MORPHO MOLECULAR DIVERSITY OF PIGMENTED TRANSPLANT AMAN RICE GERMPLASM

A THESIS

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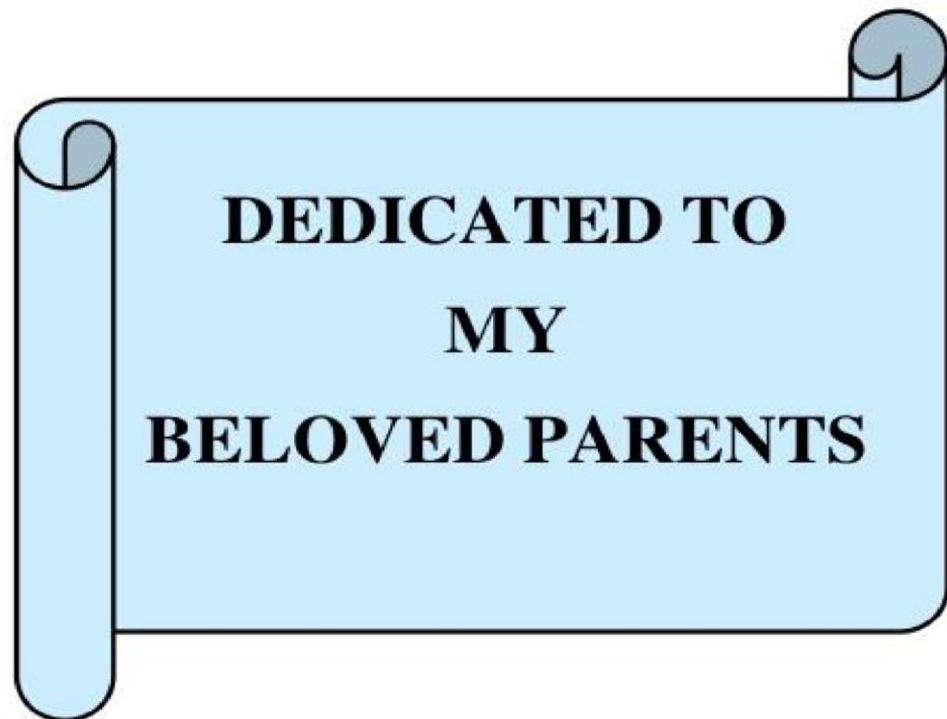Sher-e- Bangla Agricultural University

CERTIFICATE

This is to certify that the thesis entitled *"MORPHO MOLECULAR DIVERSITY OF PIGMENTED TRANSPLANT AMAN RICE GERMPLASM"* submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University (SAU), Dhaka in partial fulfillment of the requirements for the degree of **MASTERS OF SCIENCE (MS)** in **Biotechnology,** embodies the results of a piece of bona fide research work carried out by **MOMENA SULTANA**, Registration no. **13-05635** 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, 2020

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DEDICATED TO
MY
BELOVED PARENTS

ACKNOWLEDGEMENTS

June, 2020
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Abstract

Genetic divergence at morphological and molecular levels were studied in 24 pigmented Transplant Aman germplasms during the period June 2019 to December 2019 at the Genetic Resources and Seed Division (GRSD), Bangladesh Rice Research Institute, Gazipur-1701. All the pigmented germplasms studied genetic variation in respect of qualitative traits. Large dissimilarity and genetic diversity also detected in quantitative traits. The germplasm Black rice-2, Black Rice-3, Black Rice-4, Biroin (Habai), Kach Badal, Murali, Jhoria, Butu Balam (2), Latisail were identified as highly polymorphic and could be used for future breeding programs. A total of 70 alleles were detected from 16 microsatellite markers across 24 rice germplasms. The number of alleles per locus ranged from one (RM510) to eight alleles (RM1), with an average of 4.38 alleles across the 16 loci obtained in the study. The highest band size for a given microsatellite locus varied between 171bp to 350bp (RM1337). Gene diversity ranged from 0.15 to 0.82 with an average of 0.4843. The polymorphism information content (PIC) values which ranged from 0.14 (RM307) to 0.80 (RM1), with an average of 0.445 revealed much variation among the studied germplasm. Primer RM1 had the highest PIC value (0.80) and the highest number of alleles (8), therefore it detected the highest level of polymorphism. So, RM1 is considered to be the best marker for characterizing the 24 pigmented T. Aman rice germplasms. Besides, PIC value revealed that RM1, RM206, RM1337, RM5 and RM334 can also be considered as additional markers for characterizing 24 pigmented rice germplasms. Eighteen (16) unique alleles were detected by 9 SSR markers. The genetic distance was calculated using the Nei distance. The highest genetic distance was 0.8750 which was observed between Biruin (Tola) and Lal Balam. The genetic distance-based results seen in the unrooted neighbor-joining tree revealed three groups with six sub-clusters in the 24 test germplasm. Evaluation of morpho-molecular characters demonstrated that the pigmented T. Aman rice germplasm under the present study possess a high genetic diversity and showed unique features of valuable genes for safe conservation in Gene banks and their sustainable utilization in future breeding programs.

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ABBREVIATIONS AND ACRONYMS

FULL WORD	ABBREVIATION
Transplant Aman	T. Aman
Hectare	Ha
Accession	Acc.
Analysis of Variance	ANOVA
New Collection	NC
Milimeter	mm
Centimeter	cm
Maximum	Max.
Minimun	Min.
Flag Leaf Area	FLA
Culm Diameter	CD
Total Tiller	TT
Effective Tiller	ET
Panicle Length	PL
Plant Height	PH
Days to 50% Flowering	DF
Days to Maturity	DM
Filled Grains per Panicle	FG/P
Grain Length	GL
Grain Breadth	GB
Decorticated Length Breadth Ratio	DL/B
Yield per Hill	Y/H
Coefficient of Variation	CV
Standard Deviation	SD
Standard Error	SE
Least Significance Difference	LSD
Chromosome	Chro.
Frequency	Freq.
Bangladesh Rice Research Institute	BRRI
Bangladesh Bureau of Statistics	BBS
Base pair	Bp
Cetyl Trimethyl Ammonium Bromide	CTAB
Double Distilled Water	ddH_2O
Distilled Water	dH_2O
De-oxy ribonucleic Acid	DNA
Ethylene Diamine Tetra Acetic Acid	EDTA
And others	et al.
Etcetera	etc.
Food and Agriculture Organization	FAO
Gram	G
Genotype	G
Genetic Distance	GD
Gram per Liter	g/l

Mili liter	Ml
Sodium chloride	NaCl
Sodium salt of ferric Ethylene Diamine Tetra Acetic Acid	Na2EDTA
Polymerase Chain Reaction	PCR
Negative Logarithm of Hydrogen ion concentration	Ph
Simple Sequence Repeats	SSR
Single Nucleotide Polymorphism	SNP
Sodium Dodecyle Sulphate	SDS
Thermophilus aquaticus	Taq
Tris Boric Acid EDTA	TBE
Tetra Methyl Ethylene Diamine	TEMED
Tris-EDTA	TE
Tons	T
Unweighted Pair Group of Arithmetic Mean	UPGMA
Ultraviolet	UV
Volt	V
Namely	Viz.

CHAPTER I

INTRODUCTION

Rice (*Oryza sativa* L.) is a self-pollinated cereal crop belonging to the family Gramineae which have chromosome number 2n=24 (Hooker, 1979). Rice (*Oryza sativa*) is one of the most important staple crops that offer food for more than 50% of the world's population (Palanog *et al*., 2019). Because of its wide range of geographical, ecological, and climatic regions adaption, rice is one of the most diversified crop species as a major cereal crop. (Yadav *et al.,* 2013).

Bangladesh is the third largest rice producer country in the world. Rice is the staple food for her people and will keep on so in the future. More than 90% of the people depend on rice for their diet. Rice is covered about 75% of the total cropped area and more than 60% of the total agriculture labour force is employed in rice production, processing, marketing and distribution. This crop provides about 75% calorific and 55 % protein content of the average daily human diet (Bhuiyan *et al*., 2002). It also confirms political stability for the country and provides food security sense to the people. There are three different ecotypes of rice such as Aus, T. Aman and Boro in Bangladesh. Among them, T. Aman rice is the most suitable for growing rice in this country.

Rice is a major component of the Bangladeshi diet. It increases the value of nutrition of rice varieties would greatly benefit the health of the population. However, high market demand for white rice has resulted in running down of pigmented varieties. Most pigmented rice varieties are low yielding, being grown for local markets (Mau *et al*., 2017; Islam *et al*., 2018a). Rice can produce grain with brown, red, purple and even black pericarps. Pericarps are become black and purple due to the result of accumulation of anthocyanin, while red pericarps are due to proanthocyanidins (Samyor *et al*., 2017). Pigmented rice varieties be likely to have a higher protein content with a well-balanced amino acid composition, a better glycemic index and higher levels of fats, fiber and vitamin E (tocopherols and tocotrienols) (Kushwaha, 2016). Due to high levels of phenolic compounds that is anthocyanin, proanthocyanidin and phenolic acids, pigmented rice varities also exhibit strong antioxidant and free radical scavenging capacity. As people now a days are more

concerned about their healthy lifestyle, their demand for highly nutritious and healthier food is also increasing. Bangladeshi pigmented traditional rice varieties offer potential novel genes for rice genetic upgrading (Islam *et al*., 2018a).

Genetic diversity is mainly measured on the basis of the morphological diferences of quantitative important traits. Morphological and biochemical markers were used for genetic diversity analysis and for establishing a relationship among cultivars. However, this method has some drawbacks in terms of time, space, and labour cost. In addition, additive gene act on the expression of the traits so this method cannot define the exact level of genetic diversity among the germplasm, thus making environmental factors mask their true phenotypic performance (Islam *et al*., 2016). Molecular characterization by using DNA marker provides more precise, convenient and reliable results for genetic variability assessment.

With the initiation of PCR based molecular marker technology, genetic characterization of crop plants has moved into a new era. At present, there are different types of molecular markers existing for assessing genetic diversity in crop species. Among them, simple sequence repeats (SSRs) or microsatellites are very convenient for analyzing the structure of germplasm collections since they are abundant, codominant, multi-allelic, highly polymorphic, chromosome specific and easy genotype by PCR (Islam *et al*., 2018a).

 The SSR markers are particularly appropriate for assessing genetic diversity and relationships among plant species, populations, or individuals, germplasm conservation or utilization; marker assisted selection; cultivar identification; hybrid purity analysis, gene mapping studies and parents selection in breeding programs. In rice, SSR markers have been widely used in evaluating genetic diversity (Thomson *et al*., 2007; Salgotra *et al.*, 2015; Islam *et al*., 2019).

More than 1,27,000 rice accessions and wild relatives can be found in the world's largest gene bank for rice at International Rice Research Institute (IRRI) located in the Philippines (http://irri.org/our-work/research/genetic-diversity). Until now, Bangladesh Rice Research Institute (BRRI) has collected and preserved a Gene bank of about 8700 varieties/landraces/ cultivars/wild types from indigenous and exotic sources. Out of these, more than 8500 germplasms have been registered in BRRI Gene bank. Among these, 24 T. Aman rice germplasms have been taken for morpho-molecular characterization. It may be distinguished that few studies of genetic diversity within pigmented rice varieties have been

testified in Bangladesh and involve small numbers of germplasms and markers (Islam *et al*., 2018b).

Therefore, the present research work has been planned and designed to assess the genetic diversity at morphological and molecular level in twenty four pigmented Transplanted Aman rice germplasms for proper characterization and utilization of rice diversity.

OBJECTIVES:

1. To characterize qualitative and quantitative traits of the pigmented rice germplasm through morpho-agronomic characters for assessment of variability.

2. To differentiate selected germplasm of pigmented rice germplasm at the molecular level using microsatellite markers.

3. To study the polymorphism, molecular diversity and DNA profiling of pigmented T. Aman rice germplasm.

CHAPTER II
REVIEW OF LITERATURE

Rice is the main food for more than half of the world's population and is the staple food for the people of Bangladesh, constituting over 91% of the food grain production, and providing 62% of the caloric as well 46% of the protein intake in the average daily diet. The rice production area in Bangladesh is approximately 11.4 million hectares (ha) producing 51.64 million tons of rice annually. Pigmented rice like red and black rices are now becoming more popular and the demand for this rice is expected to rise in the future along with the increased level of community welfare and awareness on the importance of healthy food. Red and black rices have also long been known to be very useful for preventing and curing diseases caused by deficiencies in vitamin A and vitamin B (Kristamtini and Purwaningsih, 2009). Thus, the red and black rices are not only staple foods of carbohydrate sources but also health-promoting functional foods. The current improvement in the community welfare and prosperity has caused a shift in the community lifestyle, which nowadays is beginning to put more concern on health by adjusting their diet. This is done by consuming foods according to their nutritional values. Red and black rices are staple food that many consumers are looking for today due to its great health-promoting benefits (Kushwaha, 2016). Various studies indicated that red and black rices possess better efficacy compared to the common white rice. In addition to carbohydrate, protein, vitamins, and minerals, red and black rices are also sources of anthocyanins that are beneficial to health of antioxidants (Satue-Gracia *et al*., 1997; Nam et al. 2006; Chutipaijit *et al*., 2011; Huang and Lai, 2016), anti inflammation (Tsuda *et al*., 2002) and anti-cancer (Shao *et al*., 2004; Hyun *et al*., 2004) properties. High anthocyanin content is found in both red rice and black rices. The anthocyanin content of dark-red rice and black rices had been proved to be higher because the reddish dark to the blackish brown color is partly caused by differences in the anthocyanin content (AbdelAal, 2006; Shao *et al*., 2011; Suliartini, 2011). So morphological diversity analysis is important for pigmented rice germplasm Molecular characterization and genetic diversity analysis of rice is essential for pigmented rice germplasm development and other improvement program. Molecular markers are the influential tools to identify 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). Studies on genetic variation and molecular characterization of different rice genotypes using Simple Sequence Repeats (SSR) markers have been carried out throughout the world. Therefore, the literature related to the present study on rice and their improved varieties of Bangladesh as well as in abroad are reviewed in this chapter below:

Mau *et al*., (2017) studied 40 upland red and black rice accessions with qualitative and quantitative agro-morphological characters. A total of 26 qualitative and 16 quantitative characters were observed. Qualitative characters were descriptively analyzed and quantitative characters were analyzed with variance. Both data were also subjected to cluster analysis. Research results found a significant difference among rice accessions in both qualitative and quantitative characters. Cluster analysis employing qualitative variables classified the rice accessions into 4 clusters and 15 sub-clusters. The same analysis using quantitative characters placed the 40 rice accessions into 5 clusters and 8 sub-clusters. After agromorphological characters evaluation it can be demonstrated that the rice germplasm under the present study possessed a high genetic diversity.

Ahmed *et al*., (2015b) studied for 21 qualitative agromorphological characters of Kartiksail rice of Bangladesh at BRRI during T. Aman 2009-11 seasons. Only the presence and shape of ligule showed no variation. The genotypes showed strong surface pubescence on penultimate leaf blade (57%), erect type flag leaf (81%) and lateral tiller (71%), no anthocyanin color in nodes (86%) straw color of apiculus (76%) and awnless (81%) grain and late and slow type leaf senescence (71%), respectively. Again, four types of leaf blade (pale green, green, purple tip and purple margin), five types of lemma and palea (yellowish to straw, gold and or gold furrows on straw, brown spots/furrows on straw, brown and light purple on straw) and four types of apiculus (straw, gold, brown and purple) were observed among the germplasm. The studied germplasm also showed features for developing varieties with unique identification like KS14 had purple (medium) color in basal leaf sheath, KS5 had purple tip and KS14 had purple margin leaf blade, KS5 and KS6 had purple stigma and apiculus, KS5 had brown (tawny) lemma and palea.

