“MOLECULAR DIVERSITY ANALYSIS OF AMAN RICE GERMPLASM USING SSR MARKERS”

MD. ROKIBUL HASAN

DEPARTMENT OF BIOTECHNOLOGY

SHER-E-BANGLA AGRICULTURAL UNIVERSITY

DHAKA-1207

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MOLECULAR DIVERSITY ANALYSIS OF AMAN RICE GERMPLASM USING SSR MARKERS

BY

MD. ROKIBUL HASAN

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Approved by:

Dr. Mohammad Zahidul Islam
Senior Scientific Officer
And Head
Bangladesh Rice Research Institute
Regional Station, Gopalganj
Supervisor

Dr. Md. Ekramul Hoque
Professor
Department of Biotechnology
Sher-e-Bangla Agricultural University
Dhaka-1207
Co-Supervisor

Dr. Mohammad Nazrul Islam
Chairman
Examination Committee
Department of Biotechnology
Sher-e-Bangla Agricultural University
Dhaka-1207

DEPARTMENT OF BIOTECHNOLOGY
Sher-e-Bangla Agricultural University
Sher-e -Bangla Nagar, Dhaka -1207

CERTIFICATE

*This is to certify that the thesis entitled in "**MOLECULAR DIVERSITY ANALYSIS OF AMAN RICE GERMPLASM USING SSR MARKERS**" submitted to Faculty of Agriculture Sher-e-Bangla Agricultural University, Dhaka, in 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 "**MD. ROKIBUL HASAN**", Registration no.**19-10112** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

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

Dated: June, 2021
Dhaka, Bangladesh

Dr. Mohammad Zahidul Islam
Senior Scientific Officer
And Head
Bangladesh Rice Research Institute
Regional Station, Gopalganj

Supervisor

DEDICATED TO

MY

BELOVED PARENTS

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SAU, Dhaka

The Author

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ABSTRACT

An experiment was carried out at the laboratory of Genetic Resources and Seed Division (GRSD), Bangladesh Rice Research Institute (BRRI), Joydebpur, Gazipur, during the period of January 2020 to August 2020 for genetic diversity analysis within 18 Aman rice germplasm. Eleven rice genome specific SSR Primers were used for molecular diversity analysis. All of the eleven primers were capable to produce polymorphic bands. A maximum (6) number of bands was observed in SSR primers RM206. Thirty Eight alleles were detected with an average of 3.45 alleles. The Polymorphic Information Content (PIC) of SSR markers ranged from 0.10 (RM455) to 0.75 (RM206) with an average value of PIC = 0.42. Gene diversity ranges from 0.10 (RM455) to 0.78 (RM206) with a mean value of 0.45. The RM206 marker can be considered as the best marker among the studied markers for 18 Aman rice germplasm. Four primers showed high value of genetic diversity, five primers showed moderate diversity and two primers showed low diversity value on the basis of PIC value. Nei’s genetic distance (D) among 18 rice germplasm was computed from combined data for the 11 primers, ranged from 0.09 to 0.82 with an average of 0.46. Dendrogram based on Nei’s genetic distance using Unweighted Pair Group Method of Arithmetic Mean (UPGMA) indicated the segregation of 18 genotypes into five main clusters. This result could be further used for molecular diversity analysis and identify parent for the development of high yielding variety of rice.

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ABBREVIATIONS AND ACRONYMS

ABBREVIATION	FULL WORD
BRRI	Bangladesh Rice Research Institute
BBS	Bangladesh Bureau of Statistics
Bp	Base pair
CTAB	Cetyl Trimethyl Ammonium Bromide
ddH_2O	Double Distilled Water
dH_2O	Distilled Water
DNA	De-oxy ribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
et al.	And others
etc.	Etcetera
FAO	Food and Agriculture Organization
G	Gram
G	Genotype
GD	Genetic Distance
g/l	Gram per Liter
Ml	Mili liter
NaCl	Sodium chloride

ABBREVIATION	FULL WORD
Na_2EDTA	Sodium salt of ferric Ethylene Diamine Tetra Acetic Acid
PCR	Polymerase Chain Reaction
pH	Negative Logarithm of Hydrogen ion concentration
SSR	Simple Sequence Repeats
SNP	Single Nucleotide Polymorphism
SDS	Sodium Dodecyle Sulphate
Taq	Thermophilus aquaticus
TBE	Tris Boric Acid
TE	Tris-EDTA
T	Tons
UPGMA	Unweighted Pair Group of Arithmetic Mean
UV	Ultraviolet
V	Volt
Viz	Namely

CHAPTER-I

INTRODUCTION

Rice (*Oryza sativa L.*) is a self-pollinated cereal crop belonging to the family of Gramineae, subfamily Oryzoidae and having 2n=24 chromosomal number (Hooker, 1979). Rice is a monocot annual plant. It has been grown in Asia since ancient past times and for generations to generation farmers have maintained thousands of different varieties (Jackson, 1995). It is one of the most important staple food for almost 50% of the global population, it is grown exclusively for human consumption (Garris *et al.* 2005; Ramkumar *et al.* 2010). Some major rice producing countries are China, India, Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Pakistan, Philippines. Among them, Bangladesh has ranked 4th position in world rice production (FAO, 2016). Rice provides near about 80% of the food necessity of the common people of Bangladesh despite the shortfall of the annual production of 2 to 4 million metric tons. (Jalaluddin *et al.* 2007). According to BBS, Yearbook, (2018) a total of 13,993 metric tons Aman rice was grown in 14,035 acres of land. Almost 87% of the world total rice production was accounted by Asian farmers. Now-a-days, world 90% of rice is produced in Asia in an area of almost 150 million hectares.

Almost 80% of planet nutrition is supplied by rice and it contributes 50% of agricultural income in Asia. In Bangladesh, more than 70% of the rural people engages with rice and it is central to agriculture and the national economy (Anonymous 2002). This crop provides about 75% calories and 55% protein content of the average daily diet for the people of Bangladesh (Bhuiyan *et al.* 2002).

Rice is a very good source of Vitamin B1, thiamin, riboflavin, fiber, and iron. Besides these, it provides 20% of the world's dietary energy supply. Cooked, un-enriched, white, long-grained rice is composed of 68% water, 28% carbohydrates 3% protein and negligible fat. The health benefits of rice are providing instant energy, regulating bowel movements, maintain the level of blood sugar and mitigating the aging process.

This is very alarming that the population of Bangladesh is increasing at a terrible rate and the cultivable land of Bangladesh is decreasing day by day due to urbanization and industrialization, as a result, shortage of food is occuring. Every year the nation is still adding about 2.3 million

mouth to its total of 160 million people (Momin and Husain, 2009). In 2030 and 2050 world population is anticipated to reach 8.6 billion and 9.8 billion. A total of 83 million people is expected to be added in every year (UN DESA, 2017). As a result, world food production must be increased to 70 percent more to feed this huge sum/amount of the population (Hunter, 2017). An additional 400,000 metric tons of rice are required/ indispensable every year. So, the highest priority should be given on rice production and cultivation.

In Bangladesh, rice is mostly grown in three seasons, such as Aus, Aman and Boro. Among these seasons, Aman rice occupied the highest area coverage (48.74 % of total rice cropped area) (Anonymous, 2009). Another report, Aman rice covers about 50.92% of the rice areas of Bangladesh of which modern Aman varieties cover 60% (BBS, 2005). During mid-June to mid-July, the seedlings of the Transplanted Aman (T. *Aman*) are raised. Transplanting starts from mid-July and lasts up to October. During mid-October to mid-December harvesting is done. The highest varietal diversity is observed in the landraces of the *Aman* rice varieties. Photoperiod sensitivity, seed dormancy and adaptation to cold temperature at the reproductive phase are the major features of the Aman rice.

More than 1,13,000 gemplasm can be found in the world's largest gene bank for rice, the International Rice Research Institute (IRRI) located in the Philippines. Until now, BRRI has been collected and preserved in the genebank near about 8,700 varieties from indigenous and exotic sources. Out of these, nearly 8000 varieties have been registered in the gene bank (Khalequzzaman *et al.* 2012). BRRI Genebank is enriched with about 4,990 rice accessions for Aman. 1,670 rice accessions for Boro and 1,350 rice accessions Aus season. They are successfully adopted in different parts of the country, some of which have very nice quality, fineness, aroma, taste and high protein content (Dutta *et al.* 1998).

There is huge genetic variability available within existing varieties and wild relatives of rice providing wide scope for further improvement (Chakravarthi and Naravaneni 2006; Rahman *et al*. 2006, 2007a, b, c). For the improvement of an effective breeding program to improve high yielding Aman rice varieties, morphological and molecular characterization are very important. Otherwise, genotypes of rice have already been lost from the farmer's field. Several genetic diversity studies have been successfully utilized in different crop species based on quantitative and qualitative traits

to select genetically distant parents for hybridization (Bedoya *et al.* 2017; Islam *et al.* 2016; Malek *et al.* 2014; Khodadadi *et al.* 2014). Genetic diversity is mainly evaluated through morphological traits. But it has some limitations in the context of absorbing time, cost, space and environmental factors. For this reason, it also misguides the scientist to recognize a particular genotype.

To avoid the varietal loss, recently advent molecular characterization along with morphological traits would be the best solution. Molecular markers can reveal abundant differences among genotypes at the DNA level, providing a more direct, reliable and efficient tool for germplasm characterization, screening and evaluation in contrast to morphological traits. To avoid these drawbacks DNA markers are widely used. Markers are technically easy, recognizable, time saving, highly informative, reliable and require a small amount of DNA and independence from effects related to environmental conditions of the plant.

For more accurate and reliable characterization and discrimination of rice genotypes, the application of DNA markers has been suggested (Karkousis *et al.* 2003). There is many countries in the world that have characterized their indigenous different crop landraces at both the molecular level and morphological level. This has been accomplished for keeping their crop identity and for searching new genes for further crop development as well as breeding programme.

Molecular characterization and genetic diversity analysis of rice are very important for high yielding rice variety development and other improvement programs. Molecular markers are powerful tools to detect genetic variation and genetic relationship within and among species. DNA markers are unmasking new genes for the improvement of crop varieties (Causse *et al.* 1994).

Molecular markers have been proved as the most efficient and effective tool for characterization, conservation and management of germplasm. Among all the PCR based DNA markers, microsatellites or SSRs (simple sequence repeats) are highly preferred ones. The SSRs markers are most suitable for rice because of their reproducibility, multi-allelic nature, hyper-variability, co-dominant inheritance, relative abundance, and genome-wide coverage (Powell *et al.* 1996). Due to co-dominance, abundance, highly reproducibility and polymorphism, SSRs are an excellent molecular marker for various genetic analyses of rice.

The SSR markers are especially appropriate for evaluating genetic diversity and relationships among plant species, populations, or individuals (Kostova *et al.* 2006 and Tu *et al.* 2007), studying rice germplasm for either conservation or utilization (Sharma *et al.* 2007); marker-assisted selection breeding (Perez-Sackett *et al.* 2011 and Rani and Adilakshmi 2011); cultivar identification; hybrid purity analysis and gene mapping studies (Weising *et al.* 1997; Altaf-Khan *et al.* 2006; Rajendrakumar *et al.* 2009 and Sarao *et al*. 2010). In Bangladesh, for appropiate genetic diversity of rice (Oryza sativa L.) (Shahriar *et al.*2014); For molecular screening and allelic diversity of major riceblast resistant genes (Yasmin *et al.* 2021), Agro-morphological and physico-chemical characterizations (Ahmed *et al.*2016), molecular characterizations and genetic diversity (Khalequzzaman *et al.*2016). SSR markers have been widely applied in the genetic diversity analysis, genotypic identification and population structure estimation in several rice genetic studies (Salgotra *et al.* 2015).

Several research strategy has been taken from Genetic Resources and Seed Division (GRSD), Bangladesh Rice Research Institute (BRRI), Gazipur for identification and characterization of most divergent germplasm collected at their Gene Bank. The objective is to use the polymorphic genotype for rice improvement program. Morpho-molecular diversity, DNA fingerprint and DNA profiling document will be the baseline for the selection of most genetic diverse germplasm. Therefore, from the large collection of Aman germplasm is different accession rice, local genotypes were used for the present study.

Diversity analysis of germplasm will provide the information to plant breeders for helping them select the parent and best variety for the varietal improvement program. Therefore, the research emphasis has been given on to characterize and to evaluate the genetic diversity of 18 Bangladeshi Aman rice germplasms using molecular markers.

Objectives:

- Polymorphism study among the different Aman rice germplasms.
- DNA Profiling of different Aman germplasms.
- Genetic diversity analysis among different germplasms.
- To establish dendrogram for classifying germplasms on the basis of their genetic distances.

CHAPTER-II

REVIEW OF LITERATURE

The literature is relevant to the present study on rice and their improved varieties of Bangladesh as well as in whole world are reviewed in this chapter under the following headings:

Ahmed *et al*. (2016) evaluated a total of 350 alleles varied from 3 (RM277) to 14 (RM21) with an average of 7.8 per locus.45 microsatellite loci were detected across the 31 rice accessions. The gene diversity ranged from 0.48 to 0.90 with an average of 0.77.Again, the polymorphism information content values from 0.44 (RM133) to 0.89 (RM206) with an average of 0.74.

Ahmed *et al*. (2018) conducted an experiment about 54 T. Aman rice landraces for 11 morphological and yield contributing characters at BRRI during T. Aman seasons. The highest variation was observed for yield per hill with 53.6% CV, followed by 1000 grain weight (29.9), number of effective tillers per hill (22.8), culm diameter (18.8), leaf width (18.4), leaf length (18.1) and days to maturity (6.7) respectively. The prolonged leaf was recorded as 82.2 cm and that of culm diameter as 7.57mm, grain length as 7.2 mm and LB ratio as 3.48. The shortest days to maturity (110 days) was observed in Kajal lata and plant height (86.6 cm) in Haijam. Indursail possessed the longest panicle (31.6 cm) and the highest yield per hill (24.3 g). All the germplasm were grouped into 15 clusters using Mahalonobis D2 statistic. The maximum numbers of germplasm (7) were grouped into the clusters IV with VI, whereas clusters III and XIII contained the minimum (1).The germplasm under clusters VIII may be selected for crossing with the germplasm from clusters XIII, IX, II, XV and I for developing high yielding varieties with improved panicle length, effective tillers per hill, growth duration and grain type.

Aljumaili *et al.* (2018) carried out an experiment and evaluated genetic diversity of 50 aromatic rice accessions from three regions (Peninsular Malaysia, Sabah, and Sarawak) with 3 released varieties as a control using the 32 SSR markers. Genetic diversity index among the three population ranged from 0.25 in control to 0.98 in Sabah population. The mean numbers of effective alleles and Shannon's information index as 0.36 and 64.90%, respectively. Nei's gene diversity index was 0.36. The dendrogram based on UPGMA and Nei's genetic distance categorized the 53 rice accessions into 10 clusters. Analysis of molecular variance (ANOVA) revealed that 89% of the

total variation noticed in this germplasm came from within the populations, while 11% of the variation arised among the populations. These results reflects the maximum genetic differentiation existing in this aromatic rice germplasm.

Bajracharya *et al.* (2004) carry out a diversified study on a collection of landrace accessions from three agro-ecozones using SSR markers to understand and evaluate the value and extent of genetic diversity prevalent in landraces. 70 rice accessions (21 from Jumla- high hill, 24 from Kaski-mid-hill and 25 from Bara-plain) were analysed for genetic diversity at 39 SSR marker loci. The patterns of genetic diversity revealed by SSR polymorphisms shows variations between the study sites and among the varieties. Landraces from Kaski and Bara showed a high genetic variation with 0.34 average Nei's gene diversity (PIC) and 0.45 genetic dissimilarity coefficient and 88% of the markers were polymorphic.

