated from combined data sets for 16 loci. The value ranged from 0.18 to 0.87 (Table 15). The highest Nei's genetic distance value 0.87 was found in RM-16(N)-10 vs RM-40(C)-4-2-8. The lowest genetic distance value 0.18 was found in LD-200-1-3-3-8 vs LD-200-1-3-2-4. The lowest value of pair wise difference among rice genotypes was likely due to their genetic relatedness. On the other hand, higher value of pair-wise difference was observed among those rice lines developed from genetically distal parental. More or less similar result was found by Lakhanpaul *et al*. (2000).

Table 15. Summary of genetic distance values among 12 rice genotypes using 16 SSR markers

Gen.*	P1	P10	P11	P12	P2	P3	P4	P5	P6	P7	P8	P9
P1	0.00											
P10	0.75	0.00										
P11	0.56	0.56	0.00									
P12	0.81	0.62	0.56	0.00								
P2	0.81	0.81	0.68	0.56	0.00							
P3	0.50	0.68	0.31	0.50	0.50	0.00						
P4	0.75	0.62	0.43	0.50	0.43	0.43	0.00					
P5	0.62	0.68	0.43	0.56	0.43	0.31	**0.18**	0.00				
P6	**0.87**	0.75	0.56	0.62	0.37	0.68	0.37	0.43	0.00			
P7	0.75	0.68	0.50	0.62	0.68	0.62	0.43	0.43	0.62	0.00		
P8	0.75	0.62	0.62	0.56	0.62	0.68	0.50	0.62	0.50	0.50	0.00	
P9	0.68	0.50	0.43	0.62	0.62	0.62	0.50	0.62	0.50	0.43	0.37	0.00

Here, P1= RM-16 (N)-10, P2= RM- Kas-80(C)-1, P3= LD-200-1-3-3-5, P4= LD-200-1-3-3-8, P5= LD-200-1-3-2-4, P6= RM-40(C)-4-2-8, P7= SSB-3, P8= Magic-10, P9= Magic-72, P10= Magic-86, P11= BRRI dhan49, P12= Luxmidigha.

Gen.*= Genotypes used in this study.

4.1.7 Genetic similarity analysis using UPGMA

Dendogram based on Nei's (1973) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated differentiation of the 12 rice genotypes by 16 markers. All the 12 rice genotypes could be easily distinguished. The UPGMA cluster analysis led to the grouping of 12 rice genotypes in six major clusters at 70% cut off (Figure 18).

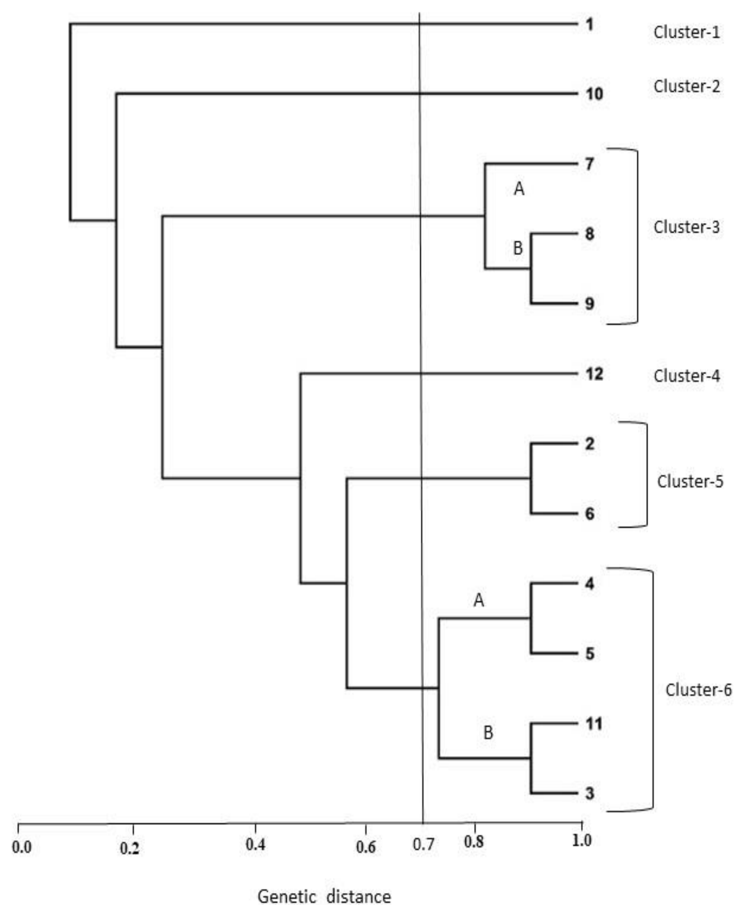


Figure 18. UPGMA Dendogram based on Nei's Genetic Distance according to SSR analysis

Legend:1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Luxmidigha.

Cluster-1, Cluster-2 and Cluster-4 each considered only one genotype and they were RM-16 (N)-10, Magic-86 and Luxmidigha respectively, which differed from other genotypes in amylose and protein content.

Cluster-3 consists of 3 genotypes having two sub clusters (3A and 3B). In 3A, SSB-3 and in 3B, Magic-10 and Magic-72 were clustered. These genotypes found to contain moderate amylose and protein content.