Islam *et al.*, (2018a) worked with diversity and population structure of 113 rice germplasm based on phenotypic and genotypic traits. Considerable variation existed across the germplasm in phenotypic traits. Based on Shannon–Weaver index, the most variable phenotypic trait was lemma-palea color. Detecting 140 alleles, 11 were unique and suitable as a germplasm diagnostic tool. Signifcant correlation coefficient was detected between phenotypic and genotypic traits.

Ahmed *et al.*, (2016) studied 21 qualitative agro-morphological characters of 40 Balam rice during T. Aman 2009 and T. Aman 2011 seasons at BRRI, Gazipur. The qualitative characters were recorded using procedure of DUS tests for inbreed and hybrid rice of Bangladesh. The presence and split or two-cleft shape type penultimate leaf ligule showed no variation. In total, 24 land races (60%) showed colorless leaf sheath and basal leaf sheath, 14 (35%) had green leaf blade, 16 (40%) had strong surface pubescence of penultimate leaf blade, 33 (82%) showed colorless auricles and collar, 30 (75%) had white color of stigma, 33 (82%) showed erect blade of flag leaf, 31 (77%) had erect curvature of lateral tiller, 30 (75%) showed colorless nodes, 17 (42%) had weak intensity of color in internodes, 14 (35%) showed yellowish to straw color lemma and palea and 18 (45%) had its medium intensity, 16 (40%) showed yellowish/straw color apiculus, 35 (87%) had awnless grain and 23 (58%) showed intermediate type of leaf senescence. Besides, Balam rice along with BR7, BR16, BRRI dhan50 and Nizersail were grouped into four major clusters by the UPGMA clustering method based on Dice coefficient. Cluster III was the largest group with thirty genotypes. The dendrogram revealed that the genotypes B7 (acc. no. 853) and B23 (acc. no. 878) was 100% duplicate or similar. The genetic distance ranging from 0.0 to 9.995 also indicated wide genetic variation among the germplasm.

Aljumaili *et al.*, (2018) worked with 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 simple sequence repeat (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 were 0.36 and 64.90%, respectively. Nei's gene diversity index was 0.36. The dendrogram based on UPGMA and Nei's genetic distance classified the 53 rice accessions into 10 clusters. Analysis of

molecular variance (ANOVA) revealed that 89% of the total variation observed in this germplasm came from within the populations, while 11% of the variation emanated among the populations. These results reflect the high genetic differentiation existing in this aromatic rice germplasm.

Islam *et al*., (2018b) studied 50 red rice germplasm from the Bangladesh Rice Research Institute (BRRI) genebank. The genotypes were characterized both morphologically and genetically using 50 simple sequence repeat (SSR) markers. Overall, 162 alleles were detected of which 22 were unique alleles. The highest and lowest polymorphic information content (PIC) indices were 0.75 and 0.04 found in markers RM282 and RM304 respectively. Genetic diversity was moderate, varying from 0.05 to 0.78 (average: 0.35). They suggested that the diverse germplasm and polymorphic trait-linked SSR markers of red rice are suitable for the detection of economically desirable trait loci/genes for use in future molecular breeding programs.

Singh *et al*., (2016) evaluated a set of 729 Indian rice varieties. To assess the genetic diversity and genetic relationship the varieties were genotyped using 36 HvSSR markers. A total of 112 alleles was amplified with an average of 3.11 alleles per locus with mean Polymorphic Information Content (PIC) value of 0.29.

Roy *et al*., (2016) studied with a valuable set of hill rice germplasm 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 gene diversity or expected heterozygosity (He) ranged from 0.41 (RM55) to 0.94 (RM259) and the average observed heterozygosity (Ho) was 0.051. The polymorphism information content (PIC) values perceived with a range from 0.37 (RM338 and RM507) up to 0.93 (RM259) with an average of 0.65.

Ahmed *et al*., (2016) estimated a total of 350 alleles varied from 3 (RM277) to 14 (RM21) with an average of 7.8 per locus.45 microsatellite loci were noticed across the 31 rice accessions. The gene diversity extended 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.

Siddique *et al*., (2016) assessed 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 extended from 0.86 (RM237) to 0.95 (RM163) with an average of 0.90 shown much variation among the studied landraces. 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 shown seven distinct clusters with a resemblance coefficient of 0.09.

Singh *et al*., (2016) evaluated genetic diversity in a set of 132 Indian wild rice accessions belonging to *Oryza nivara* and *Oryza rufipogon* and eight cultivated rice varieties. 25 highly variable simple sequence repeat (HvSSR) markers and agarose gels were used in the study. A total of 106 SSR alleles were amplified with an average of 4.24 allele per locus, PIC values for HvSSR markers extended from 0.27 (HvSSR 11-24) to 0.71(HvSSR 05-39) with an average of 0.52.

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

Travis *et al*., (2015) studied genetic diversity among 511 cultivars from Bangladesh and North East India using a 384-SNP microarray assay. They recognized 191, 229 and 142 SNPs clearly differentiate indica, japonica and aus accessions, respectively. The aus group has been further fixed into two subpopulations aus1 and aus2.

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.

Chen *et al*., (2017) worked with 30 polymorphic SSR markers to assess the genetic diversity and molecular fingerprints of 53 rice genotypes of *Oryza sativa*, *Oryza glaberrima*, and NERICA. In total, 180 alleles were identified with average polymorphism information content and Shannon's information index were 0.638 and 1.390, respectively. Population structure and neighbor-joining phylogenetic tree shown 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).

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

Nadia *et al*., (2014) studied 26 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%.

Rahman *et al*., (2012) studied the genetic diversity of 21 rice cultivars using 34 microsatellite primer pairs. The number of alleles per locus ranged from 2 to 11 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 identified at 20 loci. 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.

SSR markers are deliberated to be appropriate for assessment of genetic diversity and variety identification because of their ability to identify large numbers of discrete alleles repeatedly, accurately and efficiently. (Ijaz, 2011).

310 rice varieties of both traditional and HYV have been analysed through DNA fingerprinting with SSR markers in Bangladesh. These have been studied in four volumes in Plant Varieties of Bangladesh; morphological and molecular characterization was published by the seed wing, MOA (Rahman *et al*., 2007, 2008 and 2010).

Mia *et al*., (2010) worked with 22 aromatic rice genotypes using SSR markers Aroma was identified by 1.7% KOH as a sensory test. In this study, three SSR primers viz. RM223, RM515 and RM342 were used for identifying fgr gene locus in those genotypes. All the three markers recognized fifteen (15) rice genotypes having fgr gene locus. It was decided that the genotypes could be used in breeding program to progress new aromatic rice varieties.

Kibria *et al*., (2009) described the genetic diversity among aromatic rice genotypes using simple sequence repeat (SSR) and randomly amplified polymorphic DNA (RAPD) markers through marker aided selection (MAS).Three SSR primers (RM223, RM342A and RM515) exhibited 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 highest polymorphism (66.67%).

A set of 48 simple sequence repeat (SSR) markers were used to assess the genetic diversity of 11 Venezuelan rice cultivars. The average genetic diversity (H) over all SSR loci for the 18 genotypes was 0.524, ranging from 0.105 to 0.815. UPGMA cluster analysis based on

genetic distance coefficients showed that the Venezuelan rice varieties are closely related (Herrera *et al.*, 2008).

Islam *et al.*, (2008) used one hundred polymorphic SSR markers to characterize 21 rice genotypes. The highest number of alleles (12) were found for RM418 followed by RM10793 (11), RM3412, RM400 and RM26809 (10). The highest PIC value (0.86) was found for RM10793 followed by RM418 and RM3412 (0.85), RM26809, RM490 and RM287 (0.84), RM16 (0.83), RM493, RM562 and RM253 (0.83). These proposed their greater usefulness for characterization of rice varieties. Two main distinct clusters/groups were identified from cluster analysis. One cluster consists of mostly improved and adapted genotypes while the second cluster had mostly salt tolerant donors with few exceptions. The result exposed to broaden the genetic base for wider adaptability.

The genetic diversity among 35 rice accessions, which included 19 landraces, 9 cultivars and 7 wild relatives were assessed by using microsatellite (SSR) markers distributed across the rice genome. The mean number of alleles per locus was 4.86, showing 95.2% polymorphism. The average polymorphism information content was 0.707. Cluster analysis based on microsatellite allelic diversity clearly demarcated the landraces, cultivars and wild relatives into different groups. Genetic diversity was the highest among wild relatives (0.436), followed by landraces (0.356), and the lowest for cultivars. Allelic variability among the SSR markers was high enough to categorize cultivars, landraces and wild relatives of the rice germplasm, and to catalogue the genetic variability observed for future use (Ram *et al.*, 2007).

Thomson *et al.*, (2007) investigated 330 rice accessions, including 246 Indonesian landraces and 63 Indonesian improved cultivars for better understanding the rich source of genetic diversity, using 30 fluorescently-labeled microsatellite markers. A total of 394 alleles were detected at the 30 simple sequence repeat loci, with an average number of 13 alleles per locus and an average polymorphism information content value of 0.66. The results shown that genetic diversity analysis characterized the Indonesian landraces as 68% indica and 32% tropical japonica, with an indica gene diversity of 0.53 and a tropical

japonica gene diversity of 0.56 and Fast of 0.38 between the two groups. All of the improved varieties sampled were indica and had an average gene divers.

Lapitan *et al*., (2007) studied twenty-four rice cultivars 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, 89 generated a total of 147 rare alleles. 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.

Sujatha *et al*., (2006) studied the genetic diversity of 30 aromatic rice for identifying distinct varieties and selecting suitable parents for breeding by using six SSR primers (RM42, RM 44, RM 155, RM 156, RM 223 and RM 586) amplified 17 alleles and reported molecular markers provide a more accurate estimation of genetic diversity as compared to morphological data.

The genetic diversity of 38 traditional indigenous rice cultivars were evaluated using twelve microsatellite primer pairs. A total of 32 different reproducible bands were amplified of which 26 (81.25%) were polymorphic. The number of bands per primer ranged from one to six with an average of 2.6 bands per primer. Polymorphism information content ranged 0.00 to 0.83. A dendogram based on cluster analysis by microsatellite polymorphism grouped all the 38 rice genotypes into three major clusters. (Joshi and Behera, 2006).

The genetic diversity and DNA fingerprinting of 15 elite rice genotypes were investigated using 30 SSR primers on chromosome numbers 7-12. All the primers showed distinct polymorphism among the cultivars studied indicating the robust nature of microsatellites in revealing polymorphism. Cluster analysis grouped the rice genotypes into 10 groups. The information obtained from the DNA fingerprinting studies helped to distinctly identify and characterize nine varieties using 18 different RM primers (Chakravarthi and Naravaneni, 2006).

Jain *et al*., (2004) evaluated 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 simple sequence repeat (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, polymorphism information content (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.

Bajracharya *et al*., (2004) made a diversity study on a collection of landrace accessions from three agro-ecozones using microsatellite (SSR) markers to understand and assess the value and extent of genetic diversity prevalent in landraces. Seventy rice accessions (21 from Jumla- high hill, 24 from Kaski-mid-hill and 25 from Bara-plain) were assayed for genetic diversity at 39 SSR marker loci. The patterns of genetic diversity revealed by SSR polymorphisms varied 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.

CHAPTER III

MATERIALS AND METHODS

The chapter organizes the materials and methods used in the experiment. Two experiment were conducted for the mentioned objectives. These were

1. **Experiment 01:**

   Genetic diversity analysis of some qualitative and quantitative traits in pigmented T. Aman germplasm.

2. **Experiment 02:**
   Molecular characterization of pigmented T. Aman germplasm through SSR marker.

The details of the methodology have been followed is described below:

3.1 Experiment 01: Genetic diversity analysis of some qualitative and quantitative traits in pigmented T. Aman germplasm

3.1.1 Experimental site and time duration

The present investigation was conducted during July 2019 to December 2019. The field experiments for morphological characters of pigmented rice germplasm were carried out at the research field and and the molecular laboratory of the Genetic Resources and Seed Division (GRSD), Bangladesh Rice Research Institute (BRRI), Joydebpur, Gazipur-1701.

3.1.2 Plant materials

A total of 24 pigmented T. Aman rice accessions of Bangladesh were used in the experiment (Table 1).

3.1.3 Crop cultivation

Single seedling (25 days aged) per hill with a spacing of 20×20 cm between rows and plants, respectively was planted where single row of 5.4 m long per entry/accession were used. Fertilizers were applied @ 60:20:40 kg NPK/ha. All the fertilizers except urea were applied at time of final land preparation. Urea was applied in three equal splits at 10, 25 and 35 days after transplanting. Appropriate control measures were taken for insect pests, diseases and weeds as and when necessary.

3.1.4 Method of morphological data collection

Data on the agronomical and morphological characters were collected from ten randomly selected hills excluding border rows from plots. Ten qualitative and fourteen quantitative traits were recorded using "Germplasm Descriptors & Evaluation Form" by Bangladesh Rice Research Institute (BRRI).

3.1.5 Morphological character observations

Observed variables included both qualitative and quantitative morphological traits of leaf, culm and grain. A total of 24 pigmented T. Aman rice germplasm were observed for qualitative and quantitative characters. Morphological descriptors are presented in Table 11.

Observed qualitative characters are

1. Blade colour with classes

 (a) Green, (b) purple

2. Basal leaf sheath colour with classes

 (a) Green, (b) Light Purple, (c) Purple

3. Ligule colour with classes

(a) White, (b) Purple

4. Ligule shape with classes

(a) 2- cleft 5. Awn: distribution with classes (a) None (awnless), (b) Tip only,(c) Upper quarter only, (d) Whole length

6. Apiculus colour with classes

 (a) Straw, (b) Brown (towny), (c) .Purple

 7. Lemma and palea colour with classes

 (a) Straw, (b) Gold and gold furrows on straw, (c) Brown, (d) Purple spots on straw, (e) .Black, (f) Purple furrows on straw

8. Seed coat (bran) colour with classes

 (a) Speckled brown, (b) brown, (c) Red, (d) variable purple, (e) purple

9. Decorticated grain: Scent (aroma) with classes

(a) Non scented

 10. Leaf senescence with classes

(a) Intermediate, (b) Late and slow.

The observed quantitative characters included 14 traits and ten plants from each entry were randomly selected for recording data on (1) flag leaf area (cm^2), (2) culm diameter (mm), (3) total tiller no., (4) effective tiller no., (5) Panicle length (cm)) (6) plant height (cm)), (7) days to 50% flowering (DF), (8) days to maturity (DM), (9) filled grains per panicle, (10) 1000 gwt(g)), (11) grain length (mm)), (12) grain breadth (mm), (13) Decorticated length breadth ratio, (14) yield per hill.

Table 1. Names of 24 pigmented T. Aman rice germplasms with their accession numbers

SL. NO.	ACCESSION NO.	NAME	SEASON
1.	NC-1	Black rice-1	T. Aman
2.	NC-2	Black rice-2	T. Aman
3.	NC-3	Black Rice-3	T. Aman
4.	NC-4	Black Rice-4	T. Aman
5.	826	Mural(Uba)	T. Aman
6.	827	Murali	T. Aman
7.	831	Birun	T. Aman
8.	832	Birui Sail	T. Aman
9.	833	Biruin	T. Aman
10.	834	Biroin Kalarmur	T. Aman
11.	835	Biroin(Makri)	T. Aman
12.	837	Biroin (Habai)	T. Aman
13.	838	Makri Biroin	T. Aman
14.	841	Balam Dhan	T. Aman
15.	842	Balam	T. Aman
16.	847	Birpak	T. Aman
17.	853	Lal Balam	T. Aman
18.	854	Butu Balam	T. Aman
19.	859	Latisail	T. Aman
20.	860	Jhoria	T. Aman
21.	861	Biroi Dhan	T. Aman
22.	862	Makri Biroin	T. Aman
23.	863	Jhual Biroin	T. Aman
24.	867	Kach Badal	T. Aman

*NC: New collection

3.1.6 Morphological Data analysis

Mean quantitative data of the morphological characters were subjected to univariate analysis. Univariate analysis of the individual characters were carried out using Excel 2007 software.