Behera and Joshi *et al.* (2006) evaluated that the genetic diversity of 38 traditional indigenous rice cultivars by using 12 microsatellite primer pairs. Total 32 different reproducible bands were amplified among them 26 (81.25%) were polymorphic. The number of bands per primer ranged from 1 to 6 with an average of 2.6 bands per primer. PIC value ranged from 0.00 to 0.83. A dendogram based on cluster analysis by microsatellite polymorphism categorized all the 38 rice genotypes into 3 major clusters.

Chen *et al.* (2017) carriedout an experiment with 30 polymorphic SSR markers to evaluate the genetic diversity and molecular fingerprints of 53 rice genotypes of *O. sativa, O. glaberrima,* and NERICA. In total, 180 alleles were identified with average PIC value 0.638 and Shannon's information 1.390. Population structure and neighbor-joining phylogenetic tree indicate that 53 genotypes grouped into three distinct subpopulations conforming to the original three groups, excluding three varieties (IR66417, WAB450-4, MZCD74). NERICA showed a smaller genetic distance from *O. sativa* genotypes (0.774) than from *O. glaberrima* genotypes (0.889).

Chakravarthi and Naravaneni *et al.* (2006) conducted an experiment and investigated the genetic diversity and DNA fingerprinting of 15 elite rice genotypes by using 30 SSR primers on chromosome numbers 7-12. All the primers exhibits distinct polymorphism among the cultivars studied showing the robust nature of microsatellites in revealing polymorphism. Cluster analysis classified the rice genotypes into 10 different groups. The information gained from the DNA 13

fingerprinting studies helped to distinctly identify and characterize nine varieties using 18 different RM primers.

Ghneim *et al. (*2008) evaluated the genetic diversity in 11 Venezuelan rice cultivars by using SSR markers, the results indicated that all the 48 SSRs were polymorphic across the 11 genotypes and a total of 203 alleles were detected.

Gupta *et al.* (2013) conducted an experiment on characterization of 53 accessions of rice germplasm from IGKV, Raipur, Chhattisgarh germplasm. These germplasm accessions were evaluated for 14 morphological and seventeen agronomical characters. The specific genotypes S: 663, K: 1514, J: 311 were identified for agronomic characteristics. These may be used in hybridization programme to achieve desired segregants for higher yield.

Halder *et al.* (2016) conducted an experiment on about the genetic diversity of 12 Bangladeshi local Boro rice (*Oryza sativa* L.) germplasm using morphological traits and molecular markers. Eight (8) morphological traits (*viz.,* days to 50 percent flowering, growth duration, plant height, filled grain/panicle, 1000 grain weight and grain yield) and eight Simple Sequence Repeat (SSR) markers were used for this analysis. The plant morphological traits presented more variation among the genotypes tested. A set of eight (8) SSR primer pairs was applied for molecular characterization resulting forty-nine (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.

Hien *et al.* (2007) investigated that the Genetic diversity of morphological responses and the relationships among Asia aromatic rice (*Oryza sativa* L.*)* cultivars. Characterization for 22 morphological characters with 101 morphometric descriptors was carried out and most traits were polymorphic except to ligule color. Grain size, grain shape, culm strength, plant height and secondary branching contributed the highest mean diversity indices.

Hossain *et al.* (2007) directed an experiment by using thirty (30) microsatellite markers across 21 rice genotypes for their characterization and discrimination. The number of alleles per locus ranged

from three (RM165, RM219, RM248, RM463, RM470 and RM517) to nine (RM223), with an average of 4.53 alleles across the 30 loci obtained. (PIC) values ranged from 0.30 (RM219) to 0.84 (RM223) in all 30 loci. RM223 was found as the best marker. The frequency of the most common allele at each locus ranged from 24% (RM223 and RM334) to 81% (RM219The pair wise genetic dissimilarity coefficients indicated that the highest genetic distance was obtained between Thakurbhog and Supper Basmoti (0.81) as well as between Benaful and Keora (0.81). Basmati (Basmoti D, Super Basmati, Basmati 370) and Kalijira (Kalijira 11, 12, 13, 14) genotypes had close similarity among them but showed wide dissimilarity with other local genotypes. Being grouped into distant clusters, SupperBasmoti, Thakurbhog, Keora, and Benaful could be utilized as potential parents for the improvement of fine grain aromatic rice varieties. Genotypes Kolgochi and Buchi (having zero dissimilairty) might be possessed same genetic background.

Islam *et al.* (2018) found that the agro-morphological traits of 113 accessions of aromatic germplasm (*Oryza sativa* L.) based on qualitative agro-morphological descriptors. No duplicates were identified among the studied accessions for qualitative traits in the cluster analysis, which means there is a high diversity among the accessions for these traits. Following UPGMA cluster analysis, 113 accessions of aromatic germplasm formed ten distinct clusters. The highest numbers of germplasm (96) were found in cluster IXd, 2 were found in cluster III, IV and VI, 3 were found in IXc and the lowest number of germplasm (1) in cluster I, II,V, VII, VIII, IXa, IXb and X, respectively. Germplasm namely Begun bichi, Elai, Chinigura, Basmati 370, Ranisalut, Sakkorkhora, Jirakatari, Raduni Pagal, Kalijira (long grain), Black TAPL-554, Kalgochi, BRRI dhan34, BRRI dhan50, Badshabhog-2, Tulsimala-2, Kataribhog, BU dhan2R , Sakkorkhana, Maloti, Bashful could be used for further improvement for incorporating aroma to the high yielding varieties.

Islam *et al.* (2018) studied about 36 similar named aromatic rice landraces of Bangladesh to assess the genetic variation for the agro-morphological traits. The landraces were grouped into four clusters. The inter-cluster distances were higher than intra-cluster distances indicating wider genetic diversity among the landraces of different clusters. The intra-cluster distances were lower in all the cases reflecting homogeneity of the landraces within the clusters. The principal component analysis showed that the first five components with vector values > 1 contributed 76.51% of the total variations. The highest number (13) of landraces was constellated in cluster III

and the lowest (3) in cluster I. The intra- and inter-cluster distances were the maximum in cluster I (0.63) and between clusters I and IV (17.13), respectively and the minimum in cluster II (0.03) and between clusters III and IV (3.86), respectively. The maximum value of inter cluster distance indicated that the landraces belonging to cluster IV were far diverged from those of cluster I. Besides, the cluster mean revealed that the crosses between the genotypes of cluster I with those of clusters IV would exhibit high heterosis for maximum good characters. Hence, yield, grain breadth, days to maturity, culm diameter, ligule length had maximum contribution towards genetic divergence.

Jain *et al.* (2004) investigated the genetic relationships among 69 Indian aromatic and quality rice (*O. sativa*) germplasm using 30 fluorescently labelled rice microsatellite markers and reported that a total of 235 alleles were detected at the 30 SSR loci, 62 (26.4%) of which were present only in Basmati and other scented/quality rice germplasm accessions. The number of alleles per locus ranged from three to 22, (PIC) values ranged from 0.2 to 0.9, with an average of 0.6 and the size range between the smallest and the largest allele for a given microsatellite locus varied between 3bp and 68bp.

Kibria *et al.* (2009) studied the genetic diversity among aromatic rice genotypes by using SSR and RAPD markers through marker aided selection (MAS).Three SSR primers (RM223, RM342A and RM515) showed forty six bands among the genotypes and the average number of effective allele ranged from 1.78 to 2.49. The marker RM223 show the maximum polymorphism (66.67%).

Lapitan *et al.* (2007) conducted an experiment on 24 rice cultivars by using 164 SSR markers. A total of 890 alleles were detected by 151 polymorphic markers with an average of 5.89 per locus. Out of these markers, a total of 147 rare alleles and 89 generated. Polymorphism information content (PIC) values of the markers ranged from 0.18 (RM420) to 0.91 (RM473B) with an average of 0.68 per marker. The UPGMA dendrogram divided the cultivars into 3 clusters.

Mamunur *et al.* (2012) worked on 34 microsatellite markers across 21 types of rice to characterize and discriminate among different varieties. The number of alleles per locus ranged from 2 (two) to 11(eleven), with an average of 4.18 alleles across 34 loci. A total of 57 rare alleles were detected at 24 loci, whereas 42 unique alleles were detected at 20 loci. The results revealed that 14 rice varieties produced unique alleles that could be used for identification, molecular characterization,

and DNA fingerprinting of these varieties. Polymorphic information content (PIC) values ranged from 0.157 to 0.838, with an average of 0.488, which revealed that much variation was present among the studied varieties. The PIC values revealed that RM401 might be the best marker for identification and diversity estimation of rice varieties, followed by RM566, RM3428, RM463, and RM8094 markers. The UPGMA cluster dendrogram created in this study identified five clusters with a similarity coefficient of 0.50. The findings of this study should be useful for varietal identification and could help in background selection in backcross breeding programs.

Mehla and Kumar (2008) investigated that various morphological characters responsible for identification of rice cultivars, they concluded that there exists large variation among the rice cultivars in respect to morphological characters viz. awn length, panicle length, leaf blade colour and leaf sheath colour, node base colour, awning, distribution of awns, stigma colour, anthocyanin colouration of stem nodes and internodes, hence, these characters can be used for identification of rice cultivars.

Nachimuthu *et al*. (2015) studied the population structure and genetic diversity among 192 diverse rice germplasm lines using 61 SSR markers. The number of alleles per loci varied from 2 to 7 with an average of 3 alleles per locus and PIC values ranged between 0.146 to 0.756 for for RM17616 and RM316, respectively.

Nadia *et al.* (2014) studied twenty six landraces rice and four high yielding rice accessions to observe genetic diversity assessment and identification of superior genotypes for crop improvement program. Genetic diversity was also assessed using a set of 27 SSR markers which generated 321 polymorphic alleles and polymorphism information content (PIC) values ranged between 0.6806 (RM 11) and 0.9416 (RM 474) with an average of 0.8414. Genetic similarity analysis using UPGMA, all 30 accessions were grouped into 6 clusters based on SSR markers' data at a cut-off similarity coefficient 0.17%.

Okello *et al.* (2017) conducted an experiment on 48 rice germplasm with 18 simple sequence repeat (SSR) markers to study their genetic diversity and phylogeny structure. Each primer exhibits 100% polymorphism. A total of 275 alleles were generated by 18 primers and each primer produced on an average 15.27 alleles of the size ranging from 172.22 bp to 329.44 bp. The number of alleles increased for each primer pair was ranged from 5 to 35. The markers pTA-248 generated a

maximum number of alleles (35), while the primer RM-309 produced minimum number of alleles (5). The PIC value of primers ranged from 0.58 (RM206) to 0.85 (RM-140) with an average PIC value of 0.77. It was also observed that there was no correlation between percentage polymorphism and PIC value as SSR primer RM-206 showed minimum PIC value but were 100% polymorphic. The higher the PIC value, the more informative is the primer. Primers RM-140 and RM-122 were found to be more informative.

Roy *et al.* (2016) conducted an experiment with a valuable set of hill rice germplasm by using 35 SSR Markers. A total, 297 alleles were detected at the 35 SSR markers, ranging from 2 alleles (RM338) up to 21 alleles (RM259), with an average of 8.49 alleles across the loci. The expected heterozygosity (He) ranged from 0.41 (RM55) to 0.94 (RM259) and the average observed heterozygosity (Ho) was 0.051. The PIC values perceived with a range from 0.37 (RM338 and RM507) up to 0.93 (RM259) with an average of 0.65.

Siddique *et al.* (2016) studied about genetic diversity of 96 Aman (rainfed, partially irrigated) rice landraces of Bangladesh 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 identification 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.

Singh *et al.* (2016) evaluated that, a set of indian rice variety genotypes using 36 HvSSR markers to assess the genetic diversity and genetic relationship. Total of 112 alleles was increased with an average of 3.11 and PIC value is 0.29.

Sinha *et al.* (2015) has carried out an experiment on 55 traditional rice varieties of West Bengal, and examine for grain morphological characters. A wide range variation of grain characters, like size and shape of grain, anthocyanin coloration of lemma-palea and kernels, presence or absence of aroma, awning characteristics, were found among the investigated varieties. Wide range of

variation among the grain morphological characters indicated large genetic variation present among these varieties, which may be utilized for the selection of the parents for the plant breeding and production of new improved variety.

Sajid *et al.* (2015) has conducted an experiment and he has characterized 30 indigenous rice germplasm on the basis of 32 different agro-morphological traits among these 15 are qualitative and 17 are quantitative traits. For the traits of flag leaf length, flag leaf breadth, culm length, days to 50% flowering, panicle length, length of primary branches panicle-1secondary branches panicle-1, grain length, grain width, awn length highly significant difference ($p<0.01$) were observed and percent leaf lesion while significant differences($p<0.05$) were observed for peduncle length and primary branches. The rice germplasm exhibits adequate genetic variation for majority of the qualitative and quantitative traits.

Siddique *et al.* (2014) conducted an experiment on 24 rice genotypes for their characterization and discrimination by using five microsatellite markers. In his experiment he found that the number of alleles per locus ranged from 6 alleles (RM153) to 14 alleles (RM151), with an average of 9.6 alleles across the 5 loci. The maximum PIC value is 0.91 (RM151) and minimum PIC value is ranges as 0.65 (RM153). The best marker for identification of 24 genotypes as revealed by PIC value is RM151. The most common allele maximum frequency level 50% (RM153) and minimum frequency level is 4.17% (RM1, RM153 and RM335) which is comparable with Thomson *et al*. (2007). The two and three dimensional principal coordinate analysis (PCoA) with 24 genotypes indicated that the genotypes Chaulamaghi, Sackorkhana, Chinikanai, Chata Bazail, Dudshail, Paizra, Beto, Orgoja, Beti Chicon, Kasrail were found far away from centroid of the cluster and rest of the genotypes were placed more or less around the centroid.

Sarawgi *et al.* (2013) conducted an experiment of about seven hundred eighty two rice germplasm accessions on the basis of morphological and agronomical traits. Most of the morphological characters showed variation in different accessions except leaf : collar leaf : ligule and leaf : shape of ligule. For most of the agronomical traits significant amount of variation was observed. After evaluation of 782 accessions for eight quantitative characters, on the basis of mean values, top ten accessions were identified for the yield ancillary traits. These can be used to identify phenotypically divergent sources for traits of interest in breeding programmes.

Sajib *et al.* (2012) conducted an experiment of total 24 SSR markers across 12 elite aromatic rice genotypes for their characterization and discrimination. Nine (09) microsatellite markers were showed polymorphism among these selected 24 markers. During the experiment he found the number of alleles per locus ranged from 2 alleles (RM510, RM244, and RM277) to 6 alleles (RM 163), with an average of 3.33 alleles across 9 loci he was obtained. The maximum polymorphic information content is 0.71(RM163) and minimum polymorphic information content range is 0.14 (RM510) in all 9 loci with an average of 0.48. The best marker for the identification of 12 genotypes as revealed by PIC values is RM163. The most common allele frequency level at each locus is ranges from 41% (RM163, RM590, and RM413) to 91% (RM510).

Seetharam *et al.* (2009), reported that 30 rice genotypes including landraces, pure lines, soma clones, breeding lines and varieties specifically adapted to coastal saline environments were characterized by Simple Sequence Repeat markers and morphological characters, among 35 primers of SSR markers 28 were polymorphic.