In cluster-5, 2 genotypes namely RM- Kas-80(C)-1 and RM-40(C)-4-2-8 clustered together and they all were grouped in relatively low protein rice.

Cluster-6 considered two sub clusters (6A and 6B). Sub cluster 6A comprised of 2 genotypes, LD-200-1-3-3-8 and LD-200-1-3-2-4, those were found as moderate amylose and high protein content. Sub cluster 6B contains BRRI dhan49 and LD-200-1-3-3-5, those were identified as moderate amylose containing rice.

Based on the above result, it can be concluded that the genotypes showing diverse ranges of amylose and protein content tend to cluster together in the dendogram with some exceptions. Siddique *et al*. (2016) also observed six clusters among 20 rice genotypes of Bangladesh using 30 SSR markers.

CHAPTER 5

SUMMARY AND CONCLUSION

The study was carried out in the laboratory of Biotechnology Division and the laboratory of Soil Science Division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensigh and the laboratory of Biochemistry Department, Bangladesh Agricultural University (BAU), Mymensigh. Twelve rice (*Oryza sativa*) genotypes (10 advanced lines, 1 released variety and 1 landrace) were used in this study for biochemical and molecular characterization. The main objective of this investigation was to estimate amylose and protein content as well as to characterize and evaluate the genetic diversity of 10 mutant rice lines with two released variety using molecular markers. Eventually this study will pave the way of better utilization of these genotypes in further breeding program.

Amylose content is considered to be the most important factor influencing cooking and processing characteristics of rice. Rice breeders invariably are concerned with, and routinely select for amylose content in new lines. Among 12 rice genotypes, the amylose content varied from 20.3% to 25.6 %. The highest amylose content (25.6%) was found in Magic-86; followed by RM-40(C)-4-2-8 (25.1%), Magic-10 (24.4%), BRRI dhan49 (23.9%), Magic-72 (23.9%), LD-200-1-3-3-8 (23.3%), SSB-3 (22.7%), RM-Kas-80(C)-1 (22.1%), LD-200-1-3-2-4 (22.1%), LD-200-1-3-3-5 (21.5%), Luxmidigha (20.9%) and RM-16(N)-10 (20.3%).

Rice protein is a very important source of nutrition and energy for 50 % of the world's population. Therefore, detection of protein content in rice has received progressively increasing attention to the rice breeders. The protein content among 12 rice genotypes was varied widely. The highest protein content (8.86%) was found in SSB-3; followed by Magic-86 (8.78%), Luxmidigha (8.73%), Magic-72 (8.50%), LD-200-1-3-3-8 (8.41%), LD-200-1-3-3-5 (8.32%), LD-200-1-3-2-4 (8.28%), BRRI dhan49 (7.49%), Magic-10 (7.47%), RM-16(N)-10 (6.12%), RM-Kas-80(C)-1 (5.79%) and RM-40(C)-4-2-8 (5.04%).

In this study, genetic diversity and relationship among 12 rice genotypes at molecular level was addressed using SSR markers. 16 SSR markers were used those were RM

217, RM 42, RM 237, RM 431, RM 307, RM 105, RM 171, RM 228, RM 206, RM 536, RM 519, RM 286, RM 111, RM 475, RM 168 and RM 11. The size of various major alleles at different loci ranges from 115bp (RM286) to 322bp (RM171). Among all the genotypes, on an average, 54% of them shared a common major allele ranging from 25% (RM105 and RM519) to 83% (RM307 and RM111) at each locus. The highest gene diversity (0.81) was observed in RM519 and the lowest (0.28) was in RM111, having an average diversity of 0.55. the level of polymorphism among 12 rice genotypes was evaluated by calculating PIC values for each of the 16 SSR loci. The PIC values ranged from 0.24 to 0.78, having an average of 0.51 per locus. The pair wise comparison values of Nei's (1973) genetic distance among 12 rice genotypes were calculated from combined data sets for 16 loci. The value ranged from 0.18 to 0.87 (Table 15). The highest Nei's genetic distance value 0.87 was found in RM-16(N)-10 vs RM-40(C)-4-2-8. The lowest genetic distance value 0.18 was found in LD-200-1-3-3-8 vs LD-200-1-3-2-4. A dendogram was constructed based on Nei's similarity coefficient using UPGMA method to group the selected genotypes and observe relationship among them. Six major clusters were formed at 0.7 cut off similarity coefficient. The highest number of genotypes found in cluster-6 (4 genotypes) and the lowest number of genotypes were observed in cluster-1, cluster-2 and cluster-4 (each contained 1 genotype). Cluster-3 and cluster-5 contained 3 and 2 genotypes respectively. The genotypes showing diverse ranges of amylose and protein content condition during biochemical analysis, tend to cluster together in the dendogram with some exceptions.

Based on the results found in this study, it can be concluded that, Magic-86, SSB-3 and Magic-10 can be used to develop better quality rice varieties. On the other hand, LD-200-1-3-3-8, LD-200-1-3-2-4 and RM-40(C)- 4-2-8 can be used to develop promising deep water rice varieties. Field trial of the selected genotypes may be examined to evaluate their production. Molecular characterization of the selected genotypes using larger number of SSR markers may help breeders to prepare a complete genetic map. In conclusion, all the other information can be used to develop potential rice varieties in the future.

CHAPTER 6

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