3.2 Experiment 02. Molecular characterization of pigmented T. Aman germplasm through SSR marker.

3.2.1 Plant materials for molecular work

A total of 24 T. Aman rice germplasm accessions were used in this experiment. All of them were collected from the Gene Bank of Bangladesh Rice Research Institute (BRRI), Joydebpur, Gazipur-1701. A list of germplasm accessions used in this experiment has given in Table 1.

3.2.2 Collection of leaf samples

Five grams of seed from each genotype was sown in the earthen pot and twenty one days old seedlings were collected for DNA extraction. After collecting 3 cm long leaf tips they were 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.

3.2.3 Reagent preparation for DNA Extraction

DNA was extracted using the modified method of Ferdous *et al.*, (2012). Nanodrop (Origin, Germany) is used for the quantification of DNA samples. 0.8% agarose gel electrophoresis was used for the evaluation of the quality of DNA. High concentration of DNA samples was further diluted in 10:1 (DDH$_2$O-DNA) to a working concentration of 50 ng/µl and kept at 4°C for PCR based marker analysis. The laboratory procedures and chemical preparation of this method are described below in a detail.

3.2.4Tris (1 M Tris solution, pH=8.0)

The formula weight (FW) of Trisma base is 121.14 with chemical formula C$_4$H$_{11}$NO$_3$ was used for Tris-buffer preparation. 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 can be 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 was 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.

3.2.5 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 was dissolved in around 60 ml of autoclaved and distilled water and pH was adjusted by adding NaOH pellets (or 5M NaOH) as Na_2EDTA is acidic in nature.

The final volume was made to 100 ml in a graduated measuring cylinder by adding sterile H_2O and the solution was autoclaved.

3.2.6 NaCl (5M NaCl solution)

The formula weight (FW) of this chemical is 58.44 with the chemical formula NaCl. The required concentration is 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 can be 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 was dissolved in around 175 ml of autoclaved and distilled water and the final volume was 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 was finally autoclaved.

3.2.7 SDS (Sodium Dodecyl Sulphate) solution

The another name of this chemical is Lauryl Sulphate with the formula weight (FW) 288.4 and chemical formula $C_{12}H_{25}O_4SNa$. The essential concentration is 10%. The performance of SDS is like a detergent agent and supports 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 was prepared by dissolving 25 g SDS in 200 ml water first. Then the final volume was made to 250 ml by adding sterile H_2O. This chemical was not autoclaved and mask was worn during the preparation of this chemical.

3.2.8 Chloroform

This chemical is used in the extraction method because it is available in the market. The role of this chemical is to form precipitation after disrupting two-dimensional structure of protein. This chemical was used under fume hood and was not inhaled.

3.2.9 Ethanol

Ethanol solutions are essential in 70% and 100% form. The chemical 100% ethanol precipitates or coagulates DNA and 70% ethanol acts as both in the precipitation of DNA and dissolving of salts. Ethanol (70%) also helps to decontaminate the surface in the laboratory.

3.2.10 1X TE Buffer

This is a secondary chemical. 100 ml 1X TE Buffer was prepared as follows:

1 M Tris pH 8.0	10 ml
0.5 M Na$_2$EDTA pH 8.0	200 μl

Finally water was added up to 100 ml. This chemical redissolved DNA into solution and acted as DNA preserving solution.

3.2.11 Extraction buffer (200ml)

40mL of 1M Tris-HCL (pH 8) was mixed with 10 mL of 0.5M EDTA for 200ml extraction buffer preparation, 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.

Table 2: 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

3.2.12 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.

3.2.13 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 de-ionized water. EDTA alone will not dissolve unless NaOH is added.

3.2.14 3.5M NaCl (250 mL)

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

3.2.15 5% SDS (Sodium Dodecyl Sulphate) (100mL)

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

3.2.16 2X CTAB (Cetyl Trimethyl Ammonium Bromide) (200mL)

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.

Table 3: 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$_2$O	92mL

3.2.17 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 4^{0}C.

Table 4: Composition and preparation of the Chloroform: Isoamyl Alcohol: Phenol= 24:1:5 (100mL

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

3.2.18 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 dH$_2$O was added to make the volume 1000mL.

Table 5: Composition and preparation of the 10X TBE Buffer (1000mL)

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

3.2.19 1X TBE buffer

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

Table 6: Composition and preparation of the 1X TBE buffer

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

3.2.20 1% PVP

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

3.2.21 70% ethanol (1000mL)

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

3.2.22 Chronological steps for DNA extraction from leaf sample of pigmented T. Aman rice germplasm

Total DNA was isolated using a quick modified CTAB DNA 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 24 different pigmented T. Aman rice germplasm.

2. Firstly, young, healthy leaves were washed thoroughly by running tap water followed by de-ionized 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. 600 µl of extraction buffer 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. 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 4^0C refrigerator overnight.

11. Finally, DNA samples were stored at -20^0 C refrigerator.

3.2.23 Synthesis of SSR markers

Rice genome specific 16 well known SSR primers viz. RM1, RM5, RM205, RM206, RM 209, RM 213, RM214, RM277, RM304, RM307, RM510, RM338, RM342, RM334, RM1337 (Siddique *et al*., 2016, Islam *et al*., 2018b, Islam *et al*., 2018c) were selected and synthesized for molecular diversity analysis in 24 pigmented T. Aman rice germplasm. A list of the primer used is showed in (Table 7).

3.2.24 Amplification of SSR markers by PCR

Principle of the amplification of SSR marker

Microsatellites or SSR are tandem repeats of 1-6 nucleotides. For example, $(A)_n$, $(AT)_n$, $(ATG)_n$, $(GATT)_n$, $(CTACG)_n$, $(TACGAC)_n$ 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.

3.2.25 Polymerase chain reaction (PCR) amplification

PCR analysis was performed in 10 µl reaction sample containing 10-20 ng 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 using a GeneAtlas G (Astec, Japan) 96-well thermal cycler. Twelve-channel pipette was used for transferring DNA from dilution plate to PCR plate. 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 8.

Table 7: List of SSR markers used for diversity analysis of pigmented T. Aman rice germplasm.

Sl. No.	Primer Name	Chromosome No.	Forward primer (5′–3′)	Reverse primer (5′–3′)	Annealing Temp.
1	RM1	1	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC	55
2	RM5	1	TGCAACTTCTAGCTGCTCGA	GCATCCGATCTTGATGGG	55
3	RM125	6	ATCAGCAGCCATGGCAGCGACC	AGGGGATCATGTGCCGAAGGCC	55
4	RM205	9	CTGGTTCTGTATGGGAGCAG	CTGGCCCTTCACGTTTCAGTG	55
5	RM206	11	CCCATGCGTTTAACTATTCT	CGTTCCATCGATCCGTATGG	55
6	RM209	11	ATATGAGTTGCTGTCGTGCG	CAACTTGCATCCTCCCCTCC	55
7	RM213	2	ATCTGTTTGCAGGGGACAAG	AGGTCTAGACGATGTCGTGA	55
8	RM214	7	CAACGTGATCGAGGATAGATC	GGATTTGCTTACCACAGCTC	54
9	RM277	12	CGGTCAAATCATCACCTGAC	CAAGGCTTGCAAGGGAAG	55
10	RM304	10	TCAAACCGGCACATATAAGAC	GATAGGGAGCTGAAGGAGATG	55
11	RM307	2	GTACTACCGACCTACCGTTCAC	CTGCTATGCATGAACTGCTC	55
12	RM510	7	AACCGGATTAGTTTCTCGCC	TGAGGACGACGAGCAGATTC	55
13	RM338	3	CACAGGAGCAGGAGAAGAGC	GGCAAACCGATCACTCAGTC	55
14	RM342	8	CCATCCTCCTACTTCAATGAAG	ACTATGCAGTGGTGTCACCC	55
15	RM334	5	GTTCAGTGTTCAGTGCCACC	GACTTTGATCTTTGGTGGACG	55
16	RM1337	12	GTGCAATGCTGAGGAGTATC	CTGAGAATCTGGAGTGCTTG	55

Table 8: 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$_2$O	1.5
Total	**10**

Table 9: Temperature Profile of PCR (Easy shortcut new method)

Step	Temperature	Time	No. of Cycle
Initial denaturation	94^{0}C	2.0 min	
Denaturation	95^{0}C	30s	
Annealing	55 ^{0}C	30s	32 cycle
Extension	72^{0}C	25s	
Final extension	72^{0}C	5min	
Hold at	4^{0}C	99:99 (overnight)	

3.2.26 Polyacrylamide Gel Electrophoresis (PAGE)

Three microlitres of PCR products 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:

3.2.27 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 lain 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.

3.2.28 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 10: Composition and preparation of polyacrylamide gel

Reagents	Final conc.	8% gel
Sterile Nano pure 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.

3.2.29 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. 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 50 bp DNA ladder was loaded for size determination. Thermo Scientific Gene Ruler 1Kb Plus DNA Ladder was used from Thermo Scientific company.

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.

3.2.30 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 detached 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.

3.2.31 SSR data analysis

The size of the band for each marker was scored by AlphaEaseFC-4.0 software. The summary statistics, including the number of alleles, major allele size and frequency, gene diversity and polymorphism information content (PIC) value were determined using Power Marker version 3.25 (Liu and Muse 2005) and Software MEGA 5.1 was applied to construct the neighbor joining tree (Tamura *et al*., 2011; Hall, 2013) based on Nei's (1983) genetic distance.

Some pictorial view of experimental work

Figure-1: Leaf sample kept in ice bucket

Figure-2: Leaf cutting for grinding

Figure-3: Sample prepared for centrifuge tube

Figure-4: Chemical loaded in eppendorf
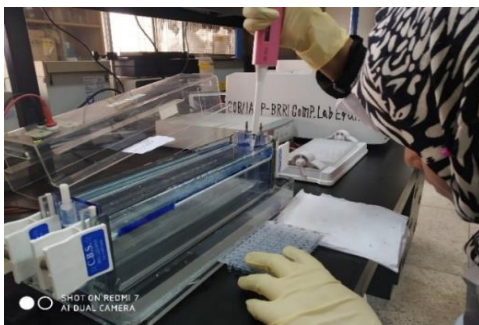
Figure-5: DNA loaded for electrophoresis

Figure-6: Acrylamide gel used for DNA visualization

Figure-7: Stained acrylamide gel

Figure-8: Rice kernel arrangement

CHAPTER IV

RESULTS AND DISCUSSION

Morphological study of a germplasm may not be sufficient for varietal identification. It seems important to start DNA finger printing of the rice germplasms, so that the microsatellites can indicate the allelic constitution of a germplasm in addition to its morphological traits that are now being used as criteria for variety identification. Highly polymorphic and repeatable PCR based markers Simple Sequence Repeats (SSRs), were used here to assess the polymorphism, diversity and similarity identification within studied local germplasms. Results which were found from the experiment have been presented below under the following headings.

4.1 Experiment 01: Genetic diversity analysis of some qualitative and quantitative traits in pigmented T. Aman germplasm

4.1.1 Qualitative character description

Qualitative characters are important for plant description (Kurlovich, 1998, Islam *et al*., 2018b) and mainly influenced by the consumers preferences, socioeconomic scenario (Hien *et al*., 2007). Frequency distribution for 10 qualitative traits of 24 pigmented T. Aman rice germplasms are shown in Table 11 and its graphical representation of frequency distribution shown in Figure 9 and Figure 10. Results of the present study in Table 11 revealed that most of the tested rice accessions possessed blade colour: green (95.83%), purple (4.17%) ; basal leaf sheath colour: green (83.33%), light purple (12.5%), purple (4.17%); ligule colour: white (95.83%), purple (4.17%); ligule shape: 2-cleft (100%); awn distribution: awnless (79.17%), tip only (12.5%), upper quarter only (4.17%), whole length (4.17%); apiculus colour: straw (50%), brown (towny)(37.5%), purple (12.5%); lemma and palea colour: straw (20.83%), gold and gold furrows on straw (20.83%), brown (25%), purple spots on straw (12.5%), black (4.17), purple furrows on straw (16.66%); seed coat (bran colour): speckled

Table 11. Characterization of 24 pigmented T. Aman rice germplasm based on qualitative characters