Surapaneni *et al.* (2016) evaluated that genetic diversity among 23 rice genotypes including wild species and cultivars of indica, japonica, aus and aromatic type. After using 77 polymorphic SSRs, 253 alleles were detected. In there, Polymorphism information content (PIC) were extended from 0.31 to 0.97 with a mean of 0.79.

Thimmanna *et al.* (2000) found that some characters such as leaf length and width, pubescence of leaf, leaf angle, ligule shape and colour, panicle type, secondary branching, exertion, awning, seed length and width and 1000 grain weight can be used in differentiating the parental lines of rice cultivar.

Vabna. F.A. *et al.* (2018) reported that genetic diversity and relationship among 24 boro rice germplasm by using 12 ssr markers. During the experiment, she found that the number of alleles per locus ranged from 1 to 8 alleles, with an average of 4.5 alleles. The highest PIC value is 0.84 (RM206) and lowest PIC value is ranges as 0.08 (RM447), with an average of 0.49. The best marker for identification of 24 genotypes as revealed by PIC value is RM206. The maximum genetic diversity is 0.86 (RM206) and the minimum genetic diversity is 0.08 (RM447), with an average of 0.52. The UPGMA dendrogram based on Nei's genetic distance revealed to three different clusters.

Venkatesan *et al.* (2015) evaluated the genetic diversity and relationship among 40 aromatic rice through microsatellite marker (SSR) analysis. The used 24 primer pairs, of which 22 (91.6%) were polymorphic. Overall, 51 alleles were identified for 22 polymorphic primer-pairs, with an average of 2.3 alleles per locus and PIC values ranged from 0.05 to 0.57 with an average of 0.33.

CHAPTER-III

MATERIALS AND METHODS

The chapter describes the materials and methods used during conducting the experiment. The details of the methodology have been followed is described below:

3.1 Experimental site and time duration

The present experiment was carried out in the laboratory of the Genetic Resources and Seed Division (GRSD), Bangladesh Rice Research Institute (BRRI). The experiment was carried out during the period of January 2020 to August 2020.

3.2 Collection of materials for molecular work

A total of 18 Aman rice germplasm were used in this experiment. All of them were collected from the greater Faridpur District of Bangladesh. A list of germplasm used in this experiment has given in Table 1.

Table 1. List of Aman rice germplasm

Sl. No	Genotypes	Upazilla	District	Season
1	Baila digha	Muksudpur	Faridpur	B. Aman
2	Bashiraj	Muksudpur	Faridpur	B. Aman
3	Dudlucky	Muksudpur	Faridpur	B. Aman
4	Balam dhan	Alfadanga	Faridpur	T. Aman
5	Dolkachu	Boalmari	Faridpur	T. Aman
6	Aijan-1	Boalmari	Faridpur	T. Aman
7	Ranga Jabra	Gopalganj sadar	Gopalganj	T. Aman
8	Aijan-2	Nagarkanda	Faridpur	T. Aman
9	Kachkolom	Gopalganj sadar	Gopalganj	T. Aman
10	Sorna dhan	Charbhrasanad	Faridpur	T. Aman
11	Jol kowcha	Tungipara	Gopalganj	T. Aman
12	Kuriaguni	Alfadanga	Faridpur	T. Aman

Sl. No	Genotypes	Upazilla	District	Season
13	Kartiksail	Tungipara	Gopalganj	T. Aman
14	Begunbichi	Gopalganj sadar	Gopalganj	T. Aman
15	Debmoni	Alfadanga	Faridpur	T. Aman
16	Sissumoti	Gopalganj sadar	Gopalganj	B. Aman
17	Shial leji	Alfadanga	Faridpur	T. Aman
18	Jhul dhan	Charbhrasanad	Faridpur	T. Aman

3.3 Collection of leaf samples

Leaf samples were collected from young green leaves from the rice plants at 10-15 DAT (Days after transplanting). After collecting leaf it was kept inside 1.5 ml microfuge tubes. The microfuge tubes containing leaf samples were immediately preserved in ice buckets which were carried to the transplanting field. The microfuge tubes containing the leaf samples were kept in poly bags and sited in the chamber of -80°C freezer. The leaf samples were crushed immediately for DNA extraction. Before collecting leaf samples, microfuge tubes were labeled properly.

3.4 Reagent preparation for DNA Extraction

Genomic DNA was extracted following modified method of Ferdous *et al.* (2012). Nanodrop (Origin, Germany) was used for the quantification of DNA samples. The quality of the DNA was evaluated by using 0.8% agarose gel electrophoresis. High concentration of DNA samples was further diluted in 10:1 (DD H_2O-DNA) to a working concentration of 50 ng/µl and stored at 4°C for PCR based marker analysis. The detailed laboratory procedures of this method including chemical preparation are described as given follow.

3.4.1 Tris Buffer (1 M Tris solution, pH=8.0)

Tris is existing in different forms viz. Tris HCL, Trisma base, etc. The formula weight (FW) of Trisma base is 121.14 with chemical formula $C_4H_{11}NO_3$ was used. The compulsory concentration for this chemical is 1 M with pH=8.0. The main functions of Tris are to maintain pH of the DNA solution and to afford buffering capacity. By keeping pH steady at 8.0 tris performs the central operation.

However, for an example, 250 ml 1M Tris with pH 8.0 was prepared as follows:

Here, We Know, N= CV (N= mole number, C= Conc. in Molar, V= Volume in liter) i.e. N =1 M X 250 ml= 1 M X 0.25 liter = 0.25

Again, Mass = N X FW = 0.25 X 121.14 = 30.29 g

So, 30.29 g Tris was dissolved in around 180 ml of autoclaved and distilled water and pH is adjusted by adding conc. HCl (5N HCl) as Tris was basic in nature. The final volume was made to 250 ml in a graduated measuring cylinder by adding sterile H_2O and the solution was autoclaved.

3.4.2 Na_2EDTA (0.5 M Na_2EDTA solution, pH=8.0)

When dissolved in water Na_2EDTA makes the solution acidic. The formula weight (FW) of the chemical is 372.24 with the chemical formula $C_{10}H_{14}N_2Na_2O_8.2H_2O$. The required concentration is 0.5 M with pH=8.0. The Na_2EDTA acts as chelating agent which chelates inorganic or metal ion. It deactivates endonuclease enzyme by chelating with its co-factor (e.g. Mg++). However, for an example, 100 ml 0.5 M Na_2EDTA with pH 8.0 can be prepared as follows:

Here, We Know, N= CV (N= mole number, C= Conc. in Molar, V= Volume in liter) i.e. N =0.5 M X 100 ml= 0.5 M X 0.1 l = 0.05

Again, Mass = N X FW = 0.05 X 372.24 = 18.61 g

So, 18.61 g Na_2EDTA is dissolved in around 60 ml of autoclaved and distilled water and pH is adjusted by adding NaOH pellets (or 5M NaOH) as Na_2EDTA is acidic in nature. The final volume is made to 100 ml in a graduated measuring cylinder by adding sterile H_2O and the solution is autoclaved.

3.4.3 NaCl (5M NaCl solution)

The formula weight (FW) of this chemical is 58.44 with the chemical formula NaCl. The required concentration was 5 M. NaCl digest cellular components and helps to burst out cell wall, cell membrane through generating osmotic pressure.
However, for an example, 250 ml 5 M NaCl was prepared as follows:
Here We Know, N= CV (N= Mole number, C= Conc. in Molar, V= Volume in liter) i.e. N =5 M X 250 ml= 5 M X 0.25 l = 1.25

Again, Mass = N X FW = 1.25 X 58.44 = 73.05 g

So, 73.05 g NaCl is dissolved in around 175 ml of autoclaved and distilled water and the final volume is made to 250 ml in a graduated measuring cylinder by adding sterile H_2O. This chemical is generally not dissolved completely until final volume is made. The chemical is finally autoclaved.

3.4.4 SDS (Sodium Dodecyl Sulphate) solution

The alternate name of this chemical is Lauryl Sulphate with the formula weight (FW) 288.4 and chemical formula $C_{12}H_{25}O_4SNa$. The required concentration is 10%. SDS acts as a detergent agent and helps in the digestion of protein by breaking disulphide bond (– S–S–). It also helps in the lysis of cell wall. However, 10% 250 ml SDS can be prepared by dissolving 25 g SDS in 200 ml water first. Then the final volume is made to 250 ml by adding sterile H_2O. This chemical was not autoclaved and mask was worn while preparing this chemical.

3.4.5 Chloroform

The function of this chemical is to disrupt two-dimensional structure of protein forming its precipitation. This chemical was used under fume hood and was not to inhale.

3.4.6 Ethanol

Ethanol solutions were required in 70% and 100% form. The chemical 100% ethanol precipitates or coagulates DNA and 70% ethanol functions as both in the precipitation of DNA and dissolving of salts. Ethanol (70%) also acts as decontaminating surface acting chemicals in the laboratory.

3.4.7 1X TE Buffer

This is a secondary chemical. 100 ml 1X TE Buffer can be prepared as follows: 1 M Tris pH 8.0 10 ml 0.5 M Na_2EDTA pH 8.0 200 ml. Finally water is added up to 100 ml. This chemical redissolves DNA into solution and acts as DNA preserving solution.

3.4.8 Extraction buffer (200ml)

For the preparation of 200ml extraction buffer, 40mL of 1M Tris-HCL (pH 8) was mixed with 10 mL of 0.5M EDTA and added to 11.4mL 3.5M NaCl in a 200mL measuring cylinder. Finally,

sterilized distilled water was added to make the volume up to the mark, then mixed well and autoclaved.

3.4.9 1M Tris HCL (pH= 8.0) (200mL)

At first, using 24.23g Tris-HCl dissolved in 100ml deionized water and adjusted to pH 8.0 using concentrated HCl. Then top up the total volume to 200mL with de-ionized water.

3.4.10 0.5M EDTA (pH= 8.0) (1000mL)

0.5M EDTA was prepared using 186.12 g of EDTA dissolved in 800 ml de-ionized water. Ten molar (10 M) NaOH solution was used to adjust the pH to 8.0. Then top up the total volume to 1 L with deionized water. EDTA alone will not dissolve unless NaOH is added.

3.4.11 3.5M NaCl (250 mL)

204.54 g NaCl was added into 800 ml of de-ionized water and adjusted the final volume to 1 L with de-ionized water.

3.4.12 5% SDS (Sodium Dodecyl Sulphate) (100mL)

5 g SDS was dissolved into 100 ml of de-ionized water in a 100mL conical flask.

3.4.13 2X CTAB (Cetyl Trimethyl Ammonium Bromide) (200mL)

19 4 g CTAB, 20mL Tris-HCL, 8 mL EDTA (pH 8), 2g PVP was dissolved into deionized water. All items should be added except NaCl. Because NaCl does not dissolve if mixed together. 80mL of NaCl was added later.

3.4.14 Chloroform:

Isoamyl Alcohol :Phenol= 24:1:5 (100mL) At first 5 mL phenol was taken in a 100mL volumetric flask. Then 91.2 mL Chloroform and 3.8mL Isoamyl alcohol was added and mixed well. The solution was stored at 40C.

3.4.15 10X TBE Buffer (1000mL)

108g Tris-HCL was taken in a volumetric flask (1000mL).Then, 9.3g of EDTA and 55g Boric acid was added. Sterilized dH2O was added to make the volume 1000mL.

3.4.16 1X TBE buffer

100mL of 10X TBE buffer was taken in 900mL de-ionized water and autoclaved.

3.4.17 1% PVP

1 g PVP was added into 100 ml 2X CTAB solution and stored.

3.4.18 70% ethanol (1000mL)

71.5 ml 95% ethanol was mixed with 28.5 ml de-ionized water and stored.

3.5 Chronological steps for DNA extraction from leaf sample of Aman rice germplasm

Total genomic DNA was isolated using a quick modified CTAB extraction method (Ferdous *et al.* 2012)**.** The steps are given below:

1. For genomic DNA extraction, young, vigorous, actively growing leaf tissues were collected from 18 different Aman rice germplasm.

2. Firstly, young, healthy leaves were washed thoroughly by running tap water followed by deionized water. Then the leaves were sterilized by ethanol to ensure the removal of wastes and any foreign DNA material source and then dried on tissue paper.

3. Approximately, 250mg of leaf sample were cut into small pieces and then taken into morter. Extraction buffer of 600 µl was added to it and grinded gently with the help of pestle. Then the ground samples were taken into the 2 ml eppendorf tube.

4. Near about 400µl of 2XCTAB solution was added to the eppendorf tube. Equal volume (400 µl) of Choloroform: Isoamyl Alcohol: Phenol (24:1:5) was added there and it was vortexed for 15 seconds in a vortex mixture.

5. The solution was centrifuged at 8,400 rpm for 10 minutes.

6. The supernatant was transferred into the new eppendorf tube and the lower layer was discarded. Approximately, 800-900 µl was taken.

7. Two-third volume of the supernatant (465 µl) isopropanol was added to it and mixed gently by inverting.

8. Then, the eppendorf tubes were allowed to incubate for 10-15 minutes at room temperature.

9. Again, the solution was centrifuged at 8,400 rpm for 5 minutes .The liquid was discarded completely and DNA pellet was washed with 70% ethanol .The DNA pellet was then air dried for 1 hour.

10. After air drying, DNA pellet was re-suspended with 50 µl of TE Buffer. It was spinned for 4-5 seconds. Then it was stored at 4°C refrigerator overnight.

11. Finally, DNA samples were stored at -20° C refrigerator.

3.6 Synthesis of SSR markers

Rice genome specific 11 well known SSR primers viz. RM170, RM190, RM205, RM206, RM208, RM228, RM252, RM283, RM314, RM455, RM447 were selected and synthesized for molecular diversity analysis in 18 Aman rice germplasm. A list of the primer used is showed in (Table 2).

Table 2. List of SSR markers used for diversity analysis of Aman genotypes

Sl. No.	Primer Name	Chro. No.	Forward primer sequence	Reverse primer sequences	Annealing Temp.
1	RM170	6	TCGCGCTTCTTCCTCGTCGACG	CCCGCTTGCAGAGGAAGCAGC	55
2	RM190	6	CTTTGTCTATCTCAAGACAC	TTGCAGATGTTCTTCCTGATG	55
3	RM205	9	CTGGTTCTGTATGGGAGCAG	CTGGCCCTTCACGTTTCAGTG	55
4.	RM206	11	CCCATGCGTTTAACTATTCT	CGTTCCATCGATCCGTATGG	55
5.	RM208	2	TCTGCAAGCCTTGTCTGATG	TAAGTCGATCATTGTGTGGACC	55
6.	RM228	10	CTGGCCATTAGTCCTTGG	GCTTGCGGCTCTGCTTAC	55
7.	RM252	4	TTCGCTGACGTGATAGGTTG	ATGACTTGATCCCGAGAACG	55
8	RM283	1	GTCTACATGTACCCTTGTTGGG	CGGCATGAGAGTCTGTGATG	55
9	RM314	6	CTAGCAGGAACTCCTTTCAGG	AACATTCCACACACACACGC	55
10	RM455		AACAACCCACCACCTGTCTC	AGAAGGAAAAGGGCTCGATC	55
11	RM447	8	CCCTTGTGCTGTCTCCTCTC	ACGGGCTTCTTCTCCTTCTC	55

3.7 Amplification of SSR markers by PCR

3.7.1 Principle of the amplification of SSR marker

Microsatellites or SSR are tandem repeats of 1-6 nucleotides. For example, (AT)n, (ATG)n,(GATT)n , (CTACG)n , (TACGAC)n and so on. They are abandoned in genomes of all organisms. The sequence of unique flanking regions of SSR can be used to design primers and carry out PCR to amplify SSR containing sequences.

3.8 Polymerase chain reaction (PCR) amplification

PCR analysis was performed in 10 µl reaction sample containing 3 µl of DNA template, 4.5 µl of Go Taq G2 Green Master Mix (Promega), 1.5 µl of Nuclease-Free Water, 0.5µl each of 10 µM forward and reverse primers a GeneAtlas G (Astec, Japan) 96-well thermal cycler was used for PCR amplification. The mixture was overlaid with 10 µl of mineral oil to prevent evaporation. The PCR plate was wrapped with adhesive film. The ingredients of PCR reaction for SSR markers showed on Table 3.

Table 3: Composition and preparation of PCR Cocktail (master mix).

Reagent	Amount (µl)
DNA	3.0
Primer (F)	0.5
Primer (R)	0.5
Master mix	4.5
DDH_2O	1.5
Total	**10**

Reagent Amount (µl) DNA 3.0 µl Primer (F) 0.5 µl Primer (R) 0.5 µl Master mix 4.5 µl DDHO 1.5 µl Total 10 µl. After initial denaturation for five minutes at 94°C, each cycle comprised 30 sec denaturation at 95°C, 30 sec annealing at 55°C, and 30 sec extension at 72°C with a final extension for 5 min at 72°C at the end of 32 cycles.

Table 4 : Temperature profile of PCR

Step	Temperature	Time	No. of Cycle
Initial denaturation	94°C	5 min	
Denaturation	95°C	30s	32 cycle
Annealing	55°C	30s	
Extension	72°C	30s	
Final extension	72°C	5 min	
Hold at	4°C	99:99 (overnight)	

3.9 Polyacrylamide Gel Electrophoresis (PAGE)

Three microliters of PCR products with SSR markers were subjected to electrophoresis using Polyacrylamide gel at 100 volt for different time settings according to EPS (Expected Product Size) to check the DNA quantification and PCR amplification. However, the detailed protocol of PAGE is given below:

3.9.1 Assembling of Glass Plates

1. Two glass plates, two spacers and one comb were washed properly using laboratory detergent (bleaching powder based) and rinsed with water. Glass plates were also washed by 0.5 M NaOH solution. Glass plates were air dried and chosen inner surfaces of the plate was sprayed with 100% ethanol and wiped with lint-free tissue.

2. The short plate (round-bottom) was hold and the rubber gasket was attached starting from one side of the plate. The notches on the gasket were aligned on the corners. The circular portion of the gasket was exposed to the inner side of the plate.

3. The short plate was laid on the table with the inner side up. Then, the spacers were put along the inside edges of the gasket.

4. The other plate was put on top of the short plate.

5. The clamps were set on both sides of the plates for tightening and the plate assembly was laid flat on the table.

3.9.2 Preparation of Polyacrylamide Gel

The following chemicals and their quantity were used to prepare eight percent of PAGE gel. The gel solution was prepared in a beaker with a magnetic stirring bar.

Table 5: Composition and preparation of polyacrylamide gel

Reagents	Final conc.	8% gel
Sterile nanopure H_2O	-	41.35 ml
10X TBE buffer	5X	6.0 ml
40% Acrylamide	8%	12 ml
10% APS	0.1%	600 µl
TEMED	1 µl/ml	50 µl
Total		**60.0 ml**

The concentration of gels used for PAGE was 8%. After adding TEMED, the solution was stirred using magnetic stirrer for few seconds on a stirrer machine at a speed to mix the chemicals properly. Then, the gel solution was poured into glass plate assembly smoothly and continuously for avoiding air bubbles, starting from one corner until it reached top portion of the short plate. The comb was inserted in the gel gently. The gel was allowed to polymerize for 30 minutes.

3.9.3 Polyacrylamide Gel Electrophoresis

After the gel was polymerized, the gasket was removed starting from one corner of the plate assembly. Around 500 ml of 0.5X TBE buffer was added in the base of the tank and around 300 ml of 0.5X TBE buffer was added on top of the tank and the comb was removed gently.

1. Two µl of 10X loading dye was added to the each well containing 10 µl PCR product and the plates were centrifuged at a speed of 3000 rpm for 30 sec in a high speed refrigerated centrifuge machine for mixing the dye equally. Around 2 µl of the mixer was loaded in the wells of PAGE gel with the help of 2-2.5 µl pipette. DNA size marker like 1Kb Plus (Thermo Scientific GeneRuler) DNA ladder was loaded for size determination.

2. The cover of the tank was put and the electrodes were connected to the power supply and the gel was run for about 2.0-2.5 hours at 100 volts. It was noted that running time depended on the size of PCR fragments.

3.9.4 Staining and Visualization of the Gel

1. The power supply unit was turned off and the plates were removed from the tank. The glass plates were separated using a knife. The acrylamide gel was removed carefully and transferred in the SYBR Safe staining solution (0.5 mg/ml) for around 20 minutes.

2. The stained gels were put in the exposure cabinet of the gel documentation system (Molecular Imager Gel Doc XR System, BIO-RAD, Korea). The gel was viewed in the computer monitor by exposing it first to white light. The necessary adjustments were made by moving the gel inside the exposure box. The gel was exposed to UV light and photograph (gel image) was taken and saved as a JPEG file.

3.10 SSR data analysis

The summary statistics including the number of alleles per locus , major allele frequency, gene diversity and Polymorphism Information Content (PIC) values were determined using POWERMAKER version 3.25 (Liu and Muse, 2005), a genetic marker data analysis software. Molecular weight for each microsatellite products, in base pairs were estimated with AlphaEaseFC (Alpha Innotech Corporation) version 4.0 software. Polymorphic information content (PIC) values were calculated with the following formula (Anderson *et al.* 1993): $PICi = 1-\sum^{n}_{j=1} P^2 ij$

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

Shannon diversity index was quantified using following formula: $I = -\sum^{n}_{j=1} Pi \ In \ Pi,$

Where, Pi is the frequency of the ith allele of a particular locus containing a total of n alleles. The individual fragments were assigned as alleles of the appropriate microsatellite loci. Genetic diversity also was assessed, and the phylogeny trees were drawn using MEGA 5.1 (Tamura *et al.* 2011) based on Nei's (1983) genetic distance.

3.11 Precautions

- ✓ To maintain a safe distance from all types of contaminations and keep DNA pure, all dishes, micropipette tips, eppendorf tubes, glass pipettes, de-ionized water and buffer solutions were legitimately autoclaved. Metal supplies i.e., scissors, forceps were cleaned with help of absolute ethanol.
- ✓ Since Ethidium Bromide (Et-Br) is an intense mutagen and carcinogenic in nature, hand gloves were utilized when taking care of anything that has been presented to Et-Br.
- ✓ Always power pack was kept turn off and the leads were unplugged before opening the electrophoresis unit to avoid electrical hazard.
- ✓ Eye protector was used while working with trans-illuminator as it produces UV radiation of 254 nm range which can cause eye damage.
- ✓ The common safety measures were kept up when performing PCR responses. All the disposables such as PCR tubes, tips, eppendorf tubes and reagents used during preparation of PCR reactions were autoclaved. Freezing condition was maintained when necessary. Hand gloves were worn amid treatment of PCR segments. Contamination of PCR segments was maintained a strategic distance from.

Some pictorial view during conducting the research

Fig 01: Leaf were cutting for grinding

Fig 02: Grinding of the sample for extraction of DNA

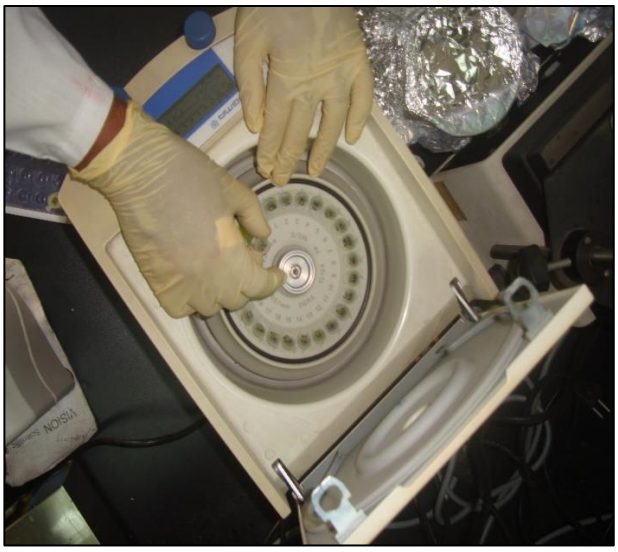

Fig 03: Sample were placed on centrifuge machine

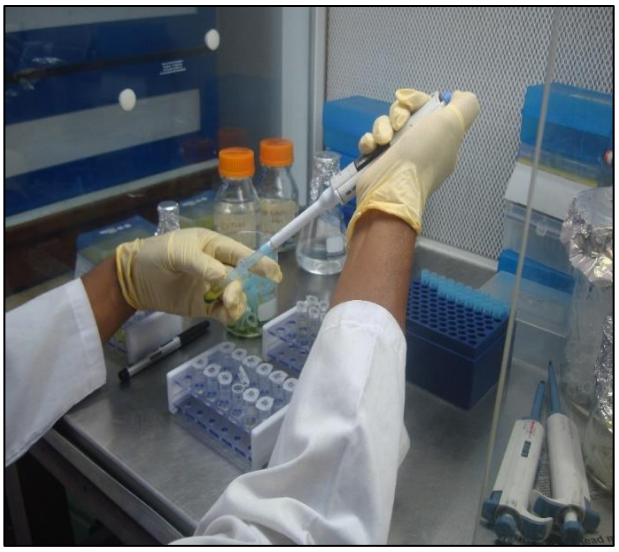

Fig 04: Seperation of Supernatant from eppendrof tube

Some pictorial view during conducting the research

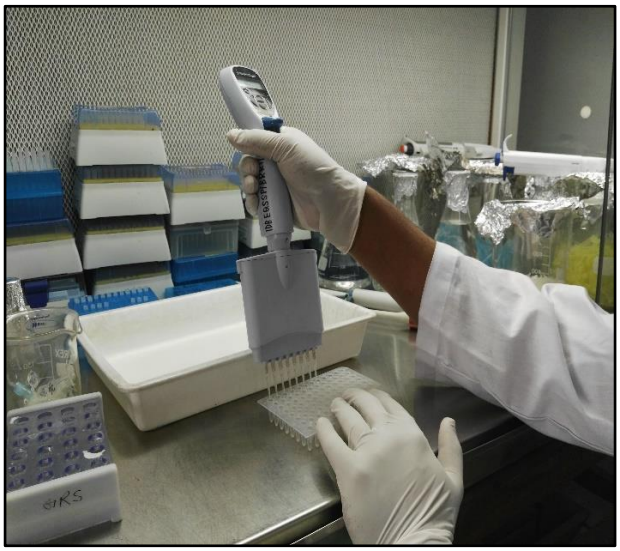

Fig 05: Sample preparation for PCR amplification

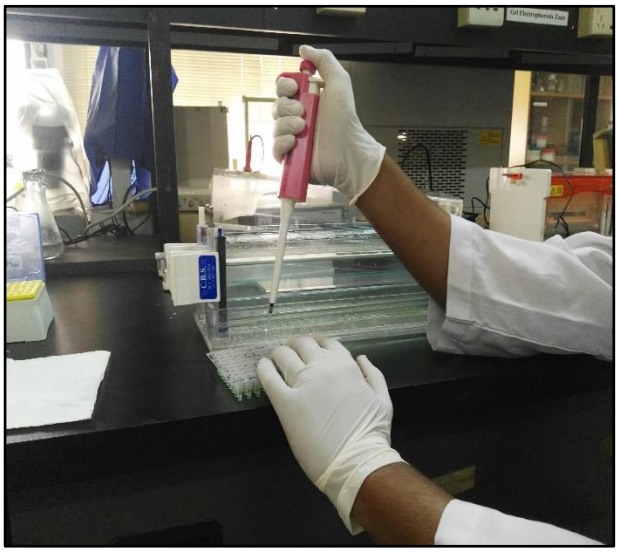

Fig 06: Loading sample in PAGE gel

Fig 07: Gel staining with SYBER Safe Dye

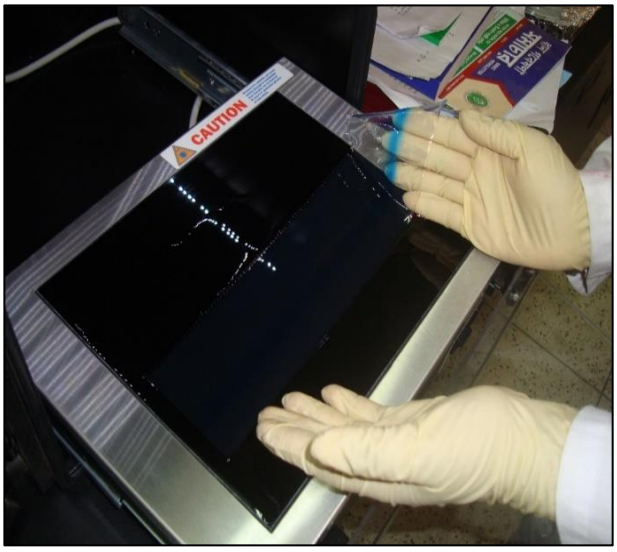

Fig 08: Stained gels were put in the exposure cabinet of the gel

CHAPTER-IV

RESULTS AND DISCUSSION

The present study was conducted to analyze the molecular diversity of Aman rice genotypes. Highly polymorphic and repeatable PCR based markers Simple Sequence Repeats (SSRs) marker were used to assess the polymorphism, diversity and similarity identification within local genotypes. Results obtained from the present study had been presented below under the following headings.

4.1 DNA amplification by SSR markers and its polymorphism

Eleven SSR primers *viz*. RM170, RM190, RM205, RM206, RM208, RM228, RM252, RM283, RM314, RM447 and RM455 produced different banding patterns separately with 18 Aman rice genotypes. The amplification of each SSR primer is presented in Table 6 and Plate 1 to 4.

The SSR primer RM206 showed polymorphic band. Different DNA amplifications were obtained which were distributed throughout all the genotypes under study. The size of amplification ranged from 100 bp to 170 bp. The genotype Balam dhan showed amplification at 100 bp. Most of genotypes have given amplification at 110 bp and 115 bp. Baila digha, Dudlucky, Aijan-1, Aijan-2, Sorna dhan and Jol kowcha showed amplification at 110 bp. Dolkachu, Ranga Jabra, Kachkolom, Begunbichi and Debmoni showed amplification at 115 bp. Sissumoti showed amplification at 130 bp. Again, Kuriaguni, Kartiksail has given amplification at 165 bp. Shial leji and Jhul dhan shows amplification at 170bp. The amplification is presented in Plate 1.

The SSR Marker RM208 also produced polymorphic banding patterns. The SSR Marker RM208 amplification ranges from size 155 to 190 bp. (G1) Baila digha and (G2) Bashiraj showed amplification at 155 bp. (G10) Sorna dhan showed its amplification at 160 bp. Most of genotypes have given their amplification at 170 bp. **(**G3) Dudlucky, (G4) Balam dhan, (G6) Aijan-1, (G7) Ranga Jabra, (G8) Aijan-2, (G11) Jol kowcha, (G12) Kuriaguni, (G13) Kartiksail, (G14) Begunbichi, (G15) Debmoni, (G16) Sissumoti, (G17) Shial leji and (G18) Jhul dhan showed amplification at 170 bp. (G5) Dolkachu and (G9) Kachkolom shoed its amplification at 190bp. The amplification is presented in Plate 2.