Sl. no.	Characters	State of characters	No. of germplasm	Germplasm (Serial number in Table 1)	Frequency %
1	Blade colour	02. Green	23	1,2,3,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24	95.83
		07.Purple	1	4	4.17
2	Basal leaf sheath colour	01. Green	20	1,2,3,5,7,8,10,11,13,14,15,16,17,18,19,20,21,22,23,24	83.33
		03. Light purple	3	6,9,12	12.5
		04. Purple	1	4	4.17
3	Ligule colour	01. White	23	1,2,3,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24	95.83
		03. Purple	1	4	4.17
4	Ligule shape	02. 2- cleft	24	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24	100
5	Awn: distribution	0. None (awnless)	19	1,2,3,4,5,7,8,9,11,12,13,15,16,17,18,20,22,23,24	79.17
		01.Tip only	3	10,19,21	12.5
		02.Upper quarter only	1	14	4.17
		05.Whole length	1	6	4.17
6	Apiculus colour	02.Straw	12	1,2,3,5,6,8,12,15,17,19,22,24	50.00
		03. Brown(towny)	9	10,11,13,14,16,18,20,21,23	37.5
		06.Purple	3	4,7,9	12.5
7	Lemma and palea colour	0. Straw	5	1,6,9,19,23	20.83
		01. Gold and gold furrows on straw	5	8,18,20,21,24	20.83
		04.Brown	6	2,3,5,14,15,17	25.00
		06. Purple spots on straw	3	4,7,16	12.5
		09.Black	1	13	4.17
		07.Purple furrows on straw	4	10,11,12,22	16.66
8	Seed coat (bran) colour	03. Speckled brown	5	5,15,17,20,21	20.83
		04. brown	5	10,11,12,13,22	20.83
		05.Red	10	6,7,8,9,14,16,18,19,23,24	41.68
		06. variable purple	2	2,3	8.33
		07.purple	2	1,4.	8.33
9	Decorticated grain: Scent (aroma)	0. Non scented	24	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24	100
10	Leaf senescence	05. Intermediate	21	1,4,5,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24	87.5
		07. Late and slow	3	2,3,6	12.5
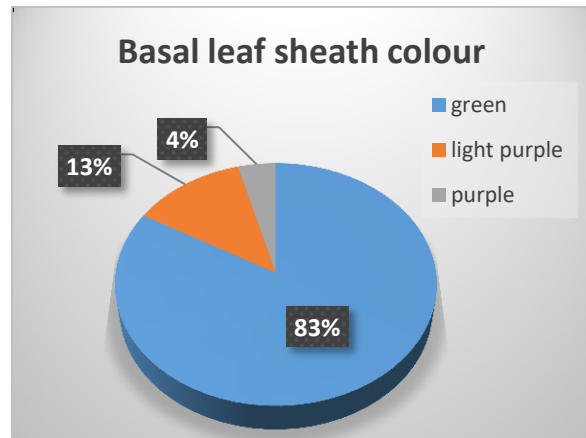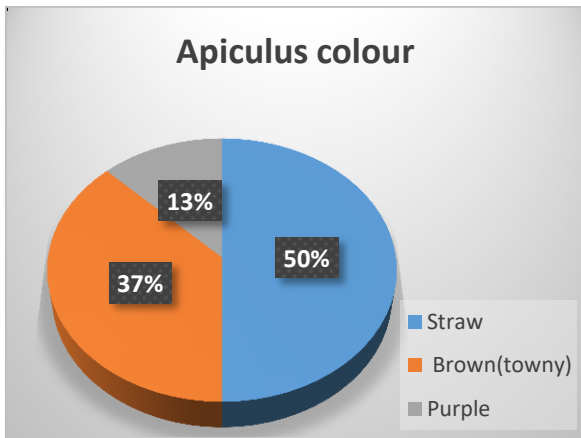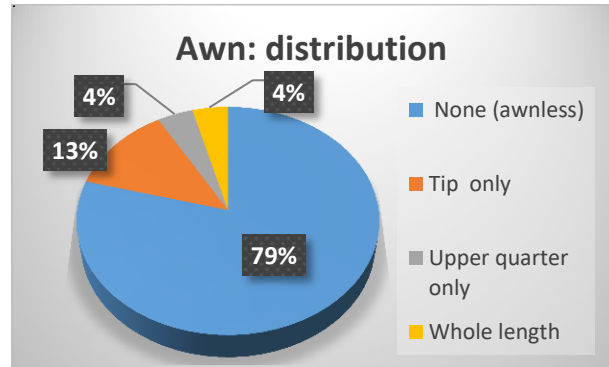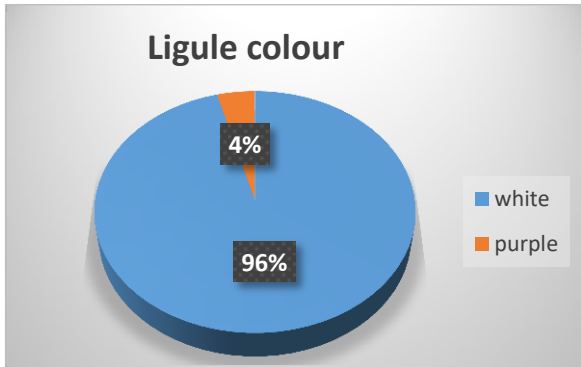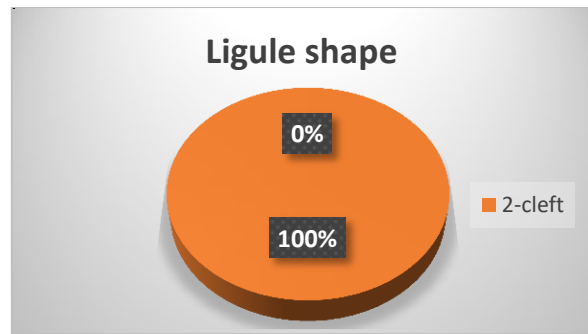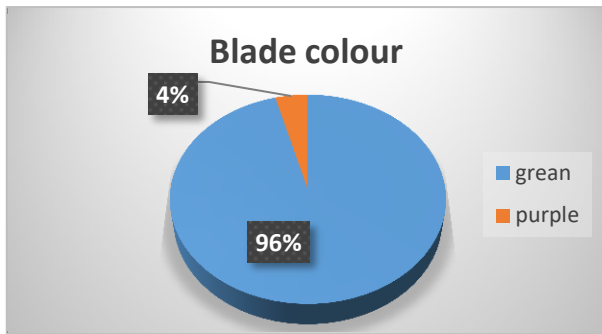
Figure 9: Frequency distribution of blade colour, ligule shape, ligule colour, awn distribution, apiculus colour, basal leaf sheath colour of 24 pigmented T. Aman rice germplasm.
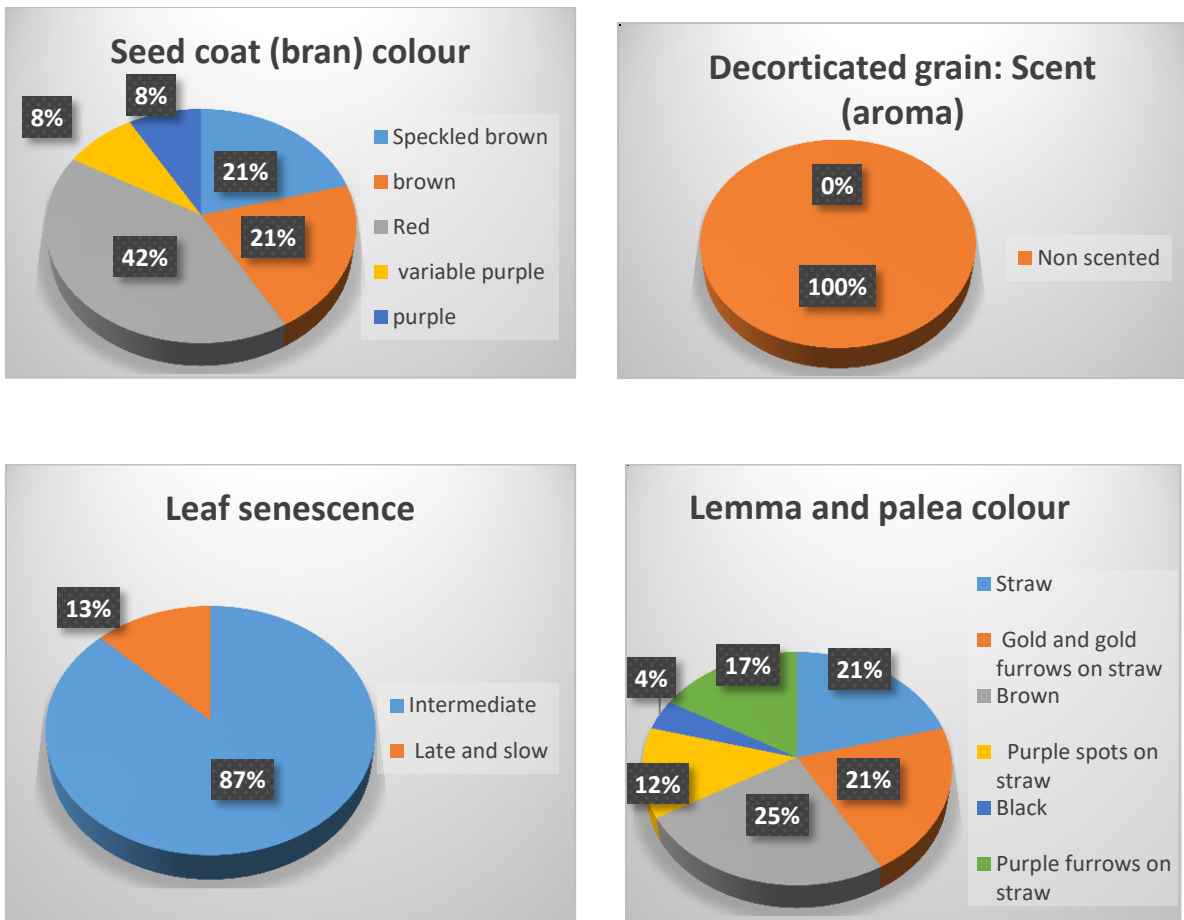
Figure 10: Frequency distribution of seed coat (bran) colour, decorticated grain:scent (aroma), leaf senescence, lemma and palea colour of 24 pigmented T. Aman rice germplasm.

brown colour (20.83%), brown (20.83%), red (41.68%), variable purple (8.33%), purple (8.33%); decorticated grain: scent(aroma) : non scented (100%), leaf senescence : intermediate (87.5%), late and slow (12.5%). The present study exhibited high variability in most of the observed qualitative traits of T. Aman rice landraces. Similar types of work was also reported by other authors (Ahmed *et al*., 2016; Akter *et al*., 2017; Islam *et al*., 2018b).

4.1.2 Identifying morphological marker of pigmented T. Aman germplasms

The unique traits of some pigmented germplasms are presented in Table no. 12. The identifying traits were given below:

NC-1 showed purple blade colour; basal leaf sheath colour of NC-4 showed purple and accession number 827, 833, 837 observed light purple; ligule colour of NC-4 displayed purple; awn distribution of 834, 859, 861 showed tip only ; 841 upper quarter only and 827 whole length whole length ; Apiculus colour of NC-4, 831, 833 showed purple; Lemma and palea colour of NC-4, 831, 847 showed purple spots on straw, 838 showed black; seed coat colour of NC-2, NC-3 is variable purple, NC-1, NC-4 showed purple; Leaf senescence of NC-2, NC -3, 827 was late and slow.

Table 12: List of germplasms showed identify morphological traits.

Sl. No.	Morphological characters	Colour pattern/ type	Germplasms
1	Blade colour	Purple	NC-4
2	Basal leaf sheath colour	Purple	NC-4
		Light purple	827,833,837
3	Ligule colour	Purple	NC-4
4	Awn distribution	Tip only	834,859,861
		Upper quarter only	841
		Whole length	827
5	Apiculus colour	Purple	NC-4,831,833
6	Lemma and palea: colour	Purple spots on straw	NC-4,831,847
		Black	838
7	Seed coat (bran) colour	Variable purple	NC-2,NC-3
		Purple	NC-1, NC-4
8	Leaf senescence	Late and slow	NC-2,NC-3,827

*NC= New collection

Figure 11: Morphological diversity showed on rice kernel, grain and panicle with their accession number

RICE KERNEL	GRAIN	PANICLE
Accession No. New Collection 1		
Accession No. New Collection 2		
Accession No. New Collection 3		
Accession No. New Collection 4		
Accession No. 826		
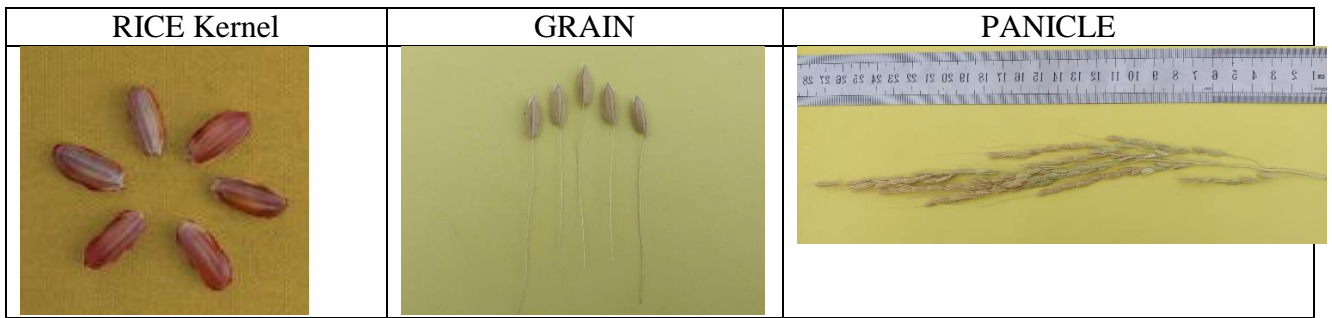
RICE Kernel	GRAIN	PANICLE
Accession No. 827		
Accession No. 831		
Accession No. 832		
Accession No. 833		
Accession No. 834		
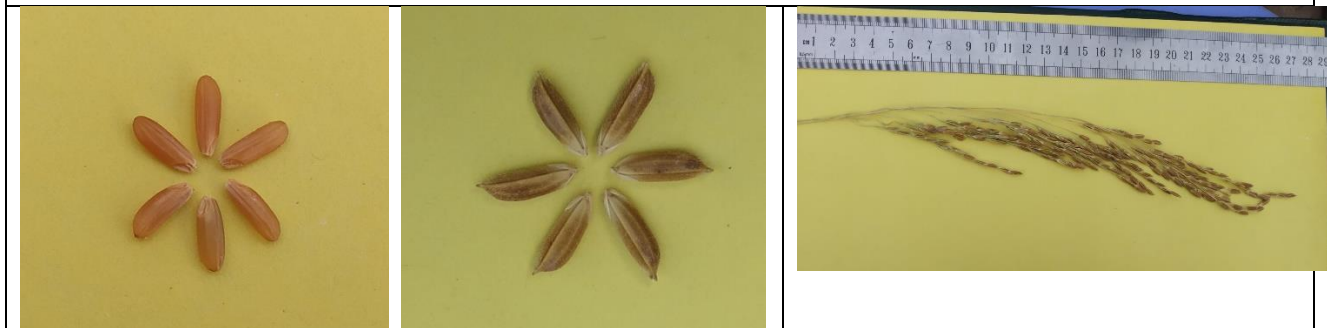
RICE Kernel	GRAIN	PANICLE
Accession No. 835		
Accession No. 837		
Accession No. 838		
Accession No. 841		
Accession No. 842		

RICE Kernel	GRAIN	PANICLE
Accession No. 847		
Accession No. 853		
Accession No. 854		
Accession No. 859		
Accession No. 860		

RICE Kernel	GRAIN	PANICLE
Accession No. 861		
Accession No. 862		
Accession No. 863		
Accession No. 867		

4.1.2 Quantitative ANOVA Table Description

Univariate analysis is the simplest form of quantitative (statistical) analysis. The analysis was carried out with the description of a single variable in terms of the applicable unit of analysis. Rice accessions were evaluated for agronomical traits viz., flag leaf area, culm diameter, effective tiller, panicle length, panicle height, days of 50% flowering, days of 50% maturity, filled grain per panicle, grain length, grain breadth, decorticated length breadth, yield per hill.

The descriptive statistics were employed to assess the variability of different quantitative characters and Analyses Of Variance are presented in Table 13. The character flag leaf area varied between 73.66cm^2 (Max.) to 17.65cm^2 (Min.) with a mean of 41.31cm^2 and having coefficient of variation is 31.83%. The highest mean performance was recorded for Kach Badal (73.66 cm) whereas the lowest mean performance was recorded for Jhual Biroin (17.65 cm). The character culm diameter (cm) varied between 5.76 (mm) to 3.16 (mm) with a mean of 4.63mm and having coefficient variation 13.93%. The highest mean performance was recorded for Black Rice-2 (5.76mm) whereas the lowest mean performance was recorded for Latisail(2) (3.16mm). The number of total tillers varied between 16 to 5 with a mean of 11 and having coefficient of variation 27.95%. Latisail showed the highest mean performance and Black Rice-4 and Birun(2) showed lowest mean performance for this trait. Effective tiller had mean of 9 and having minimum range of 5 and maximum range of 13 and having a coefficient of variation 25.63%. The highest mean performance was recorded for Butu Balam(2) and Latisail whereas the lowest mean performance was recorded for Black Rice-4 and Birun(2). The character Panicle length (cm) varied between 30.20 (cm) to 21.40 (cm) with a mean of 26.35 cm and having coefficient of variation 8.62%. The highest mean performance was recorded for Biroi Dhan whereas the lowest mean performance was recorded for Biroin (Habai)). Panicle length contributes positively yet maximum panicle length is not the only factor responsible for higher grain yield. Abbasi *et al*., (1995) observed that the rice variety DR-39 had maximum panicle length but due to lower grain fertility exhibited lower grain yield. So panicle length alone does not determine the high grain yield as traits such as grain size, grain shape, higher numbers of tillers/plant, longer panicles and greater number of grains/panicle ultimately contribute to higher grain yield. The character plant height (cm) varied between 158.20 (cm) 87.20 (cm) with a mean of 131.01 cm and having coefficient variation 10.59%. The highest mean performance was recorded for

Birun(2) (158.20 cm) whereas the lowest mean performance was recorded for Biroin (Habai) (87.20 cm).

Table 13: Variability of different quantitative characters in 24 pigmented Transplant Aman rice germplasm.