The SSR marker RM228 also produced polymorphic banding patterns. Different DNA amplifications were obtained which were distributed throughout all the genotypes under study the amplification of SSR Marker RM228 ranges from 90 bp to 140 bp. Most of the genotypes have given their amplification at 90 bp. (G1) Baila digha, (G2) Bashiraj, (G4) Balam dhan, (G5) Dolkachu, (G6) Aijan-1, (G7) Ranga Jabra, (G8) Aijan-2, (G9) Kachkolom, (G10) Sorna dhan, (G11) Jol kowcha, (G12) Kuriaguni, (G14) Begunbichi, (G16) Sissumoti, (G17) Shial leji and (G18) Jhul dhan showed its amplification at 90bp. (G3) Dudlucky showed its amplification at 105bp. (G13) Kartiksail has given it's amplification at 140 bp. (G15) Debmoni shwed its amplification at 120bp. The amplification is presented in Plate 3.

The SSR Marker RM314 also produced polymorphic banding patterns. The amplification of SSR Marker RM314 ranges from 80 to 95 bp. Most of the genotypes given amplification at 80 bp and 85bp. (G1) Baila digha, (G2) Bashiraj, (G3) Dudlucky, (G4) Balam dhan, (G5) Dolkachu, (G6) Aijan-1**,** (G12) Kuriaguni, (G13) Kartiksail, (G14) Begunbichi have given their amplification at 85 bp. (G7) Ranga Jabra, (G10) Sorna dhan, (G11) Jol kowcha, (G17) Shial leji and (G18) Jhul dhan has given their amplification at 80bp. (G8) Aijan-2, (G9) Kachkolom , (G15) Debmoni, and (G16) Sissumoti gave their amplification at 95 bp. The amplification is presented in Plate 4.

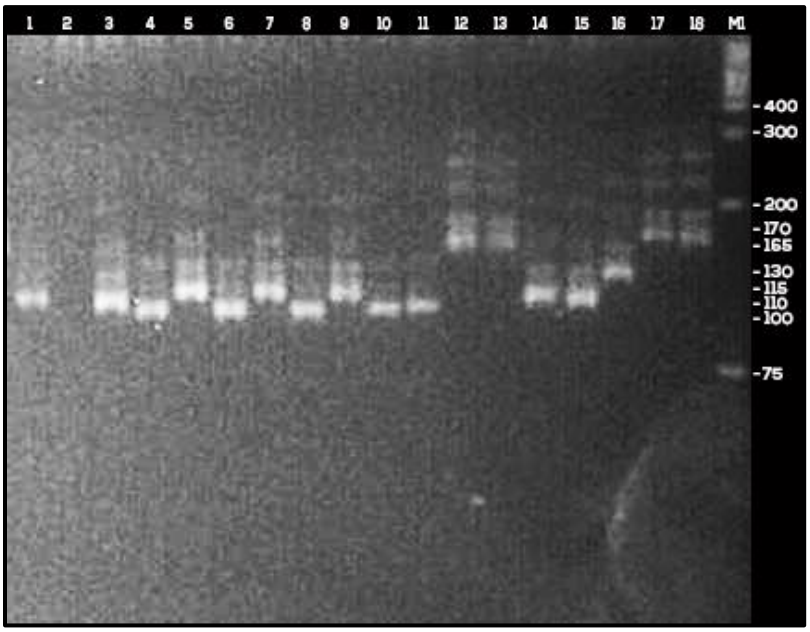

Plate 1. SSR profile of 18 Aman rice genotypes using primer RM206.

M_1 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder).

Lane: G1. Baila digha, G2. Bashiraj, G3. Dudlucky, G4. Balam dhan, G5. Dolkachu, G6. Aijan-1, G7. Ranga Jabra, G8. Aijan-2, G9. Kachkolom, G10. Sorna dhan, G11. Jol kowcha, G12. Kuriaguni, G13. Kartiksail, G14. Begunbichi, G15. Debmoni, G16. Sissumoti, G17. Shial leji, G18. Jhul dhan.

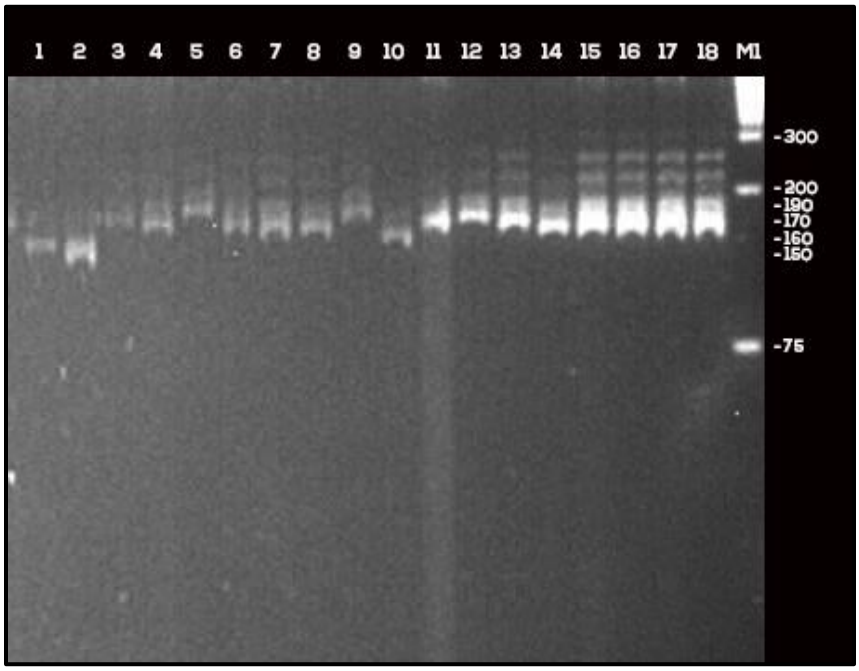

Plate 2. SSR profile of 18 Aman rice genotypes using primer RM208.

M_1 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder).

Lane: G1. Baila digha, G2. Bashiraj, G3. Dudlucky, G4. Balam dhan, G5. Dolkachu, G6. Aijan-1, G7. Ranga Jabra, G8. Aijan-2, G9. Kachkolom, G10. Sorna dhan, G11. Jol kowcha, G12. Kuriaguni, G13. Kartiksail, G14. Begunbichi, G15. Debmoni, G16. Sissumoti, G17. Shial leji, G18. Jhul dhan.

Plate 3. SSR profile of 18 Aman rice genotypes using primer RM228.

M_1 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder).

Lane: G1. Baila digha, G2. Bashiraj, G3. Dudlucky, G4. Balam dhan, G5. Dolkachu, G6. Aijan-1, G7. Ranga Jabra, G8. Aijan-2, G9. Kachkolom, G10. Sorna dhan, G11. Jol kowcha, G12. Kuriaguni, G13. Kartiksail, G14. Begunbichi, G15. Debmoni, G16. Sissumoti, G17. Shial leji, G18. Jhul dhan.

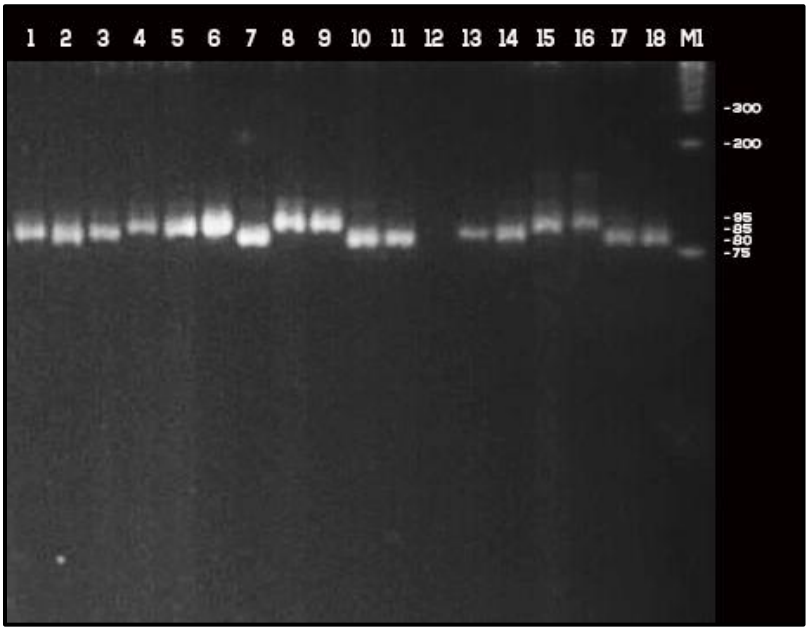

Plate 4. SSR profile of 18 Aman rice genotypes using primer RM314.

M_1 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder).

Lane: G1. Baila digha, G2. Bashiraj, G3. Dudlucky, G4. Balam dhan, G5. Dolkachu, G6. Aijan-1, G7. Ranga Jabra, G8. Aijan-2, G9. Kachkolom, G10. Sorna dhan, G11. Jol kowcha, G12. Kuriaguni, G13. Kartiksail, G14. Begunbichi, G15. Debmoni, G16. Sissumoti, G17. Shial leji, G18. Jhul dhan

4.2 Number of alleles, range of DNA fragment, number of polymorphic alleles, allele frequency, gene diversity and Polymorphism information content (PIC) in different Aman rice germplasm

Range of DNA fragments, number of alleles, and the number of polymorphic alleles, allele frequency, gene diversity and polymorphism information content (PIC) found among experimental genotypes for 11 SSR markers are presented in Table 6. A total of 38 alleles were detected for the 11 polymorphic SSR loci, with an average number of alleles of 3.45. A range of 2 to 6 no. of alleles per primer were generated (Table 6). In the current study, the average number of alleles (3.45 alleles/locus) is slightly lesser than the average number of alleles (3.88 alleles/ locus) reported by (Zhang *et al.* 2011) in rice core collection with 150 rice varieties from south Asia and Brazil. The average alleles per locus as 3.9 in 416 rice accessions collected from China reported by (Jin *et al*. 2010). The number of alleles detected by microsatellite markers varied from 2 to 6 with an average of 3.45 alleles per locus which are a little bit higher than those reported by (Wang *et al.* 2009). 2-4 allele/locus and an average of 2.6957 alleles per locus using a different set of rice germplasm reported (Jalaluddin *et al.* 2007). This value is comparable to 1-8 allele per SSR locus with an average number of alleles of 4.58 per locus for various classes of microsattelite reported by (Siwach *et al*, 2004) and 2-7 alleles per locus as reported by Chakrabarthia and Naravaneni (2006).

The length of the DNA fragments varies within a range of 90 bp to 260 bp. Primer RM170 amplified the lowest 90 bp fragment whereas primer RM252 amplified the highest 260 bp fragment of DNA. The frequency of the major allele ranged from 27.78 to 94.44. The highest frequency (94.44) of allele was noticed in the primer RM455 and the lowest frequency (27.78) was noticed in the primer RM252. Gene diversity ranged between 0.10 (RM455) to 0.78 (RM206) with an average of 0.45. Primer RM206 showed highest gene diversity (0.78) followed by RM252 (0.77), RM205 (0.62), RM314 (0.62), RM170 (0.52), RM208 (0.45), RM447 (0.35), RM228 (0.30),

RM283 (0.29), RM190 (0.20) and RM455 showed the lowest gene diversity (0.10). Total gene diversity was obtained 5.00 with an average 0.45. The average gene diversity is 0.358 reported by (Chen *et al.* 2011), which is lower than our present study. The gene diversity reported in our study is lesser than gene diversity (0.68) reported by (Liakat Ali *et al*. 2011).

Gene diversity (H_e) computed according to (Nei,1973) varied from 0.16 (RM17616) to 0.75 (RM287) with the an average of 0.52. US accession panel with average gene diversity of 0.43 reported by (Agrama and Eizenga, 2008) which is slightly lower than the present study. Polymorphic Information Content (PIC) value for 11 SSR markers ranged from 0.10 (RM455) to 0.75 (RM206) and the average PIC value was 0.42. The findings of polymorphic information content values, are comparable to these previous findings of microsatellite analysis in rice viz. 0.67-0.88 reported by (Gohain *et al.* 2006), 0.34- 0.88 reported by (Thomson *et al.* 2007), 0.20-0.90 with an average of 0.56 reported by (Jain *et al.* 2003). 0.26 to 0.65 with an average of 0.47 reported by (Singh *et al.* 2015), 0.28-0.50 with a mean of 0.45 reported by (Umadevi *et al.* 2014) and 0.239 to 0.765 with an average of 0.508 reported by (Hossain *et al.* 2012). The highest PIC value was obtained from RM206 (0.75) followed by RM252 (0.73), RM205 (0.58), RM314 (0.55), RM170 (0.48), RM208 (0.42), RM447 (0.29), RM228 (0.28), RM283 (0.27), RM190 (0.19) and RM455 (0.10) respectively. The lowest PIC value 0.10 was shown by primer RM455. From the PIC value, it is clear that RM206 can be considered as the best marker for 18 Aman rice genotypes followed by RM252, RM205, RM314, RM170, RM208, RM447, RM228, RM283, RM190 and RM455 respectively. Again, RM455 can be considered as the least powerful marker due to its lowest PIC value. The results indicate that the 18 Aman rice landraces have shown low to high genetic diversity.

Table 6: Number of alleles, range of DNA fragment, number of polymorphic alleles, allele frequency, gene diversity and Polymorphism information content (PIC) found among 18 Aman rice germplasm for 11 microsatellite markers

SL NO	Marker	Chro. No	Position (CM)	Motif*	Allele No	Size Range (bp)	Highest Size (bp)	Freq (%)	Gene Diversity	PIC
1	RM206	11	102.9	(CT)21	6	100-170	170	33.33	0.78	0.75
2	RM208	2	186.4	(CT)17	4	150-190	190	72.22	0.45	0.42
3	RM228	10	130.3	(CA)6(GA)36	4	90-140	140	83.33	0.30	0.28
4	RM314	6	33.6	GT)8(CG)3(G)5	3	80-95	95	50.00	0.62	0.55
5	RM170	6	2.2-7.4	(CCT7)	3	75-90	90	66.67	0.52	0.48
6	RM190	6	7.4	(CT)11	2	85-100	100	88.89	0.20	0.19
7	RM205	9	114.7	(CT)25	4	100-140	140	55.56	0.62	0.58
8	RM252	4	99	(CT)19	5	200-260	260	27.78	0.77	0.73
9	RM283	1	31.4	(GA)18	3	125-150	150	83.33	0.29	0.27
10	RM447	8	124.6	(CTT)8	2	80-85	85	77.78	0.35	0.29
11	RM455	7	65.7	(TTCT)5	2	130-140	140	94.44	0.10	0.10
	Max				6			94.44	0.78	0.75
	Min				2			27.78	0.10	0.10
	Total				38			733.33	5.00	4.64
	Mean				3.45			66.67	0.45	0.42

4.2.1 Diversity profile revealed by different markers

The polymorphism information content (PIC) value is a reflection of allelic diversity and frequency among the genotypes. The used eleven markers have a different level of diversity based on PIC value. Among them, RM455, RM190 showed low diversity and the value was less than 0.25. Again, RM283, RM228, RM447, RM208 and RM170 showed moderate diversity. The diversity level of those genotypes ranged from 0.25 to 0.50. The rest of the four markers such as RM314, RM205, RM252 and RM206 showed highly diverse results. The diversity was more than 0.50 and it might be up to 1.0. The result revealed to broaden the genetic base for wider adaptability.

Table 7. Diversity level showed by different markers based on PIC value

Sl. No.	PIC Value	Diversity level	Markers
1.	≤ 0.25	Low	RM190, RM455
2.	0.25- 0.50	Moderate	RM283, RM228, RM447, RM208, RM170
3.	0.50-1.0	High	RM314, RM205, RM252, RM206

4.3 Nei's Genetic Distance

The value of pair-wise comparisons of Nei's (1983) genetic distance among 18 relatives of rice genotypes weas computed from combined data for the 11 primers which are ranged from 0.09 to 0.82 with an average value of 0.46 (Table 8). Comparatively higher Nei's genetic distance (0.82) was observed between some genotypes Sorna dhan vs Begunbichi. The maximum genetic distance between them indicated that genetically they are different and also highly diverse. The difference between the maximum and minimum genetic identity indicates the existence of variability among 18 genotypes of Aman rice. The genotypes which have higher (0.82) genetic distances are more different than those which have a lower value. Again, many pairs have shown the lowest genetic distance (0.09) which indicate their genetically much more closer among them and homogeneity in nature.

Baila digha shows maximum genetic dissimilarity 64% with Begunbichi (G14). 55% with Debmoni (G15), Sissumoti (G16), Jhul dhan (G18), Ranga Jabra (G7) and Kachkolom (G9). 45% with Sorna dhan (G10), Kuriaguni (G12), Kartiksail (G13), Shial leji (G17) and Dudlucky (G3). 36% with Jol kowcha (G11), Bashiraj (G2), Balam dhan (G4), Dolkachu (G5) and Aijan-2 (G8). Baila digha shows minimum genetic dissimilarity 27% with Aijan-1 (G6).

Sorna dhan shows maximum genetic dissimilarity 82% with Begunbichi (G14). 64% with Kartiksail (G13), Sissumoti (G16), Jhul dhan (G18), Dudlucky (G3), and Dolkachu (G5). 55% with Kuriaguni (G12), Debmoni (G15), Bashiraj (G2) and Kachkolom (G9). 45% with Jol kowcha (G11), Shial leji (G17) and Ranga Jabra (G7). 36% with Balam dhan (G4). Sorna dhan shows minimum genetic dissimilarity 27% with Aijan-1 (G6) and Aijan-2 (G8).

Jol kowcha shows maximum genetic dissimilarity 73% with Begunbichi (G14). 64% with Bashiraj (G2), Dolkachu (G5) and Kachkolom (G9). 55% with Kartiksail (G13), Debmoni (G15), Sissumoti (G16) and Dudlucky (G3). 45% with Jhul dhan (G18) and Balam dhan (G4). 36% with Kuriaguni (G12), Shial leji (G17), Aijan-1 (G6), Ranga Jabra (G7) and Aijan-2 (G8).

Kuriaguni shows maximum genetic dissimilarity 64% with Kachkolom (G9). 55% with Begunbichi (G14), Jhul dhan (G18), Dudlucky (G3) and Dolkachu (G5). 45% with Debmoni (G15), Sissumoti (G16) and Bashiraj (G2). 36% with Kartiksail (G13), Shial leji (G17) and Aijan-2 (G8). Kuriaguni shows minimum genetic dissimilarity 27% with Balam dhan (G4), Aijan-1 (G6) and Ranga Jabra (G7).

Kartiksail shows maximum genetic dissimilarity 64% with Begunbichi (G14), Sissumoti (G16) and Kachkolom (G9). 55% with Shial leji (G17), Jhul dhan (G18), Dolkachu (G5) and Ranga Jabra (G7). 45% with Bashiraj (G2) and Aijan-2 (G8). 36% with Debmoni (G15), Balam dhan (G4) and Aijan-1 (G6). Kartiksail shows minimum genetic dissimilarity 27% with Dudlucky (G3).

Begunbichi shows maximum genetic dissimilarity 73% with Sissumoti (G16), Shial leji (G17) and Jhul dhan (G18). 64% with Debmoni (G15), Dudlucky (G3) and Aijan-2 (G8). 55% with Bashiraj (G2), Begunbichi (G14), Aijan-1 (G6) and Ranga Jabra (G7). 45% with Kachkolom (G9). Begunbichi shows minimum genetic dissimilarity 36% with Dolkachu (G5).

Debmoni shows maximum genetic dissimilarity 64% with Jhul dhan (G18). 55% with Dolkachu (G5). 45% with Sissumoti (G16), Shial leji (G17), Bashiraj (G2), Dudlucky (G3) and Kachkolom (G9). 36% with Balam dhan (G4), Aijan-1 (G6) and Ranga Jabra (G7). Debmoni shows minimum genetic dissimilarity 27% with Aijan-2 (G8).

Sissumoti shows maximum genetic dissimilarity 64% with Bashiraj (G2), Dolkachu (G5) and Kachkolom (G9). 55% with Dudlucky (G3). 45% with Jhul dhan (G18), Balam dhan (G4), Aijan-1 (G6) and Ranga Jabra (G7). Sissumoti shows minimum genetic dissimilarity 36% with Shial leji (G17) and Aijan-2 (G8).

Shial leji shows maximum genetic dissimilarity 64% with Dudlucky (G3) and Kachkolom (G9). 55% with Bashiraj (G2) and Dolkachu (G5). 36% with Balam dhan (G4), Aijan-1 (G6) and Aijan-

2 (G8). 27% with Jhul dhan (G18). Shial leji shows minimum genetic dissimilarity 18% with Ranga Jabra (G7).

Jhul dhan shows maximum genetic dissimilarity 73% with Bashiraj (G2) and Kachkolom (G9). 64% with Dudlucky (G3) and Dolkachu (G5). 55% with Balam dhan (G4), Aijan-1 (G6) and Aijan-2 (G8). Jhul dhan shows minimum genetic dissimilarity 45% with Ranga Jabra (G7).

Bashiraj shows maximum genetic dissimilarity 55% with Dudlucky (G3) and Ranga Jabra (G7). 45% with Aijan-2 (G8) and Kachkolom (G9). Bashiraj shows minimum genetic dissimilarity 36% with Balam dhan (G4), Dolkachu (G5) and Aijan-1 (G6).

Dudlucky shows maximum genetic dissimilarity 73% with Kachkolom (G9). 64% with Dolkachu (G5) and Ranga Jabra (G7). 45% with Balam dhan (G4) and Aijan-2 (G8). Dudlucky shows minimum genetic dissimilarity 36% with Aijan-1 (G6).

Balam dhan shows maximum genetic dissimilarity 45% with Dolkachu (G5) and Kachkolom (G9). 36% with Ranga Jabra (G7). 18% with Aijan-2 (G8). Balam dhan shows minimum genetic dissimilarity 9% with Aijan-1 (G6).

Dolkachu shows maximum genetic dissimilarity of 55% with Ranga Jabra (G7) and Aijan-2 (G8). 45% with Aijan-1 (G6). Dolkachu shows minimum genetic dissimilarity of 18% with Kachkolom (G9).

Aijan-1 shows maximum genetic dissimilarity 45% with Kachkolom (G9). 36% with Ranga Jabra (G7). Aijan-1 shows minimum genetic dissimilarity of 9% with Aijan-2 (G8).

Ranga Jabra shows genetic dissimilarity of 36% with Aijan-2 (G8).

Shah *et al.* (2013) has evaluated that the genetic diversity of 40 rice accessions using 24 microsatellite markers are distributed over the whole rice genome and from their a total 66 alleles were detected. Polymorphism Information Content (PIC) value ranged from 0.0476 (RM315) to 0.5993 (RM252), with an average of 0.3785 per marker and average genetic diversity overall SSR loci was 0.4477 ranging from 0.0488 to 0.6638. The result is similar of the present findings regarding genetic diversity in rice germplasm.

Table 8: Genetic Distance among 18 Aman rice germplasm

OTU	G1	G10	G11	G12	G13	G14	G15	G16	G17	G18	G2	G3	G4	G5	G6	G7	G8	G9
G1	0.00																	
G10	0.45	0.00																
G11	0.36	0.45	0.00															
G12	0.45	0.55	0.36	0.00														
G13	0.45	0.64	0.55	0.36	0.00													
G14	0.64	0.82	0.73	0.55	0.64	0.00												
G15	0.55	0.55	0.55	0.45	0.36	0.64	0.00											
G16	0.55	0.64	0.55	0.45	0.64	0.73	0.45	0.00										
G17	0.45	0.45	0.36	0.36	0.55	0.73	0.45	0.36	0.00									
G18	0.55	0.64	0.45	0.55	0.55	0.73	0.64	0.45	0.27	0.00								
G2	0.36	0.55	0.64	0.45	0.45	0.55	0.45	0.64	0.55	0.73	0.00							
G3	0.45	0.64	0.55	0.55	0.27	0.64	0.45	0.55	0.64	0.64	0.55	0.00						
G4	0.36	0.36	0.45	0.27	0.36	0.55	0.36	0.45	0.36	0.55	0.36	0.45	0.00					
G5	0.36	0.64	0.64	0.55	0.55	0.36	0.55	0.64	0.55	0.64	0.36	0.64	0.45	0.00				
G6	0.27	0.27	0.36	0.27	0.36	0.55	0.36	0.45	0.36	0.55	0.36	0.36	0.09	0.45	0.00			
G7	0.55	0.45	0.36	0.27	0.55	0.55	0.36	0.45	0.18	0.45	0.55	0.64	0.36	0.55	0.36	0.00		
G8	0.36	0.27	0.36	0.36	0.45	0.64	0.27	0.36	0.36	0.55	0.45	0.45	0.18	0.55	0.09	0.36	0.00	
G9	0.55	0.55	0.64	0.64	0.64	0.45	0.45	0.64	0.64	0.73	0.45	0.73	0.45	0.18	0.45	0.55	0.36	0.00

Legend: G1. Baila digha, G2. Bashiraj, G3. Dudlucky, G4. Balam dhan, G5. Dolkachu, G6. Aijan-1, G7. Ranga Jabra, G8. Aijan-2, G9. Kachkolom, G10. Sorna dhan, G11. Jol kowcha, G12. Kuriaguni, G13. Kartiksail, G14. Begunbichi, G15. Debmoni, G16. Sissumoti, G17. Shial leji, G18. Jhul dhan.

4.4 UPGMA dendrogram

Based on the Nei's genetic distance calculation of 18 Aman rice germplasm , a dendrogram was established. Unweighted Pair Group Method of Arithmetic Mean (UPGMA) indicated the segregation of 18 Aman rice genotypes into five main clusters: The genotypes G1(Baila digha), G10(Sorna dhan), G15(Debmoni), G2(Bashiraj), G4(Balam dhan), G6(Aijan-1), G8(Aijan-2), G13(Kartiksail), G3(Dudlucky), G5(Dolkachu) and G9(Kachkolom) were grouped in cluster I. The germplasm G16 (Sissumoti), G17 (Shial leji), and G7 (Ranga Jabra) were grouped in cluster II. The local aman rice germplasm G11 (Jol kowcha) and G12 (Kuriaguni) were grouped in cluster III. The landrace G14 (Begunbichi) were grouped in cluster IV. The germplasm G18 (Jhul dhan) were grouped in cluster V. Here, "G" indicated the Aman rice genotypes.(Table 9).

In this dendrogram, Aman rice genotypes of more genetic similarity are placed in the same cluster. The dendrogram revealed that the genotypes that derivatives of genetically similar type clustered together. The genotypes are 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. Cluster-I genotypes, Cluster-II genotypes, Cluster-III genotypes, Cluster-IV genotypes and Cluster-V genotypes are more different from each other genotypes. Present study shows similarity with Yasmin *et al.* (2012). So, they can be easily used for the rice improvement program. Yasmin *et al. (*2012) reported that genetic diversity of 24 rice (*Oryza sativa*) genotypes comprising five salts tolerant, one tidal submergence tolerant, 9 high yielding inbred and 10 hybrid rice varieties with one hundred SSR markers. The highest number of alleles were produced for RM1024 followed by RM6959 (21), RM154 (20), RM540 (19) and RM2518 (19) .The polymorphism information content (PIC) value ranged from 0.588 (RM38) to 0.927 (RM1024), with an average of 0.852. UPGMA-cluster-analysis based on genetic distance coefficients separated all the genotypes into five main distinct clusters.

Principle Co-ordinate Analysis (PCoA):

From fig 10, We can say that, germplasm G2. Bashiraj, G3. Dudlucky, G4. Balam dhan, G5. Dolkachu, G6. Aijan-1, G7. Ranga Jabra, G8. Aijan-2, G10. Sorna dhan and G15. Debmoni are closely linked with each other and they shows homogenesis in nature. Again, germplasm G12

(Kuriaguni), G17 (Shial leji), G11 (Jol kowcha), G1 (Baila digha), G9 (Kachkolom), G14 (Begunbichi), G13 (Kartiksail), G18 (Jhul dhan) they are diversified from each other.

Table 9. List of aman rice germplasm with their cluster based on UPGMA dendrogram

Cluster	Genotypes
I	G1(Baila digha), G10(Sorna dhan), G15(Debmoni), G2(Bashiraj), G4(Balam dhan), G6(Aijan-1), G8(Aijan-2), G13(Kartiksail), G3(Dudlucky), G5(Dolkachu), G9(Kachkolom).
II	G16 (Sissumoti), G17 (Shial leji), G7 (Ranga Jabra)
III	G11 (Jol kowcha), G12 (Kuriaguni).
IV	G14 (Begunbichi).
V	G18 (Jhul dhan).