Sl. No	FLA (cm²)	Culm dia (mm)	Total Tiller	Eff tiller	PL (cm)	PH (cm)	DF	DM	FG/P	1000 gwt.(g)	GL (mm)	GB (mm)	D L/B	Y/hill(g)
1	46.13	5.36	8	7	25.40	129.00	114	142	117	26.10	9.50	3.23	2.53	21.38
2	37.36	**5.76**	10	9	28.60	120.00	90	117	85	26.43	10.09	2.82	**3.00**	19.09
3	36.93	5.57	9	9	28.50	119.30	90	117	68	25.91	**10.30**	2.83	2.88	15.59
4	47.76	4.65	**5**	**5**	25.40	143.20	**117**	**146**	92	26.47	9.04	3.17	2.50	**11.18**
5	32.74	4.50	8	7	25.60	118.60	101	129	105	23.29	8.49	2.59	2.63	19.03
6	38.95	3.80	11	10	24.80	121.80	**80**	**107**	85	25.27	8.73	2.86	2.45	21.00
7	23.39	4.73	**5**	**5**	28.20	**158.20**	109	139	136	24.09	8.45	3.09	2.26	18.02
8	47.05	5.20	7	7	27.40	130.80	111	138	147	**17.00**	**8.08**	**2.36**	2.86	16.49
9	32.50	4.54	10	9	27.60	153.00	109	139	106	25.40	8.51	3.01	2.22	20.23
10	31.59	4.59	12	10	25.60	137.80	110	138	57	27.20	8.93	3.21	2.25	14.98
11	39.26	4.58	11	9	27.60	132.40	111	138	67	23.84	8.37	3.03	2.21	14.41
12	72.78	3.77	7	6	**21.40**	**87.20**	114	142	74	23.74	8.61	**3.35**	2.09	12.23
13	33.92	4.78	14	12	21.40	128.80	112	139	98	25.43	8.58	3.04	2.25	18.97
14	43.03	5.44	12	11	29.40	142.80	108	137	140	29.00	9.08	2.57	2.98	**22.57**
15	59.56	3.70	11	10	28.00	120.40	82	108	82	21.13	8.61	2.50	**2.03**	17.32
16	31.15	4.06	12	10	22.80	137.80	107	135	**39**	29.67	8.98	3.30	2.25	11.82
17	48.66	4.54	10	9	27.40	121.40	106	135	86	21.54	8.19	2.53	2.70	15.86
18	36.65	5.59	15	**13**	26.60	129.20	109	139	72	22.87	8.35	2.60	2.64	21.01
19	43.93	**3.16**	**16**	**13**	25.60	132.20	98	127	51	23.84	8.28	2.57	2.64	16.11
20	45.85	4.58	9	8	27.20	138.20	109	139	**153**	21.34	8.21	2.58	2.63	21.70
21	37.08	4.56	13	11	**30.20**	137.40	110	138	137	23.50	8.73	2.81	2.54	21.05
22	33.85	4.62	15	12	27.40	140.40	108	137	71	23.89	8.55	3.07	2.30	20.75
23	**17.65**	4.61	13	11	26.00	133.00	109	137	103	23.29	8.98	2.71	2.71	17.43
24	**73.66**	4.32	13	10	24.40	131.40	99	127	89	**34.25**	9.66	3.31	2.48	17.02
Min	17.65	3.16	5	5	21.40	87.20	80	107	39	17.00	8.08	2.36	2.03	11.18
Max	73.66	5.76	16	13	30.20	158.20	117	146	153	34.25	10.30	3.35	3.00	22.57
Mean	41.31	4.63	11	9	26.35	131.01	105	133	94	24.77	8.80	2.88	2.50	17.72
Std	13.15	0.64	2.98	2	2.27	13.87	9.96	10.57	31.13	3.34	0.58	0.30	0.28	3.26
CV	31.83	13.93	27.95	25.63	8.62	10.59	9.51	7.95	33.06	13.49	6.59	10.40	11.01	18.43
SE	6.50	2.84	5.71	5.23	1.76	2.16	1.94	1.62	6.75	2.75	1.35	2.12	2.25	3.76
LSD	12.73	5.57	11.18	10.26	3.45	4.24	3.81	3.18	13.23	5.40	2.64	4.16	4.41	7.37

*FLA=Flag Leaf Area (cm²), CD=Culm Diameter (mm), TT= Total Tiller, ET= Effective Tiller, PL=Panicle Length (cm), PH=Plant Height (cm), DF=Days to 50% Flowering, DM=Days to Maturity, FG/P=Filled Grains per Panicle, GL=Grain Length (mm), GB=Grain breadth (mm), D L/B=Decorticated Length Breadth Ratio, Y/H=Yield per Hill (g)

Plant height in rice is a complex character and is the product of several genetically controlled factors called internodes (Cheema *et al*., 1987). Plant height in rice is a multifaceted character and the end product of a number of genetically controlled factors called internodes (Cheema *et al*., 1987). Reduction in plant height may develop their resistance to lodging and reduce substantial yield losses associated with this trait (Abbasi *et al*., 1995). Hien *et al*., (2007) reported that improvement of pigmented Transplant Aman also should focus on both decreasing the plant height and increasing the culm strength. The character days of 50% flowering varied between 80 to 117 days with a mean of 105 days and having coefficient of variation 9.51%. The highest mean performance was recorded for Black Rice-4 (117 days) whereas the lowest mean performance was recorded for Murali (80 days). Days of 50% maturity varied between 146 days to 107 days with a mean of 133 days and having coefficient of variation 7.95%. The highest mean performance was recorded for Black Rice-4 whereas the lowest mean performance was recorded for Murali. The number of filled grains per panicle varied between 39 to 153 with a mean of 94 and having coefficient of variation 33.06%. The highest mean performance was recorded for Jhoria whereas the lowest mean performance was recorded for Birpak. 1000 grain weight ranged from 17.00 - 32.25 with mean value 24.77. Standard Deviation (SD) for 1000 grain weight was 3.34 with coefficient of variation (CV) of 1000 grain weight was 13.49%.Grain length ranged from 8.08 – 10.30 with mean value 8.30. Grain breadth having mean value 2.88 (mm) and varied in ranges of 2.36 to 3.35 (mm). The deteriorate length and breadth had mean value 2.50 and varied in range of 2.03 to 3 and having coefficient of variation 11.01. Yield per hill ranged from 22.57 (g) – 11.18 (g) with mean value 17.72 9 (g). The highest yield per hill (22.57 g) was observed in Balam dhan and the lowest (11.18 g) was found in Black Rice-4. Standard Deviation (SD) for yield per hill was 3.26 with coefficient of variation (CV) of yield per hill was 18.43%. Similar findings were also reported by other authors (Islam *et al.,* 2016; Mau *et al.*, 2017; Islam *et al.,* 2018a)

After evaluation of 24 pigmented T. Aman rice germplasms for 14 quantitative characters, on the basis of mean values, some germplasms which showed best performance were identified like Black Rice-4, Black Rice-3, Biroin(Habai), Kach Badal, Jhoria, Butu Balam(2), Latisail. Identifying germplasm for different agronomical characters in phenotypically divergent sources would help in prebreeding and breeding programs. The variety having higher yield would be utilized in a crossing programme, if other characters satisfy the breeder's objectives.

4.1.3 Cluster analysis of 24 pigmented T. Aman rice germplasm for 14 quantitative characters

The dendrogram, constructed by using UPGMA clustering based on Eucledean method, distributed the 24 pigmented T. Aman rice germplasm into three major clusters along with minor sub-clusters, groups and sub-groups for 14 quantitative traits (Figure 12). Out of three clusters Cluster 1 was the major cluster with maximum germplasms (12) namely G10 (Biroin Kalamur), G11 (Biroin (Makri)),G18 (Butu Balam(2)), G22 (Makri Biroin), G17 (Lal Balam), G16 (Birpak), G19 ((Latisail), G24 (Kach Badal), G2 (Black Rice-2), G6 (Murali), G3 (Black Rice-3), G15 (Balam), while cluster 2 consisted of one germplasm namely G12(Biroin (Habai)) and cluster 3 consisted of 11 germplasms namely G4 (Black Rice-4, G9 (Biroin (Tola)), G5 (Mural (Uba)), G13 (Makri Biroin), G23 (Jhual Biroin), G1 (Black Rice-1), G7 (Birun(2)), G8 (Birui Sail), G20 (Jhoria), G14 (Balam Dhan), G21 (Biroi Dhan) . Besides, Cluster 1 were consisted of two sub-clusters. In cluster 1, G10 (Biroin Kalamur), G11 (Biroin (Makri)), G18 (Butu Balam(2)), G22 (Makri Biroin), G17 (Lal Balam), G16 (Birpak), G19 ((Latisail) formed sub cluster 1 and G24 (Kach Badal), G2 (Black Rice-2), G6 (Murali), G3 (Black Rice-3), G15 (Balam) formed sub cluster 2. The cluster3 consisted of two sub clusters. In cluster 3, G4 (Black Rice-4, G9 (Biroin (Tola)), G5 (Mural (Uba)), G13 (Makri Biroin), G23 (Jhual Biroin), G1 (Black Rice-1) formed sub cluster 1 and G7 (Birun(2)), G8 (Birui Sail), G20 (Jhoria), G14 (Balam Dhan), G21 (Biroi Dhan) formed sub cluster 2. The observed result are comparable to previous analysis using quantitative characters placed the 40 upland rice accessions into 5 clusters and 8 sub-clusters (Mau *et al.*, 2017). Similar types of work was also reported by other authors (Ahmad *et al*., 2015, Islam *et al.,* 2014; Islam *et al.,* 2017).

Germplasms of more genetic similarity are placed in same sub cluster. The dendrogram revealed that the germplasms that derivatives of genetically similar type clustered together.

The germplasms grouped in the same cluster due to lower genetic distance and the other germplasms having more genetic dissimilarity grouped in another cluster due to higher genetic distance.
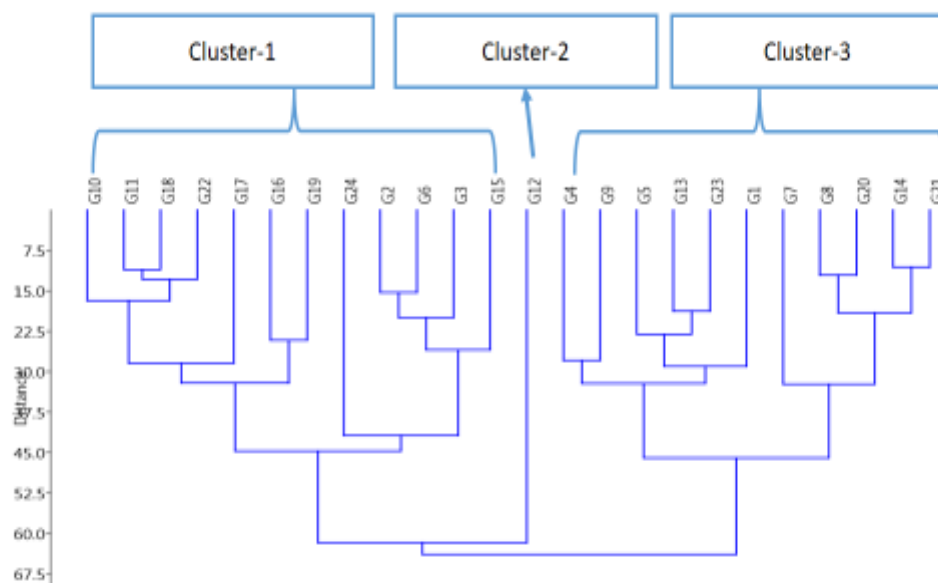
*G1=Black Rice 1, G2=Black Rice 2, G3=Black Rice 3, G4=Black Rice 4, G5=Mural(uba), G6=Murali, G7=Birun(2), G8=Birui Sail, G9=Biruin (Tola), G10=Biroin kalamur, G11=Biroin(makri), G12=Biroin(Habai), G13=Makri Biroin, G14=Balam Dhan, G15=Balam, G16=Birpak, G17=Lal Balam, G18=Butu Balam(2), G19=Latisail, G20=jhoria, G21=Biroi Dhan, G22=Makri Biroin, G23=Jhual Biroin, G24=Kach Badal

Figure 12. Unweighted pair group method of arithmetic mean (UPGMA) Cluster dendrogram of 24 pigmented T. Aman rice germplasm for 14 quantitative characters using Eucledean method.

4.2 Experiment 02. Molecular characterization of pigmented T. Aman germplasm through SSR markers.

4.2.1 Molecular diversity

Twenty four (24) pigmented T. Aman rice germplasms were successfully amplified with the 16 microsatellite marker (Table 14) each referred to as loci and DNA bands as alleles. A total of 70 alleles were detected from 16 microsatellite markers across 24 rice genotypes.

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The number of alleles per locus ranged from one (RM510) to eight alleles (RM1), with an average of 4.38 alleles across the 16 loci obtained in the study. The highest range of band sizes was found for RM1337 (171-350). Among the 16 SSR markers, the highest number of alleles (8) were found for RM1 followed by RM209 (7); RM206 (6); RM1337 (6), RM334 (6) and so on. The polymorphism information content (PIC) values which ranged from 0.14 (RM307) to 0.80 (RM1), with an average of 0.445, revealed much variation among the studied germplasm. The observed PIC values were similar to previous estimates of microsatellite analysis in rice of 0.34–0.88 (Thomson *et al*., 2007) and 0.65–0.91 (Siddique *et al*., 2014). The allele frequency ranged from 29.17% (RM206) to 91.67 % (RM307, RM510 and RM125) with an average of 64.84 alleles (Table 14). PIC value revealed that RM1 was considered as the best marker for 24 pigmented T. Aman rice germplasm.

4.2.2 DNA amplification by SSR markers and its polymorphism

Sixteen SSR primers viz. RM1, RM5, RM125, RM205, RM206, RM209, RM213, RM214, RM277, RM304, RM307, RM510, RM338, RM342, RM334, RM1337 produced different banding pattern separately with 24 pigmented Transplant Aman rice germplasm. The amplification of each SSR primers are presented in Table 14 and some SSR profiles are presented on Plate 1 to 6.

The SSR primer RM1 produced eight fragments of DNA amplification. The amplification of band ranged from 84 bp to 180 bp. The size range observed, are comparable to previous SSR amplification in rice viz. 80 bp to 175 bp (Vabna, 2018). The genotypes Black rice-1, Black rice-2 and Black Rice-3 were able to produce 123 bp. The germplasms Lal Balam, Butu Balam and balam were able to produce 117 bp. Balam Dhan, Jhoria, Biroi Dhan were able to produce 180 bp. The germplasms Birui Sail and Biruin were able to produce 104 bp. The germplasm Jhoria and Biroi Dhan were able to produce 133 bp polymorphic band. The germplasms Mural (Uba), Murali and Balam Dhan were able to produce 95 bp. Jhual Biroin and Latisail were able to produce 90 bp (Plate 1).

The SSR primer RM1337 has the ability to amplify six fragments of DNA among all the experimental materials. The band size ranged from 171 bp to 350 bp. It was noticed that 189 bp fragment was common in all the germplasms and was monomorphic for all. Only Black Rice-3 and Murali were able to produce 208 bp and 196 bp unique polymorphic band

respectively.171 bp polymorphic bands were produced by Lal Balam and Makri Biroin germplasms and 183 bp polymorphic bands were produced by Birpak, Butu Balam and Latisail germplasms. The germplasms Black rice-1, Biroin Kalarmur, Biroin (Makri), Biroin (Habai), Makri Biroin and Jhual Biroin were able to produce 178 bp. The amplification product was presented in Plate2.

Three fragments of DNA amplification were detected by the SSR primer RM213. The approximate fragment size ranged from 125 bp to 210 bp. All the bands produced 125 bp are monomorphic bands. Jhoria, Biruin, Balam Dhan, Birpak, Biroin (Makri) germplasms showed polymorphism band. The amplification product is presented in Plate 3.

The SSR primer RM277 has the ability to amplify DNA among all the experimental materials with the band size ranged from 140 bp to 175 bp. It was noticed that 140 bp was common for all germplasms and they were produced monomorphic band. The DNA fragment 165 bp polymorphic band was produced by Latisail germplasm. Again, 175 bp polymorphic band was produced by Latisail germplasm. The amplification product is presented in Plate 4.

The SSR primer RM206 has the ability to amplify DNA among all the experimental materials with the band size ranged from 140 bp to 275 bp. The size range observed, are comparable to previous SSR amplification in rice viz. 130 bp to 280 bp (Vabna, 2018). Black Rice-2, Black Rice-3 showed amplification at 172 bp. Birun(2), Balam Dhan, Balam, Birpak produced polymorphic band at 130 bp. Lal Balam, Butu Balam(2), Makri Biroin germplasms produced polymorphic band at 156 bp. The amplification product is presented in Plate 5.