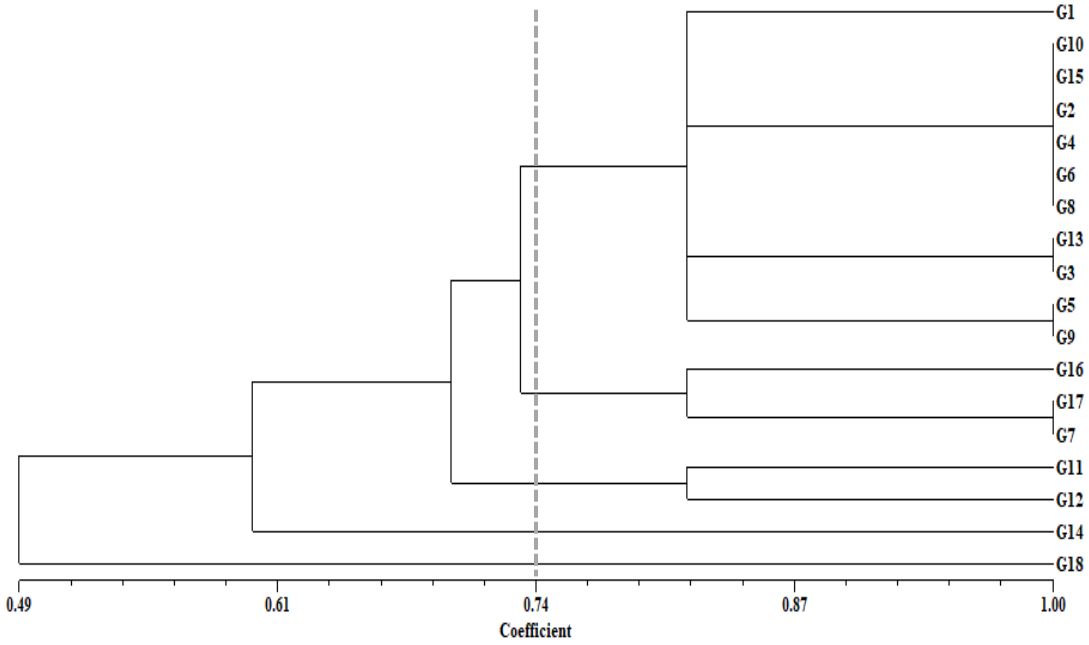

Fig 9: An UPGMA cluster dendrogram showing the genetic relationships between 18 Aman rice landraces of Bangladesh based on the alleles detected by 11 microsatellite markers

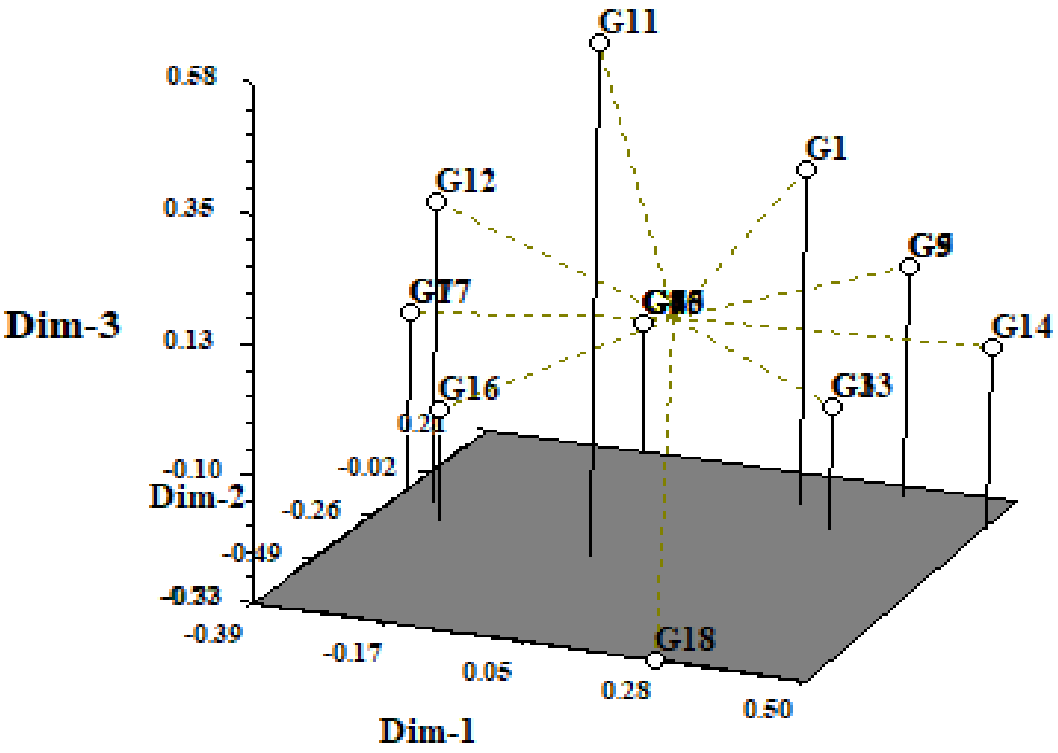

Fig 10: Three-dimensional view of principal coordinate analysis (PCA) with 11 microsatellite markers over 18 Aman rice genotypes

CHAPTER-V

SUMMARY AND CONCLUSION

Agriculture is the single largest economic sector of Bangladesh. Bangladesh is currently self-sufficient for rice production in the context of shrinking rice land and water resources. Rice diversity as well as its traits and genes, need to be considered in future breeding program for developing new varieties. Hence, molecular diversity analysis can help to select the best diverse genotype in rice improvement program.

In this present study, Aman rice genotypes were used for PCR amplification and eleven well rice genome specific SSR primers viz. RM170, RM190, RM205, RM206, RM208, RM228, RM252, RM283, RM314, RM455 and RM44 were selected and synthesized for molecular diversity analysis. All of them showed polymorphic amplification. The primers identified 38 alleles, which ranged from 02 to 06 alleles per locus and average value is 3.45. It was observed that, the frequency of the major allele ranged from 27.78 (RM252) to 94.44 (RM455). Primer RM206 showed highest polymorphic information content (PIC) value 0.75 and primer RM455 showed lowest PIC value 0.10 and mean PIC value was 0.42. Again, primer RM455 showed lowest genetic diversity 0.10 and Primer RM206 showed highest genetic diversity 0.78 and mean value is 0.45. Present study indicated that, RM206 was considered as the best marker for 18 Aman rice germplasm followed by others and RM455 was considered as the least powerful marker for those genotypes. Genetic distance obtained from this study ranged from 0.09 to 0.82 with an average of 0.46. The highest genetic dissimilarity (0.82) was observed between the genotypes Sorna dhan and Begunbichi and the lowest dissimilarity (0.09) was observed between genotypes Aijan-1 and Aijan-2. Based on Nei's (1983) genetic distance calculation of 18 relatives of Aman rice genotypes, a dendrogram was calculated. Unweighted Pair Group Method of Arithmetic Mean (UPGMA) indicated the segregation of 18 Aman rice genotypes into five major clusters.

The result obtained from the present study showed high genetic diversity among the rice germplasm. Cluster showed genetic similarity and dissimilarity among all the varieties. SSR markers are powerful tools to detect genetic variation and genetic relationships within and among different rice genotypes. The study result can be information for further research on genetic variation and diversity analysis of rice germplasm of Bangladesh.

CHAPTER-VI

RECOMMENDATION

The results obtained from the present study on molecular diversity analysis provides some useful implications for establishment of sovereignty of Bangladeshi Aman rice gene pool. It is revealed that there was a high level of genetic diversity among accessions of Aman rice. From this present study it is recommended that SSR markers were very effective in polymorphism detection. The current study is widely acceptable in all concern regarding Aman rice germplasm. The present study can be used as a guideline/suggestion for the next researchers who have concern for experimenting rice. Following points might be considered for studying the genetic qualities of Aman rice in Bangladesh.

- ✓ The large number of SSR marker could be used for attaining more accurate and diverse results.
- ✓ For getting more precise and accurate results high throughput molecular markers such as Single Nucleotide Polymorphism (SNP) could be used for genome-wide coverage of rice germplasm.

CHAPTER-VII

REFERENCES

Agrama H, Eizenga G. Molecular diversity and genome-wide linkage disequilibrium patterns in a worldwide collection of *Oryza sativa* and its wild relatives. Euphytica. 2008;160(3):339–355. doi: 10.1007/s10681-007-9535-y.

Ahmed, M.S.U., Khalequzzaman, M., Bashar, M.K., Shamsuddin, A.K.M. (2016). Agro Morphological, Physico-Chemical and Molecular Characterization of Rice Germplasm with Similar Names of Bangladesh. Rice Sci. 23(4): 211-218.

Ahmed, M.S., Rashid, E. S. M. H., Akter, N. and Khalequzzaman, M. (2018). Morphological characterization and diversity of T. Aman rice germplasm of Bangladesh. Bangladesh Rice J. 22 (2) : 13-22.

Aljumaili, S.J., Rafii, M.Y., Latif, M.A., Sakimin, S.Z., Arolu, I.W. and Miah, G. (2018). Genetic Diversity of Aromatic Rice Germplasm Revealed By SSR Markers. BioMed Res. Intl. 2018: 11.

Altaf Khan, M., Qureshi, S.N., Saha, S., Jenkins, J.N., Brubaker, C.L. and Reddy, O.U.K.(2006). Usefulness of SSR Derived from Tetraploid Gossypium spp. for Analyses of Diploid Gossypium spp. J. Crop Improv. 16:1-20. American J. of Hum. Genet. 44: 397–401.

Andrson, J. A., Churchill, G. A., Autrique, J. E., Tanksley, S. D. and Sorrels, M. E. (1993). Optimizing parent selection for genetic linkage maps. Genome. 36: 181- 186.

Anonymous, (2002). National Workshop on Rice Research and Extension-2002. Feeding the extra millions by 2025. Bangladesh Rice Res. Inst. Gazipur, p: 1.

Anonymous. (2009). Statistical Pocket Book of Bangladesh (Bangladesh Burea of Statistics), Ministry of Planning, Government of the People Republic of Bangladesh, pp: 175.

Bajracharya, J., Steele, K.A., Witcombe, J.R., Sthapit, B.R. and Jarvis, O. I. (2004). Molecular marker diversity in rice (Oryza sativa L.) landraces of Nepal. In: Proceedings of the Second

National Workshop on On-farm Conservation of Agricultural Biodiversity in Nepa1. Nagarkot, Nepal. pp. 25-27.

BBS. 2005. The statistical Yearbook of Bangladesh. Bangladesh Bureau of Statistics, Ministry of planning .Dhaka. Bangladesh.

BBS. 2018. Year book of agricultural statistics 2018. 30th ed. Bangladesh Bureau of Statistics, Statistics and Informatics Division, Ministry of Planning, Gov. of the People's Republic of Bangladesh. www.bbs.gov.bd.

Bedoya, C. A., Dreisigacker, S., Hearne, S., Franco, J., Mir,C. and Prasanna, B.M.(2017). Genetic diversity and population structure of native maize populations in Latin America and the Caribbean. PLoS ONE. 12(4): e0173488.

Behera, L. and Joshi, R.K. (2006). Identification and differentiation of indigenous non-Basmati aromatic rice genotypes of India using microsatellite markers. Afr. J. Biotech. 6(4): 348-354.

Bhuiyan, N.I., Paul, D.N.P. and Jabbar, M.A. (2002). Feeding the extra millions by 2025 challenges for rice research and extension in Bangladesh. Extended summary, Natl. Workshop on Rice Research and Extension, Jan. 29-31, BRRI, Gazipur, Bangladesh. p. 9.

Causse, M.A., Fulton, T.M., Cho, Y.G., Ahn, S.N. and Chunwongse, J. (1994). Saturated molecular map of the rice genome based on an interspecific backcross population. Genetics. 138: 1251-1274.

Chakravarthi, B.K. and Naravaneni, R. (2006). SSR marker based DNA fingerprinting and diversity study in rice (Oryza sativa L.). African J. Biotech. 5(9): 684-688.

Chen H, He H, Zou Y, Chen W, Yu R, Liu X, Yang Y, Gao Y-M, Xu J-L, Fan L-M (2011) Development and application of a set of breeder-friendly SNP markers for genetic analyses and molecular breeding of rice (*Oryza sativa L.*). Theor Appl Genet. 123(6):869–879.

Chen, C., He, W., Nassirou, T.Y., Nsabiyumva, A., Dong, X., Adedze, M.N., Jina, D. (2017). Molecular characterization and genetic diversity of different genotypes of Oryza sativa and Oryza glaberrima (research article). Elect. J. of Biotech. 30: 48-57.

Dutta, R K,: Lahiri, B.P., and Mia., M.A.B.; (1998). Characterization of some aromatic and fine rice cultivars in relation to their phsico-chemical quality of grains. Indian J. Plant Physil. 3 (1): 61-64.

FAO (2016). FAOSTAT Production Database. http://faostat3.fao.org./home/E.

Ferdous, J., Hanafi, M. M., Rafii, M. Y. and Muhammad, K. (2012). A quick DNA extraction protocol: Without liquid nitrogen in ambient temperature. Afr. J. Biotech. 11(27): 6956-6964.

Garris, A.J., Tai, T.H., Coburn, J., Kresovich, S. and McCouch S.R. (2005) Genetic structure and diversity in Oryza sativa L. Genetics 169:1631–1638.

Ghneim, H. T., Poso, D., Perez, I., Torrealba, G. N., Pieters, A., Martinez, C. and Tohme, J. (2008). Assessment of genetic diversity in Venezuelan rice cultivars using simple sequence repeats marker. Electronic J. of Biot. 11(5): 1-14.

Gohain B., A. Talukdar and M. K. Modi. (2006). Mining of allele(s) for aroma in local aromatic rice (joha) germplasm of Assam using SSR marker. Abstracts 26th International Rice Conference-2, Int. Rice Congress, October 9-13, 2006.

Gupta, R., Tetwar, S. and Nair, S. K. (2013). Agro-morphological characterization of rice germplasm of Chhattisgarh. Int. J. Plant Sci., 9(1): 257-262.

Halder,T., Hoque,M.E., Islam, M.M., Ali, L. and A. K. Chowdhury, A.K.(2016). Morpho-molecular characterization of Bangladeshi local boro rice (Oryza Sativa L.) genotypes. Bangladesh J. Pl. Breed. Genet, 29(2): 01-09.

Hien, N.L., Sarhadi, W.A., Hirata, Y. and Oikawa, Y. (2007). Genetic diversity of morphological responses and the relationships among Asia aromatic rice (Oryza sativa L.) cultivars. Tropics 16: 343–355.

Hooker, J.D. (1979). The floral of British India. Reeve. Co. Kent. England. 21: 310-315.

Hossain, M.Z., Rasul, M.G., Ali, M.S. Iftekharuddaula, K.M. and Mian, M.A.K.(2007).Molecular characterization and genetic diversity in fine grain and aromatic landraces of rice (Oryza sativa L.) using microsatellite markers. Bangladesh J. Genet Pl. Breed, 20(2): 01-10.

Hossain, M.M., Islam, M.M., Hossain, H., Ali, M.S., Jamie, A., Komamine, A. and Prodhan, S.H. (2012). Genetic diversity analysis of aromatic landraces of rice (Oryza sativa L.) by microsatellite markers. Genes, Genomes and Genomics, 6: 42-47.

Hunter, M. (2017). We don't need to double world food production by 2050 – here's why. Retrieved from: http://theconversation.com/we-dont-need-to-double-worldfood-production-by-2050-heres-why-74211.

Islam, M. Z., Khalequzzaman, M., Bashar, M.K., Ivy, N.A., Haque, M.M. and Mian, M.A.K.(2016). Variability assessment of aromatic and fine rice germplasm in Bangladesh based on quantitative traits. The Sci.World J. Volume 2016, Article ID 2796720, 14 pages.

Islam, M.Z., Akter, N., Chakrabarty, T., Bhuiya, A., Siddique, M.A. and Khalequzzaman, M.(2018). Agro-morphological characterization and genetic diversity of similar named aromatic rice (Oryza sativa L.) landraces of Bangladesh. Bangladesh Rice J. 22 (1): 45-56.

Islam, M.Z., Khalequzzaman, M., Bashar M.K., Ivy, N.A., Haque, M.M., Mian, M.A.K. and Tomita, M. (2018).Agro-morphological characterization of bangladeshi aromatic rice (Oryza sativa L.) germplasm based on qualitative traits. Bangladesh Rice J. 22 (2) : 41-54.

Jackson, M.T., 1995. Protecting the heritage of rice biodiversity. Geo J. 35, 267–274.

Jain, S., S. E. Mitchell, R. K. Jain, S. Kresovich and S. R. McCouch, (2003). DNA fingerprinting and phylogenetic analysis of Indian aromatic high-quality rice germplasm using panels of fluorescent-labeled microsattelite markers. In: Advance in Rice Genetics ed. by Khush et al. IRRI, Philippine. 162-166.

Jain, S., Rajinder, K.J. and McCouch, S.R. (2004). Genetic analysis of Indian aromatic and quality rice (Oryza sativa L.) germplasm using panels of fluorescently-labelled microsatellite markers. Theor. Appl. Genet. 109: 965-977.