The SSR primer RM510 was able to amplify only one monomorphic band among all the genotypes under studies. It was able to amplified in the position of 120 bp fragment. The amplification product is presented in Plate 6. Similar type of work also reported by other others (Joshi and Behera, 2006; Charkravarthi and Narvanti, 2006; Islam *et al*., 2008; Kibria *et al*., 2009; Nadia *et al*., 2014)
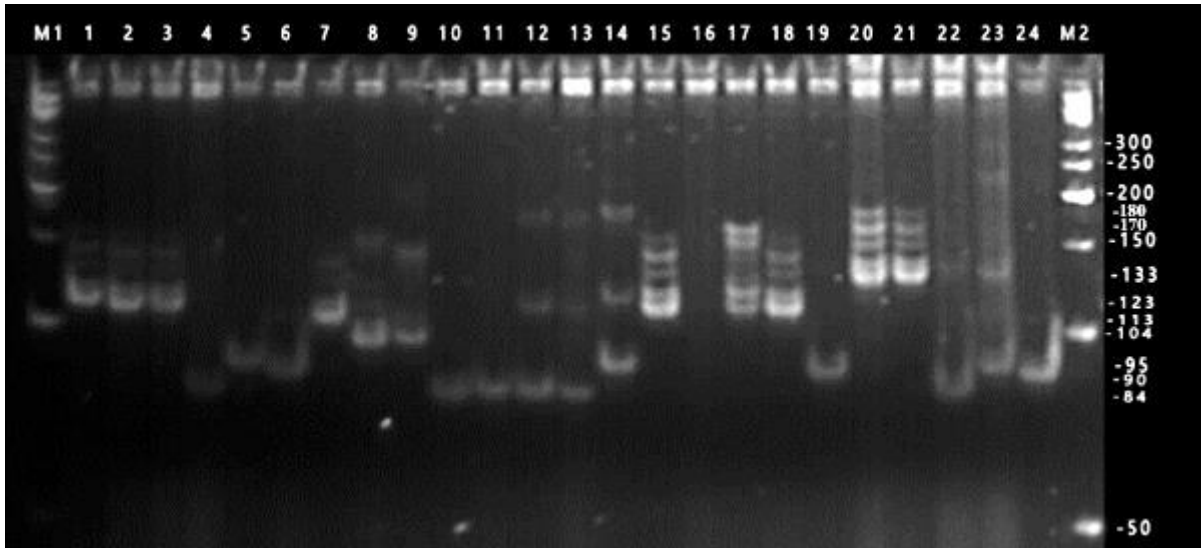
Plate 1. SSR profile of 24 pigmented T. Aman rice germplasm using primer RM1. M1 and M2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane : 1) Black rice-1, 2) Black rice-2, 3) Black Rice-3, 4) Black Rice-4, 5) Mural(Uba), 6) Murali, 7) Birun, 8) Birui Sail, 9) Biruin, 10) Biroin Kalarmur, 11) Biroin(Makri), 12) Biroin (Habai), 13) Makri Biroin, 14) Balam Dhan, 15) Balam , 16) Birpak, 17) Lal Balam, 18) Butu Balam, 19) Latisail, 20) Jhoria, 21) Biroi Dhan, 22) Makri Biroin, 23) Jhual Biroin, 24) Kach Badal
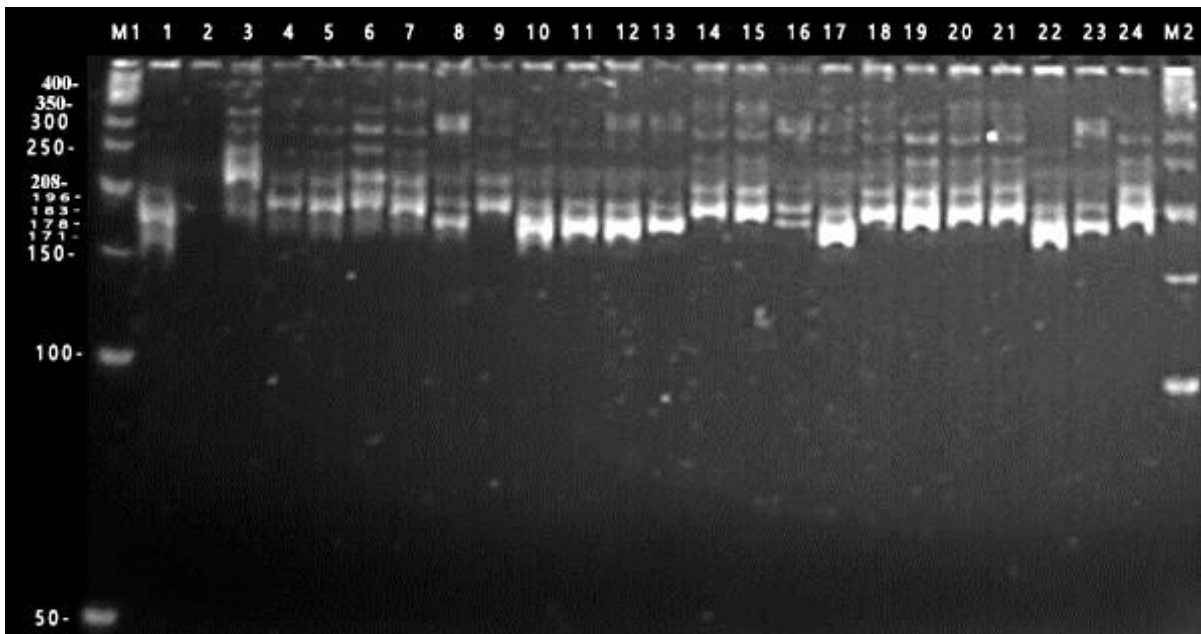
Plate 2. SSR profile of 24 pigmented T. Aman rice germplasm using primer RM1337. M1 and M2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane : 1) Black rice-1, 2) Black rice-2, 3) Black Rice-3, 4) Black Rice-4, 5) Mural(Uba), 6) Murali, 7) Birun, 8) Birui Sail, 9) Biruin, 10) Biroin Kalarmur, 11) Biroin(Makri), 12) Biroin (Habai), 13) Makri Biroin, 14) Balam Dhan, 15) Balam , 16) Birpak, 17) Lal Balam, 18) Butu Balam, 19) Latisail, 20) Jhoria, 21) Biroi Dhan, 22) Makri Biroin, 23) Jhual Biroin, 24) Kach Badal

50
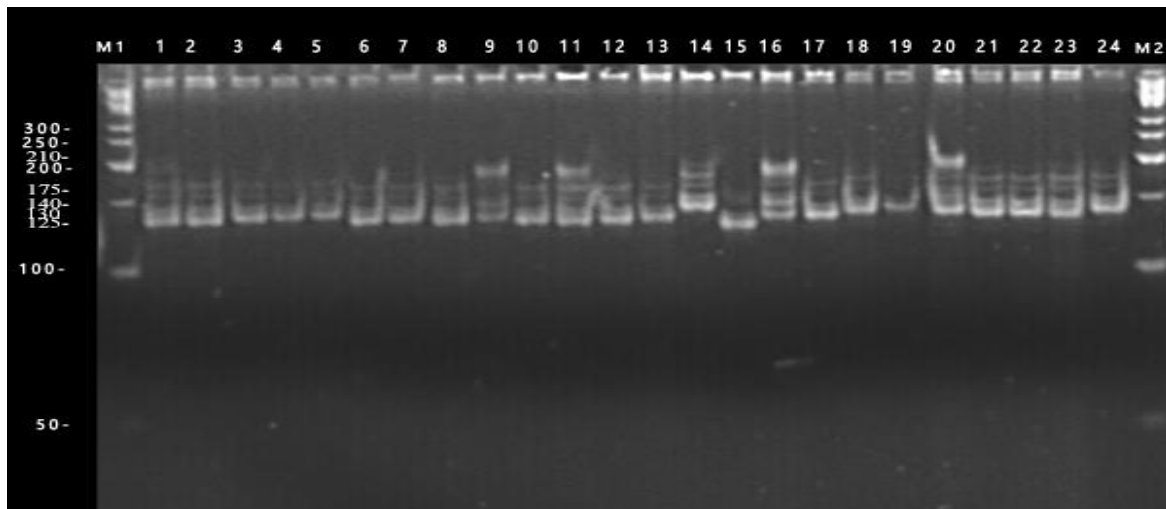
Plate 3. SSR profile of 24 pigmented T. Aman rice germplasm using primer RM213. M1 and M2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane : 1) Black rice-1, 2) Black rice-2, 3) Black Rice-3, 4) Black Rice-4, 5) Mural(Uba), 6) Murali, 7) Birun, 8) Birui Sail, 9) Biruin, 10) Biroin Kalarmur, 11) Biroin(Makri), 12) Biroin (Habai), 13) Makri Biroin, 14) Balam Dhan, 15) Balam , 16) Birpak, 17) Lal Balam, 18) Butu Balam, 19) Latisail, 20) Jhoria, 21) Biroi Dhan, 22) Makri Biroin, 23) Jhual Biroin, 24) Kach Badal.
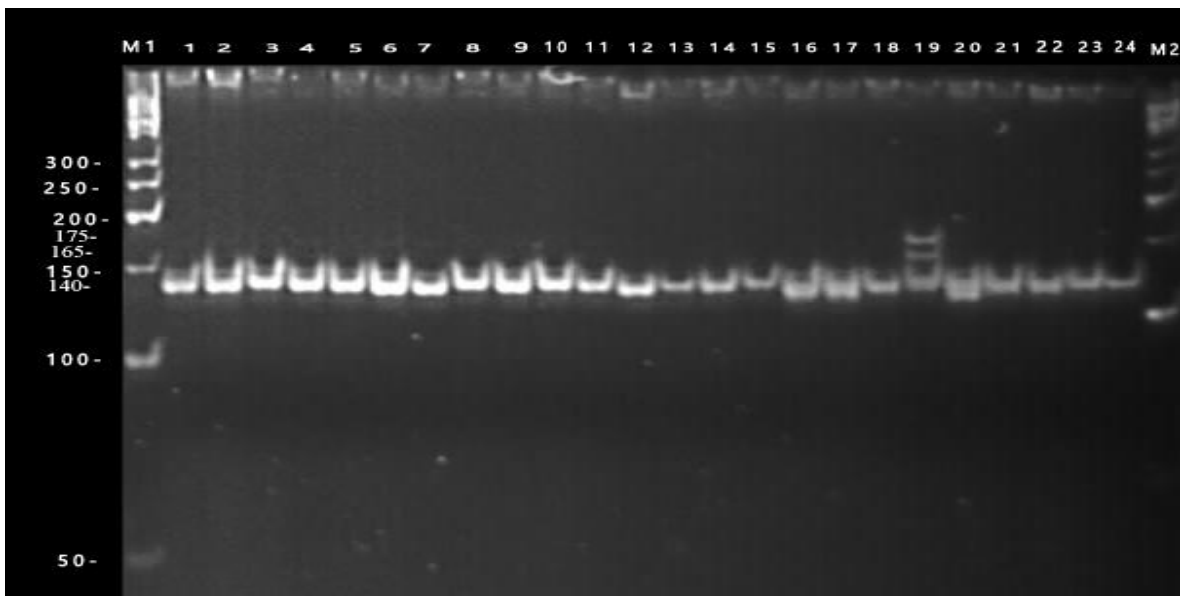
Plate 4. SSR profile of 24 pigmented T. Aman rice germplasm using primer RM277. M1 and M2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane : 1) Black rice-1, 2) Black rice-2, 3) Black Rice-3, 4) Black Rice-4, 5) Mural(Uba), 6) Murali, 7) Birun, 8) Birui Sail, 9) Biruin, 10) Biroin Kalarmur, 11) Biroin(Makri), 12) Biroin (Habai), 13) Makri Biroin, 14) Balam Dhan, 15) Balam , 16) Birpak, 17) Lal Balam, 18) Butu Balam, 19) Latisail, 20) Jhoria, 21) Biroi Dhan, 22) Makri Biroin, 23) Jhual Biroin, 24) Kach Badal.

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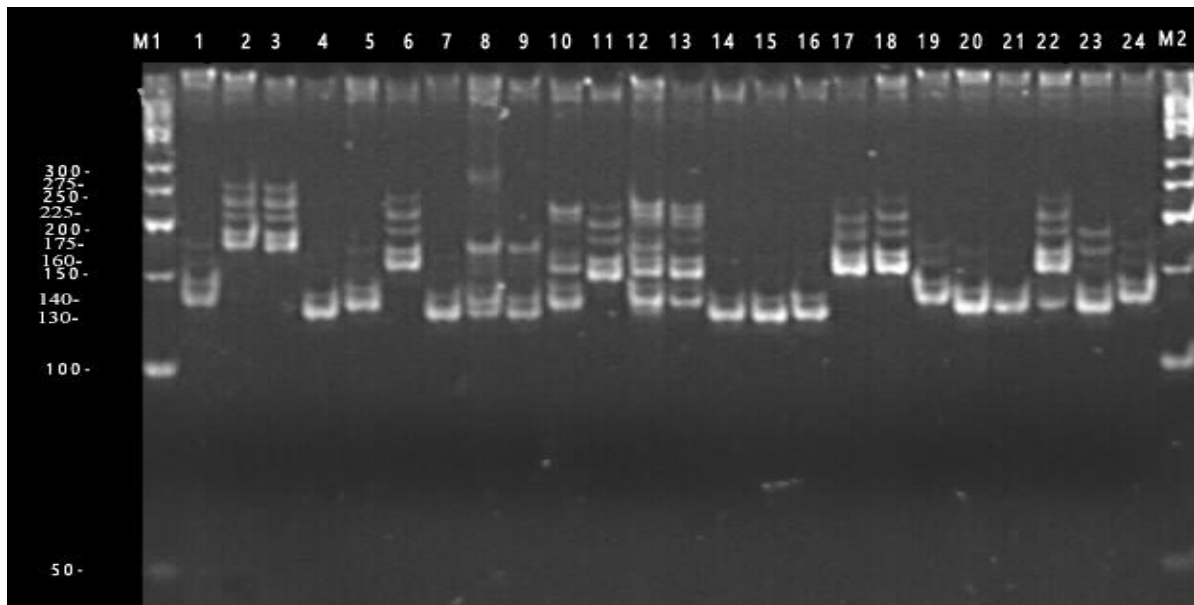
Plate 5. SSR profile of 24 pigmented T. Aman rice germplasm using primer RM206. M1 and M2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane : 1) Black rice-1, 2) Black rice-2, 3) Black Rice-3, 4) Black Rice-4, 5) Mural(Uba), 6) Murali, 7) Birun, 8) Birui Sail, 9) Biruin, 10) Biroin Kalarmur, 11) Biroin(Makri), 12) Biroin (Habai), 13) Makri Biroin, 14) Balam Dhan, 15) Balam , 16) Birpak, 17) Lal Balam, 18) Butu Balam, 19) Latisail, 20) Jhoria, 21) Biroi Dhan, 22) Makri Biroin, 23) Jhual Biroin, 24) Kach Badal.
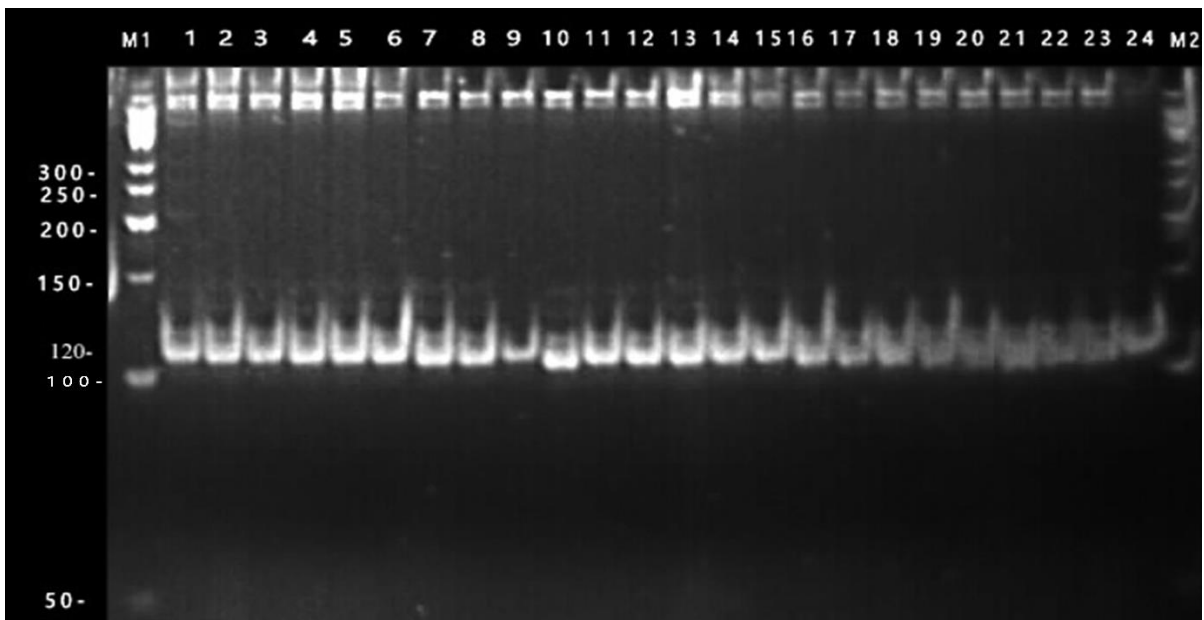
Plate 6. SSR profile of 24 pigmented T. Aman rice germplasm using primer RM510. M1 and M2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane : 1) Black rice-1, 2) Black rice-2, 3) Black Rice-3, 4) Black Rice-4, 5) Mural(Uba), 6) Murali, 7) Birun, 8) Birui Sail, 9) Biruin, 10) Biroin Kalarmur, 11) Biroin(Makri), 12) Biroin (Habai), 13) Makri Biroin, 14) Balam Dhan, 15) Balam , 16) Birpak, 17) Lal Balam, 18) Butu Balam, 19) Latisail, 20) Jhoria, 21) Biroi Dhan, 22) Makri Biroin, 23) Jhual Biroin, 24) Kach Badal.

4.2.3 Genetic analysis of pigmented T. Aman rice germplasm by different markers

A total of 70 polymorphic alleles were detected across 24 T. Aman rice germplasms using 16 SSR markers (Table 14). The highest range of band sizes was found in RM1337 (171-350 bp) followed by RM338 (114-172bp) and RM334 (154-170bp) respectively. The number of alleles per locus ranged from 8 alleles (RM1) to 1 allele (RM510) with an average of 4.38 alleles across the 16 loci, with 1 allele for 1 marker, 2 alleles for 1 marker, 3 alleles for 3 markers, 4 alleles for 5 markers, 5 alleles for 1 marker, 6 alleles for 3 markers, 7 alleles for 1 marker and 8 alleles for 1 marker. The frequency of the most common allele at each locus ranged from 29.17% (RM206) to 91.67% (RM510). On an average, 64.84% of the 24 test rice germplasms shared a common major allele at any given locus. Jain *et al.* (2004) observed that 53.6% of 69 rice genotypes shared common alleles at any locus. Thomson *et al*. (2009) indicated that on an average, 62% of the 190 rice accessions of Indonesia shared a common major allele at any given of SSR locus. Similar results were also observed by others (Saini *et al*., 2004; Lu *et al*., 2005; Jayamani *et al*., 2007; Islam *et al*, 2018b). Gene diversity ranged between 0.15 to 0.82 with an average of 0.484375. Primer RM1 showed highest gene diversity (0.82) and primer RM307 showed the lowest gene diversity (0.15).

4.2.4 PIC values

Polymorphism information content or PIC value of marker is the probability of the marker allele that can be deduced in the progeny and is a good measure of a marker's usefulness for linkage analysis. So, PIC is the reflection of allele diversity and frequency among the varieties and varied greatly for all the SSR loci tested. The PIC values for 16 SSR loci varied from 0.14 to 0.80, with an average .445 (Table 14). The PIC values observed, are comparable to three previous estimates of microsatellite analysis in rice viz. o.67-0.88 (Gohain *et al.*, 2006), 0.34-0.88 (Thomson *et al.,* 2007), 0.20-.90 with an average 0.56 (Jain *et al.,* 2003). The highest PIC values were observed for the primer of RM1 (0.80) followed by RM206 (0.74), RM 1337 (0.66), RM5 (0.58) and RM334 (.54) (Table 11). All the loci except the five primer RM1 (0.80), RM206 (0.74), RM 1337 (0.66), RM5 (0.58) and RM334 (.54) showed low PIC value (<0.50). The estimated average PIC values are similar with the average PIC values as reported by others (Joshi *et al*., 2010; Bahera *et al*., 2012; Pachauri *et al*., 2013).

Table 14: Number of alleles, allele size range, frequency, gene diversity and polymorphism information content (PIC) among 24 T. Aman rice germplasm

SL. No.	Marker	Chro. No.	Position (cM)	Motif*	Allele No.	Unique allele	Size range (bp)	Freq (%)	Gene diversity	PIC
1	RM1	1	29.7	(GA)26	8	1	84-180	33.33	0.82	0.80
2	RM5	1	94.9	(GA)14	5	-	113-124	50.00	0.64	0.58
3	RM125	7	24.8	(GCT)8	3	2	120-146	91.67	0.16	0.15
4	RM205	9	7.4	(CT)11	4	1	123-158	79.17	0.36	0.34
5	RM206	11	114.7	(CT)25	6	1	140-275	29.17	0.78	0.74
6	RM209	11	73.9	(CT)18	7	5	125-155	70.83	0.48	0.47
7	RM213	2	186.4	(CT)17	4	2	125-210	70.83	0.45	0.40
8	RM214	2	186.4	(CT)17	3	-	116-138	58.33	0.56	0.49
9	RM277	12	130.3	(CA)6(GA)36	3	-	140-175	75.00	0.41	0.37
10	RM304	10	73	(GT)2(AT)10(GT)33	4	1	152-167	70.83	0.47	0.44
11	RM307	4	0	(AT)14(GT)21	2	-	172-181	91.67	0.15	0.14
12	RM510	6	20.8	(GA)15	1	-	120	91.67	0.16	0.15
13	RM338	3	108.4	(CTT)6	4	1	114-172	62.50	0.50	0.41
14	RM342	8	78.4	(CAT)12	4	-	130-149	66.67	0.50	0.44
15	RM334	5	141.8	(CTT)20	6	-	154-170	50.00	0.61	0.54
16	RM1337	12	57.2	(AG)21	6	2	171-350	45.83	0.70	0.66
	Max.				8.00		171-350	91.67	0.82	0.80
	Min.				1.00			29.17	0.15	0.14
	Total				70	16			7.75	7.12
	Mean				4.38			64.84	0.4843	0.445

*Chro.= Chromosome, Freq.= Frequency, PIC= Polymorphism Information Content, bp= Base pair

Moreover, the SSR markers used in the study were selected on the basis of their high PIC values reported earlier. Higher PIC values for some SSRs similar to our findings were also reported in the literature (Garland *et al.*, 1999; Juneja *et al*., 2006; Giarrocco *et al*., 2007;

Jayamani *et al*., 2007; Ram *et al*., 2007). In general, higher PIC values were observed for SSRs having higher number of alleles. Lower PIC value indicates that the genotypes under study are closely related types, while the higher value of PIC indicates higher diversity of materials which is better for development of new varieties. Primer RM1 had the highest PIC value (0.80) and the highest number of alleles (8), therefore it detected the highest level of polymorphism. So, RM1 is supposed to be the best marker for characterizing the 24 pigmented T. Aman rice germplasms and to a lesser extent RM206, RM 1337, RM5, RM334 were also useful for molecular characterization of these genotypes. PIC value revealed that RM1, RM206, RM1337, RM5 and RM334 were considered as the best marker for 24 pigmented T. Aman rice germplasms.

4.2.5 Diversity revealed by different markers

Molecular diversity data was presented in Table-14. The polymorphism information content (PIC) value is a reflection of allelic diversity and frequency among the varieties. The used 16 markers have different level of diversity on the basis of PIC value. Among them, RM125, RM307, RM510 showed low diversity. Again, RM205, RM209, RM213, RM214, RM277, RM304, RM338, RM342showed moderate diversity and RM1, RM5, RM1337, RM206, RM334 showed high diversity. The results revealed that the varieties produced unique alleles that could be used for molecular characterization and DNA fingerprinting of these T. Aman rice landraces. Because, these unique alleles may have some special characteristics which may be helpful in breeding as well as other research program concerning rice development. The result revealed to broaden the genetic base for wider adaptability (Islam *et al*., 2018b).

Table 15. Diversity level showed by different markers based on PIC value

Sl. No.	PIC Value	Diversity level	Markers
1.	≤ 0.25	Low	RM125, RM307, RM510
2.	0.25- 0.50	Moderate	RM205, RM209, RM213, RM214, RM277, RM304, RM338, RM342
3.	0.50-1.0	High	RM1, RM5, RM1337, RM206, RM334

4.2.6 Unique Alleles

Eighteen (16) unique alleles were detected at 9 SSR markers (Table 16). Unique alleles are important because they may be diagnostic of a particular germplasm and useful for breeding purpose. The markers RM1, RM125, RM205, RM206, RM209, RM213, RM304, RM338 and RM1337 showed unique allele in 16 T. Aman rice germplasms namely Birun, Biruin Birpak, Black Rice -4, Murali, Black Rice-4, Latisail, Mural(Uba), Black Rice-1, Murali, Balam, Balam Dhan, Latisail, Kach Badal, Black Rice-3, Murali .The occurrence of relatively higher number of unique alleles in the pigmented T. Aman rice germplasm indicates its potentiality as a reservoir of novel alleles for crop improvement. Similar finding was also reported by (Islam *et al*., 2018b).

Table 16. Microsatellite loci that unique alleles in different rice germplasm

Sl. No.	Marker	Chro. No.	Unique allele (bp)	Name of germplasms
1	RM1	01	133 bp	Birun
2	RM125	07	120 bp, 146 bp	Biruin, Birpak
3	RM205	09	122 bp	Black Rice -4
4	RM206	11	147 bp	Murali
5	RM209	11	125 bp, 125bp, 135bp, 155bp, 145bp	Black Rice-4, Latisail, Mural(Uba), Black Rice-1, Murali
6	RM213	02	131 bp, 150 bp	Balam, Balam Dhan
7	RM304	10	167 bp	Latisail
8	RM338	03	166bp	Kach Badal
9	RM1337	12	208bp, 196bp	Black Rice-3, Murali
	Total		16	

4.2.7 Genetic Distance based Analysis

Genetic distance is a measure used to quantify the difference between two populations in relation to the frequency of particular trait as well as the relationship among the population. The value of pair-wise comparisons of Nei's (1983) genetic distance among 24 relatives of rice genotypes were computed from combined data for the 16 primers, ranged from 0.0625 to 0.8750 (Table 17). The highest inter-genotypic distance was 0.8750 which was observed between Biruin (Tola) and Lal Balam. The lowest inter-genotypic distance was 0.0625 which was observed between Biroin (Habai) and Biroin (Makri), Makri biroin and Biroin (Habai), Biroi Dhan and Jhoria. The pair-wise genetic dissimilarity coefficients indicated that Biruin (Tola) showed 87% genetic distance with Lal Balam,75 % with Murali, Latisail, Biroin (Habai) and Biroin (Makri); 68 % with Balam and Makri Biroin, 62 % with Black Rice-3. Mural (Uba), Black Rice-3 and Black Rice-2 showed 81% genetic distance with Birpak. Black Rice-3 showed 75% genetic distance with Balam and Kach Badal, 62 % with Makri Biroin, Lal Balam and Black Rice-2. Murali showed 75 % with Jhual Biroin, 62% with Mural (Uba),Balam Dhan, Balam and Birpak,Kach Badal showed 68% genetic distance with Black Rice -2 ,62% with Jhual Biroin, Black Rice-1, Birpak and Lal Balam. Jhual Biroin showed 68% genetic distance with Birpak, Latisail and Lal Balam (2), 62% with Makri Biroin ,Biroin (Habai) and Balam. Latisail showed 68% genetic distance with Birpak, Butu Balam(2) showed 68% genetic distance with Birpak Birpak showed 68% genetic distance with Balam Dhan, Balam showed 68% genetic distance with Biroin Kalamur. The highest genetic distance between them indicated that genetically they are dissimilar and also highly diverse. The difference between the highest and lowest genetic identity indicates the presence of variability among 24 germplasms of T. Aman rice. Those (germplasms) which have higher (0.8750) genetic distance are more dissimilar than those which have a lower value. Again, many pairs have showed the lowest genetic distance (0.0625) which indicates their genetically much more closeness among them.

The results obtained from this study on molecular characterization provided some useful implications for the establishment of sovereignty of the Bangladeshi rice gene pool. Considering results from marker-assisted diversity analysis, accessions that are far apart based on their genetic coefficient (like Biruin (Tola) and Lal Balam, Mural (Uba) and Birpak, Black Rice-3 and Birpak, Black Rice-2 and Birpak) could be selected as parents for

further breeding programs. This will bring about greater diversity, which will lead to a high productive index in terms of an increase in yield and overall quality.