Jalaluddin, M., Nakai, H., Yamamoto, T. (2007). Genetic diversity and DNA fingerprinting of some modern Indica and Japonica rice. SABRAO J. Breed. and Genet. 39(1): 43-52.

Jin L, Lu Y, Xiao P, Sun M, Corke H, Bao J (2010) Genetic diversity and population structure of a diverse set of rice germplasm for association mapping. Theor Appl Genet. 121(3):475–487.

Karkousis, A., Barr, A.R., Chalmers, K.J., Ablett, G.A., Holton, T.A., Henry, R.J., Lim, P. and Langridge, P. (2003). Potential of SSR markers for plant breeding and variety identification in Australian Barley germplasm. Aust. J. Agric. Res. 54(1): 197–1,210.

Khodadadi, M., Fotokian, M.H. and Miransari, M. (2014). Genetic diversity of wheat (Triticum aestivum L.) genotypes based on cluster and principal component analyses for breeding strategies. AJCS, 5(1): 17-24.

Kibria, K., Nur, F., Begum, S. N., Islam, M. M., Pau, S. K., Rahman, K. S. and Azam, S. M. M.(2009). Molecular marker based genotypic diversity analysis in aromatic rice genotypes using SSR and RAPD markers. Intl. J. Sust. Crop Prod. 4: 23-34.

Kostova, A., Todorovska, E., Christov, N., Hristov, K. and Atanassov, A. (2006). Assessment of Genetic Variability Induced by Chemical Mutagenesis in Elite Maize Germplasm via SSR Markers. J. Crop Improv. 16:37-48.

Lapitan, V. C., Brar, D. S., Abe, T. and Redofia, E. D. (2007). Assessment of genetic diversity of Philippine rice cultivars carrying good quality traits using SSR markers. Breed. Sci. 57(4): 263-270.

Liakat Ali M, McClung AM, Jia MH, Kimball JA, McCouch SR, Georgia CE (2011) A rice diversity panel evaluated for genetic and agro-morphological diversity between subpopulations and its geographic distribution. Crop Sci 51(5):2021–2035.

Litt, M. and Luty, J.A. (1989). A hyper variable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle acting gene.

Liu, K., Muse, S. V. (2005). Power Marker: Integrated analysis environment for genetic marker data. Bioinformatics. 21: 2128–2129.

Malek, M. A., Rafii, M.Y., Afroz, M.S.S., Nath,U.K. and Mondal, M.M.A. (2014). Morphological characterization and assessment of genetic variability, character association, and divergence in Soybean mutants. The Sci. World J.1-12.

Mamunur Rahman,M., Rasul, M.G., Hossain, M.A. Iftekharuddaula, K.M. and Hasegawa, H.(2012) Molecular Characterization and Genetic Diversity Analysis of Rice (Oryza sativa L.) using SSR Markers. J. of Crop Improvement, 26:244–257.

Mehla, B. S. and Kumar, S. (2008). Use of Morphological Traits as Descriptors for Identification of Rice Genotype. Agric. Sci. Digest, 28(2): 104.

Momin, S. I. and Husain, M. (2009). Technology development and dissemination to augment rice production in Bangladesh. In:'The Guardian'. pp. 33-35.

Nachimuthu, V.V., Muthurajan, R., Duraialaguraja, S., Sivakami, R., Pandian, B.A., Ponniah, G., Gunasekaran, K., Swaminathan, M., Suji, K.K., Sabariappan, R. (2015). Analysis of Population Structure and Genetic Diversity in Rice Germplasm Using SSR Markers: An Initiative Towards Association Mapping of Agronomic Traits in Oryza Sativa. Rice. 8:30.

Nadia, I., Mohiuddin, A.K.M., Sultana, S. and Ferdous J. (2014). Diversity analysis of indica rice accessions (Oryza sativa L.) using morphological and SSR markers. Annals of Biol. Res. 5(11): 20-3.

Nei M (1973) Analysis of gene diversity in subdivided populations. Proc Natl Acad Sci 70(12):3321–3323.

Okello, M., Sawardekar, S.V., Ghokhale, N. B., Waghmode, B.D. and Patil, D.M. (2017). Molecular characterization of rice germplasm (Oryza sativa L.) using simple sequence repeat (SSR) markers. Advanced Agricultural Research & Technology J. Vol. I Issue I.

Perez-Sackett, P.T., Cianzio, S.R., Kara, P.C., Aviles, M. And Palmer, R.G. (2011). QTL Mapping of Whitefly Resistance in Soybean. J. Crop Improv. 25:134-150.

Powell W., Machray G. C. and Provan J. (1996). Polymorphism revealed by simple sequence repeats. Trends Plant Sci. 1:215-222.

Rahman, L., Molla, M.R, Sultana, S., Islam, M.N., Ahmed, N.U., Rahman, M.S. and Nazim-ud-Dowla, M. (2007c). Plant varieties of Bangladesh: morphological and molecular characterization. Seed Wing, Ministry of Agriculture, Government of the Peoples' Republic of Bangladesh, Bangladesh.1:486.

Rahman, L., Molla, M.R., Sultana, S., Islam, M.N., Ahmed, N.U., Rahman, M.S., Nazim-ud-Dowla, M., Shah-E-Alam, M. and Alam, M.S. (2006). Plant varieties of Bangladeshmorphological and molecular characterization for plant variety protection. Bangladesh J. Agr. Sci. 33: 215-225.

Rahman, M.S., Easmin, F., Islam, M.S., Samad, M.A. and Alam ,M.S. (2007b). Random amplified polymorphic DNA (RAPD) analysis in some indigenous aromatic rice (Oryza sativa L.) cultivars. Bangladesh J. Crop Sci 18:331–340.

Rahman, M.S., Molla , M.R., Alam, M.S. and Rahman, L. (2007a). DNA fingerprinting of rice (Oryza sativa L.) cultivars using microsatellite markers. In: Poster presentation in the 1st conference on ''Promotion of Biotechnology in Bangladesh: National and International Perspectives'', held on 06–08 April at the University of Dhaka, Bangladesh.

Rajendrakumar, P., Biswal,A., Sakthivel,K., Madhav,M., Neeraja,C., Balachandran,S., Srinivasarao,K., Natarajkumar,P., Hari,Y., Sujatha,K. and Sundaram,R. (2009). Development and validation of class I SSR markers targeting (GATA) n repeat motifs in rice. Euphytica 169:263-271.

Ramkumar G, Biswal AK, Mohan KM, Sakthivel K, Sivaranjanj AKP, Neeraja CN, Ram T, Balachandran SM, Sundaram RM, Prasad MS (2010) Identifying novel alleles of rice blast resistant genes pikb and pita through allele mining. Int. Rice Res Notes 117:185.

Rani, M.G. and Adilakshmi, D. (2011). Genetic Analysis of Blast Resistance in Rice with Simple Sequence Repeats (SSR). J. Crop Improv. 25:232-238.

Roy. S., Marndi, B., Mawkhlieng, B., Banerjee, A., Yadav, R. and Misra, A. (2016). Genetic diversity and structure in hill rice (Oryza sativa L.) landraces from the North-Eastern Himalayas of India. BMC Genet. 17(1): 107.

Sajib, M.A., Hossain, M.M., Mosnaz, A.T.M.J., Hossain, H., Islam, M.M., Ali, M.S. and Prodhan, S.H. (2012). SSR marker based molecular characterization and genetic diversity Analysis of Aromatic Landraces of Rice (Oryza sativa L.). J. BioSci. Biotech. 1(2): 107-116.

Sajid, M., Shahid, A.K., Haris, K., Javed, I., Ali, M.N.S. and Syed, M.A.S. (2015). Characterization of rice (Oryza Sativa L.) germplasm through various agro-morphological traits. Sci. Agri., 9(2), 83-88.

Salgotra, R.K., Gupta, B.B., Bhat, J.A., and Sharma, S. (2015). Genetic diversity and population structure of Basmatirice (Oryza sativa L.) germplasm collected from North Western Himalayas using trait linked SSR markers. Plos one. 10(7): 1-16.

Sarao, N.K., Vikal,Y., Singh,K., Joshi,M.A. and Sharma,R.C. (2010). SSR marker-based DNA fingerprinting and cultivar identification of rice (Oryza sativa L.) in Punjab state of India. Plant Genet. Res. 8:42-44.

Sarawgi, A.K., Subba Rao, L.V., Parikh, M., Sharma, B. and Ojha, G.C. (2013). Assessment of Variability of Rice (Oryza sativa L.) Germplasm using Agro-morphological Characterization. J. of Rice Research, Vol. 6 No.1.

Seetharam, U., Thirumeni, S., Paramasivam, K. (2009). Estimation of genetic diversity in rice (Oryza sativa L.) genotypes using SSR markers and morphological characters. African J. of Biot. 8: 2050-2059.

Shah, S.M., Naveed, S.A. and Arif, M. (2013). Genetic diversity in basmati and nonbasmati rice varieties based on microsatellite markers. Pak. J. Biot. 45(S1): 423-431.

Sharma, R.C., Chaudhary, N.K., Ojha, B., Yadav,L., Pandey, M.P. and Shrestha, S.M.(2007). Variation in rice landraces adapted to the lowlands and hills in Nepal. Plant Genet.Res. 5:120-127.

Siddique, M.A., Khalequzzaman, M., Fatema, K., Islam, M.Z., Islam, M.M. and Chowdhury, M.A.Z. (2016). Molecular Characterization and Genetic Diversity of Aman Rice (Oryza sativa L.) Landraces in Bangladesh. Ban. Rice J. 20(2): 1-11.

Siddique, M.A., Rashid, E.S.M.H., Khalequzzaman, M., Bashar, M.K. and Khan, L.R. (2014). Molecular characterization and genetic diversity in T. Aman landraces of rice (Oryza sativa L.) Using Microsatellite Markers. Thai J. of Agri. Sci, 47(4): 211-220.

Singh, A., Saini, R., Singh, J., Arya, M., Ram, M. and Singh, P.K. (2015). Genetic diversity studies in rice (Oryza Sativa L.) using microsatellite markers. International Journal of Agriculture, Environment & Biotechnology, 8: 143-152. doi: 10.5958/2230-732X.2015.00019

Singh, N., Choudhury, D.R., Tiwari, G., Singh, A.K., Kumar, S., Srinivasan, K., Tyagi, R.K., Sharma, A.D., Singh, N.K. and Singh, R. (2016). Genetic diversity trend in Indian rice varieties: an analysis using SSR markers. BMC Genetics. 17: 127.

Sinha, A.K., Mallick, G.K. and Mishra, P.K. (2015). Grain morphological diversity of traditional rice varieties (Oryza sativa L.) in lateritic region of West Bengal. Int. J. Consv. Sci., 3(6): 419-426.

Siwach, P., S. Jain, N. Saini, V. K. Chowdhury and R. K. Jain. 2004. Allelic Diversity among Basmati and Non-Basmati long grain indica Rice Varieties using microsattelite markers. J.Pl. Biochem. Biot. 13:25-32.

Surapaneni, M., Balakrishnan, D., Mesapogu, S., Raju, A.K., Rao, Y.V., Neelamraju, S. (2016). Genetic characterization and population structure of Indian rice cultivars and wild genotypes using core set markers. Biotech. 6: 95.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA 5.1: molecular evolutionary genetics analysis using maximum likelihood, evolutionary

distance, and maximum parsimony methods. Mol. Biol. Evol. 28(10): 2731±9. https://doi.org/10.1093/molbev/msr121. PMID: 21546353.

Thimmanna, D., Jagadish, G. V. and Venkataramana, F. (2000). Diagnostic morphological characteristics of the parents of Karnataka rice hybrids. Karnataka J. of Agri. Sci. 13(3): 729-732.

Thomson, M. J, E. M. Septiningsih, F. Suwardjo, T. J. Santoso, T. S. Sililonga and S. R. McCouch. (2007). Genetic diversity analysis of traditional and improved Indonesian rice (O sativa L) germplasm using microsattelite markers. Theor. Appl. Genet. 114: 559-568.

Tu, M., B.R. Lu., Zhu.Y. and Wang.Y. (2007). Abundant within-varietal genetic diversity in rice germplasm from Yunnan Province of China revealed by SSR fingerprints. Biochem. Genet. 45:789-801.

Umadevi, M., Veerabadhiran, P. and Manomani, S. (2014). Assessment of genetic diversity of rice (*Oryza sativa*) cultivars using simple sequence repeat (SSR) markers. African J. of Biot, 13: 3547-3552.

UNDESA (United Nations Department of Economic and Social Affairs). (2017). the World Population Prospects. UN Publications.

Vabna, F.A. (2018). Molecular characterization and genetic diversity analysis of Boro rice germplasm using SSR markers. M.Sc. thesis, SAU, Dhaka, Bangladesh.

Venkatesan, K., Bhat, K.V. (2015). Microsatellite marker-based molecular characterization of small and medium-grained aromatic rice germplasm of Odisha, India. SABRAO J. of Breed and Genet. 47(3): 248-259.

Wang, Q., X. Sang, Y. Ling, F. Zhao, Z. Yang, Y. Li and G. He. 2009. Genetic analysis and molecular mapping of a novel gene for zebra mutation in rice (Oryza sativa L.). J. of Genet. and Genomics, 36: 679-684.

Weising, K., Winter, P., Hüttel, B. and Kahl,G. (1997). Microsatellite Markers for Molecular Breeding. J. Crop Prod. 1:113-143.

Yasmin, F., Islam, M. R., Rehana, S., Mazumder, R. R., Anisuzzaman, M., Khatun, H., Rayhan, R. and Gregorio, G. B. (2012). SABRAO J. of Breed and Genet. 44 (1): 163-175.

Zhang P, Li J, Li X, Liu X, Zhao X, Lu Y (2011) Population structure and genetic diversity in a rice core collection (*Oryza sativa L.*) investigated with SSR markers. PLoS One 6(12):e27565.

APPENDICES

Appendix I.: Chemical preparation for DNA extraction and PCR work

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

Reagent	200 mL preparation
Tris-HCL (pH= 8.0)	40mL
EDTA (pH= 8.0)	10mL
NaCl	11.4mL
SDS	20mL
DD H_2O	118.6mL

I(b): Composition and preparation of the 2X CTAB solution

Reagent	200 mL preparation
Tris HCl (pH=8.0)	20mL
EDTA (pH=8.0)	08mL
NaCl	80mL
CTAB	04gm
PVP	02gm
DDH_2O	92mL

I(c): Composition and preparation of the chloroform: isoamyl alcohol (24:1) with 5% phenol (CIP)

Reagent	100 ml preparation
Chloroform	91.2 ml
Isoamyl alcohol	3.8 ml
Phenol	5 ml

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

Reagent	100ml preparation
1 M Tris (pH =8.0)	10 ml
0.5 M Na_2EDTA (pH= 8.0)	200 µl

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

Reagent	1 L preparation
Tris HCL (pH= 8)	108 g
EDTA	9.3g
Boric acid	55 g
Water	Up to 1 L

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

Reagent	1 L preparation
10 X TBE	100 ml
De-ionized water	900 ml

I(g): DNA dilution: (Working sample)

From the main stock of DNA 20µL of DNA was diluted with 180µL of de-ionized water.

I(h): Primer dilution

Primer was centrifuged at 13000 rpm for 5 min.

↓

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

↓

Kept overnight at 4^0 C

↓

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

↓

Stored at -20^0 C freezer

I(i): PCR cocktail for 96 samples

Reagent	**Amount**
DNA	288
Primer (F)	48
Primer (R)	48
Master mix	432
DDH_2O	144