Table 17: Genetic distance among 24 pigmented T. Aman rice germplasm

OUT	G1	G10	G11	G12	G13	G14	G1 5	G16	G17	G18	G19	G2
G1	0.0000											
G10	0.3750	0.0000										
G11	0.3750	0.1250	0.0000									
G12	0.4375	0.1875	0.0625	0.0000								
G13	0.5000	0.1250	0.1250	0.0625	0.0000							
G14	0.5625	0.4375	0.5000	0.5625	0.5000	0.0000						
G15	0.6250	0.6875	0.6250	0.5625	0.6250	0.5000	0.0000					
G16	0.6250	0.6250	0.5000	0.5625	0.6250	0.6875	0.5625	0.0000				
G17	0.6250	0.4375	0.3750	0.3750	0.4375	0.5625	0.5000	0.6250	0.0000			
G18	0.5000	0.3750	0.3750	0.4375	0.5000	0.4375	0.6250	0.6875	0.3750	0.0000		
G19	0.5000	0.4375	0.3750	0.4375	0.5000	0.5000	0.6250	0.6875	0.5000	0.3750	0.0000	
G2	0.3750	0.4375	0.5625	0.5625	0.5000	0.4375	0.6875	0.8125	0.6250	0.5625	0.6250	0.0000
G20	0.4375	0.4375	0.4375	0.5000	0.5625	0.5000	0.5000	0.5000	0.4375	0.5000	0.5625	0.5000
G21	0.3750	0.3750	0.3750	0.4375	0.5000	0.4375	0.5000	0.5625	0.5000	0.4375	0.5000	0.4375
G22	0.5000	0.3750	0.2500	0.3125	0.3750	0.5625	0.6250	0.5000	0.3750	0.4375	0.4375	0.6250
G23	0.4375	0.4375	0.5625	0.6250	0.5625	0.5000	0.6250	0.6875	0.6875	0.5625	0.6875	0.5625
G24	0.6250	0.4375	0.3750	0.4375	0.4375	0.5625	0.5625	0.6250	0.6250	0.5625	0.4375	0.6875
G3	0.3750	0.4375	0.5625	0.5625	0.5000	0.5000	0.7500	0.8125	0.6250	0.5625	0.6250	0.0625
G4	0.5625	0.3750	0.3750	0.4375	0.3750	0.4375	0.6250	0.5625	0.6250	0.6250	0.5625	0.4375
G5	0.4375	0.5625	0.5625	0.5625	0.6250	0.4375	0.6250	0.8125	0.5625	0.4375	0.5000	0.3750
G6	0.5625	0.5000	0.3750	0.4375	0.5000	0.6250	0.6250	0.6250	0.5625	0.5625	0.5625	0.6875
G7	0.3125	0.3750	0.3750	0.4375	0.5000	0.3750	0.3750	0.4375	0.5000	0.4375	0.5000	0.4375
G8	0.3750	0.4375	0.4375	0.5000	0.5625	0.5000	0.5000	0.4375	0.5625	0.5000	0.5625	0.5000
G9	0.5625	0.6250	0.7500	0.8125	0.7500	0.5625	0.6875	0.6875	0.8750	0.6250	0.7500	0.5625

Table 17 Contd.: Genetic distance among 24 pigmented T. Aman rice germplasm

OUT	G20	G21	G22	G23	G24	G3	G4	G5	G6	G7	G8	G9
G1												
G10												
G11												
G12												
G13												
G14												
G15												
G16												
G17												
G18												
G19												
G2												
G20	0.0000											
G21	0.0625	0.0000										
G22	0.3750	0.3125	0.0000									
G23	0.4375	0.4375	0.6250	0.0000								
G24	0.5000	0.5000	0.3750	0.6250	0.0000							
G3	0.5625	0.5000	0.6250	0.5625	0.7500	0.0000						
G4	0.4375	0.3750	0.4375	0.5625	0.5000	0.5000	0.0000					
G5	0.4375	0.3750	0.6250	0.6250	0.6250	0.4375	0.5000	0.0000				
G6	0.5625	0.5000	0.4375	0.7500	0.5625	0.6875	0.5000	0.6250	0.0000			
G7	0.2500	0.1875	0.3750	0.4375	0.5000	0.5000	0.4375	0.4375	0.5000	0.0000		
G8	0.2500	0.1875	0.4375	0.4375	0.5625	0.5625	0.4375	0.4375	0.5625	0.1875	0.0000	
G9	0.5625	0.5000	0.7500	0.5000	0.6875	0.6250	0.5625	0.5000	0.8125	0.5000	0.4375	0.0000

*G1=Black Rice 1, G2=Black Rice 2, G3=Black Rice 3, G4=Black Rice 4, G5=Mural(uba), G6=Murali, G7=Birun(2), G8=Birui Sail, G9=Biruin (Tola), G10=Biroin kalamur, G11=Biroin(makri), G12=Biroin(Habai), G13=Makri Biroin, G14=Balam Dhan, G15=Balam, G16=Birpak, G17=Lal Balam, G18=Butu Balam(2), G19=Latisail, G20=jhoria, G21=Biroi Dhan, G22=Makri Biroin, G23=Jhual Biroin, G24=Kach Badal

4.2.8 UPGMA dendrogram

On the basis of the Nei's genetic distance calculation of 24 pigmented T. Aman rice germplasms a dendrogram was calculated. Unweighted Pair Group Method of Arithmetic Mean (UPGMA)-based dendrogram generated through neighbor-joining (NJ) tree analysis, which grouped all germplasms into three major clusters I, II, and III comprised of 8, 8, and 8

germplasm, respectively. G1, G10, G11, G12,G13,G14,G15,G16 were grouped in cluster I (Table 14 and Figure12); G17,G18,G19,G2,G20,G21,G22,G23 were grouped in cluster II and G24, G3, G4, G5, G6, G7, G8, G9 were grouped in cluster III. Here, "G" indicated the T. Aman rice germplasms.

Table 18: List of germplasm with their cluster based on UPGMA method

Cluster	Sub cluster	Genotypes	No. of germplam
I	1	Black Rice 1, Biroin kalamur, Biroin(makri), Biroin(Habai)	4
	2	Makri Biroin, Balam Dhan, Balam, Birpak	4
II	1	Lal Balam, Butu Balam(2), Latisail, Black Rice 2,	4
	2	jhoria, Biroi Dhan, Makri Biroin, Jhual Biroin	4
III	1	Kach Badal, Black Rice 3, Black Rice 4, Mural(uba),	4
	2	Murali, Birun(2), Birui Sail, Biruin (Tola),	4

In cluster I, G1 (Black Rice 1), G10 (Biroin kalamur), G11 (Biroin (Makri)), G12 (Biroin(Habai)) formed sub cluster 1; again,G13 (Makri Biroin), G14 (Balam Dhan), G15 (Balam), G16 (Birpak) grouped together in sub cluster 2. Here, G17 (Lal Balam), G18 Butu Balam(2)), G19 Latisail, G2 (Black Rice 2) formed sub cluster1 of cluster II.G20 (Jhoria), G21 (Biroi Dhan), G22 (Makri Biroin), G23 (Jhual Biroin) formed sub cluster 2 of cluster 2.G24 (Kach Badal), G3 (Black Rice 3), G4 (Black Rice 4), G5 (Biroin Kalamur) formed subcluster1 of cluster 3and G6 (Murali), G7 (Birun(2)), G8 (Birui) G9 (Biruin (Tola)), formed subcluster 2 of cluster 3. Genotypes of more genetic similarity are placed in same sub cluster. The dendrogram revealed that the genotypes that derivatives of genetically similar type clustered together. The germplasms grouped in the same cluster due to lower genetic distance and the other germplasms having more genetic dissimilarity grouped in another cluster due to higher genetic distance. Yasmin *et al*., *(*2012) reported that genetic diversity of 24 rice (*Oryza sativa*) germplasms comprising five salt 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 clearly separated all the germplasms into five main distinct clusters.
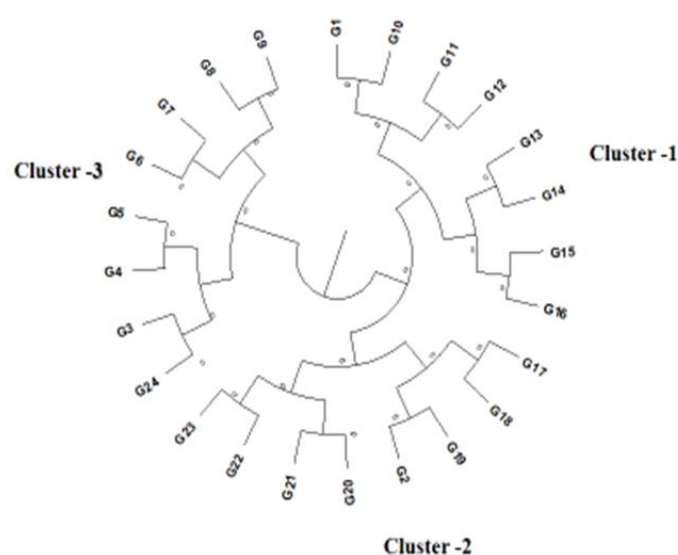
*G1=Black Rice 1, G2=Black Rice 2, G3=Black Rice 3, G4=Black Rice 4, G5=Mural(uba), G6=Murali, G7=Birun(2), G8=Birui Sail, G9=Biruin (Tola), G10=Biroin kalamur, G11=Biroin(makri), G12=Biroin(Habai), G13=Makri Biroin, G14=Balam Dhan, G15=Balam, G16=Birpak, G17=Lal Balam, G18=Butu Balam(2), G19=Latisail, G20=jhoria, G21=Biroi Dhan, G22=Makri Biroin, G23=Jhual Biroin, G24=Kach Badal

Figure 13: An unrooted neighbor-joining tree showing the genetic relationships among 24 pigmented T. Aman rice germplasm of Bangladesh based on the alleles detected by 16 microsatellite marker.

CHAPTER V
SUMMARY AND CONCLUSION

Twenty four pigmented T. Aman rice germplasms were evaluated for ten (10) qualitative and 14 quantitative characters to study genetic divergence through univariate analysis. The research was directed to study morpho-molecular diversity of 24 pigmented T. Aman rice germplasm. Based on frequency distribution, 10 qualitative traits of 24 pigmented T. Aman rice germplasms showed variation in different accessions. After the evaluation of 24 pigmented T. Aman rice germplasm for 14 quantitative characters, based on mean values, some germplasm which showed best performance were identified like Black Rice-4, Black Rice-3, Biroin (Habai), Kach Badal, Jhoria, Butu Balam (2), Latisail.

The number of total tiller varied from 5-16. Effective tiller ranged from 5 - 13 with mean value 9. The highest number of effective tillers (13) was observed in Butu Balam and Latisail. Panicle length varied from 21.40 (cm) to 30.20 (cm). Plant height (cm) ranged from 87.20 (cm) to 158.20 (cm). The shortest plant height (87.20 cm) was observed in Biroin (Habai)) and the longest (158.20 cm) in Beruin. Days to 50% flowering ranged from 80 to 117 days with mean value 105 days. The highest mean performance was recorded for Black Rice-4 (117 days). Filled grains per panicle varied from 39 to 153. The highest mean performance was recorded for Jhoria (153). 1000 grain weight ranged from 17 (g) - 32.25 (g) with mean value 24.17g. Kach Badal had the highest (32.25 g) 1000 grain weight. Yield per hill ranged from 11.18 – 22.57 with mean value 17.72. Balam Dhan showed highest mean 17.72 (g). The highest yield per hill (22.57 g) was observed in Balam Dhan and the lowest (11.18 g) in Black Rice-4. After the evaluation of 24 pigmented T. Aman rice germplasm for 14 quantitative characters, based on mean values, some germplasm which showed best performance were identified like Black rice-2, Black Rice-3, Black Rice-4, Biroin (Habai), Kach Badal, Murali, Jhoria, Butu Balam (2), Latisail. Identifying germplasm for different agronomical characters in phenotypically divergent sources would help in prebreeding and breeding programs.

The dendrogram, constructed by using UPGMA clustering based on Eucledean method, distributed the 24 pigmented T. Aman rice germplasms into three major clusters along with four sub-clusters, for 14 quantitative traits.

Using 16 SSR primers across 24 pigmented rice germplasms, a total of 70 alleles were found in the present study of which RM 1 showed the highest number of alleles (8) and RM 510 showed the lowest number of alleles (1), with an average of 4.38 alleles across the 16 loci. Gene diversity ranged from 0.15 to 0.82 with an average of 0.4843. Primer RM1 showed highest gene diversity (0.82) and primer RM307 showed the lowest gene diversity (0.15).The PIC values for 16 SSR loci varied from 0.14 to 0.80 with an average of 0.445. Primer RM1 had the highest PIC value (0.80) and the highest number of alleles (8), therefore it detected the highest level of polymorphism. So, RM1 is supposed to be the best marker for characterizing the 24 pigmented T. Aman rice germplasms.

Eighteen (16) unique alleles were detected at 9 SSR markers. The markers RM1, RM125, RM205, RM206, RM209, RM213, RM304, RM510, RM338 and RM1337 showed unique allele in T.Aman rice germplasm namely Birun, Biruin, Birpak, Black Rice -4, Murali, Black Rice-4, Latisail, Mural(Uba), Black Rice-1, Murali, Balam, Balam Dhan, Latisail, Kach Badal, Black Rice-3, Murali.

The value of pair-wise comparisons of Nei's (1983) genetic distance among 24 pigmented T. Aman rice germplasms were computed from combined data for the 16 primers, ranged from 0.0625 to 0.8750. The highest inter genotypic distance was 0.8750 which was observed between Biruin (Tola) and Lal Balam. The lowest inter genotypic distance was 0.0625 which was observed between Biroin (Habai) and Biroin (Makri), Makri biroin and Biroin (Habai), Biroi Dhan and Jhoria. The genetic distance-based results seen in the unrooted neighbor-joining tree revealed three groups with six sub-clusters in the 24 test germplasm. Cluster showed genetic similarity and dissimilarity among varieties.

SSR markers were found to be a powerful tool to detect genetic variation and genetic relationship within and among different rice germplasm. Findings from this study suggest that the diverse germplasm and polymorphic SSR markers of pigmented rice are suitable for the detection of economically desirable trait genes for use in future molecular breeding programs.

RECOMMENDATIONS

The present study exposed that there was a high level of genetic diversity among accessions of pigmented Transplant Aman rice germplasm. It was recommended that SSR markers were effective in the recognition of polymorphism. The existing study can be used as an instruction for the next researchers who have concern for pigmented rice. For studying the morphology and genetical qualities of Transplant Aman rice in Bangladesh following points might be considered.

- A large number of pigmented germplasms could be included for getting more diverse result.

- A large number of SSR markers along with other markers like RAPD, ISSR etc. could be used for procurement more precise and diverse result.

- To obtain more specific result high throughout molecular markers such as Single Nucleotide Polymorphism (SNP) could be used for genome-wide coverage of pigmented rice germplasm.

REFERENCES

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

Ahmad, F., Hanafi, M.M., Hakim, A.H, Rafi, M.Y., Arolu, I.W., Abdulla, S.N.A. (2015). Genetic divergence and heritability of 42 coloured upland rice genotypes (*Oryza sativa*) as revealed by microsatellites marker and agro-morphological traits. *PLoS One* **10** (9): e0138246. DOI: 101371/journal.

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

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.

Akter, N., Islam, M.Z., Bhuiya, A., Siddique, M.A. and Khalequzzaman, M. (2017). Distinctness of 45 irrigated rice (*Oryza sativa* L.) landraces of Bangladesh through agro-morphological traits. *Eco-friendly Agril. J*. **10**(07): 100-107.

Abdel-Aal E-SM, Young, J.C., Rabalsk, I. (2006). Anthocyanin composition in black, blue, pink, purple, and red cereal grains. *J Agric Food Chem*, **54**: 4696-4704.

Abbasi, F.M., Sagar, M.A., Akram, M. and Ashraf, M. (1995). Agronomic and quality traits of some elite rice genotypes. *Pakistan J. Sci. Indust. Re*s., **38**: 348–50

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. 25-27 August 2004, Nagarkot, Nepal.

Behera, L., Patra, B.C., Sahu, R.K., Nanda, A., Sahu, S.C., Patnaik, A., Rao, G.J.N. and Singh, O.N. (2012). Assessment of genetic diversity in medicinal rices using microsatellite markers. *Aus. J. Crop Sci.*, **6**(9): 1369-1376.

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.

Chutipaijit, S., Chaum, S. and Sompornpailin, K. (2011). High contents of proline and anthocyanin increase protective response to salinity in *Oryza sativa* L. spp. *indica. Austr J Crop Sci*., **5**(10):1191-1198.

Chen, X., Temnykh, S., Xu, Y., Cho, Y.G. and McCouch, S.R. (2001) Development of a microsatellite framework map providing genome-wide coverage in rice (*Oryza sativa* L.). *Theor Appl Genet.,* **95**:553–567.

Cheema, A.A., Awan, M.A. and Iqbal, J. (1987). Improvement of Plant Height Architecture in Basmati Rice. *Pakistan J. Agric. Res*., **8**: 371– 4.

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.

Chen, C., He, W., Nassirou, T.Y., Nsabiyumva, A., Dong, X., Adedze, M.N. and Jina, D. (2017). Molecular characterization and genetic diversity of different genotypes of 51 *Oryza sativa* and *Oryza glaberrima* (research article). *Elect. J. of Biotech*. **30**: 48– 57.

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.

Garland, S.H., Lewin, L., Abedinia, M., Henry, R. and Blackeney, A. (1999). The use of microsatellite polymorphisms for the identification of Australian breeding lines of rice (*Oryza saitva* L.). *Euphytica,* **108**: 53-63.

Giarrocco, L.E., Marassi, M.A. and Salerno, G.L. (2007). Assessment of the genetic diversity in argentine rice cultivars with SSR markers. *Crop Sci.*, **47**: 853-858.

Gohain B., Talukdar, A. and Modi, M.K. (2006). Mining of allele(s) for aroma in local aromatic rice (joha) germplasm of Assam using SSR marker. Abstracts 26th International Rice Conference-2, International Rice Congress, October 9-13, 2006, New Delhi, India. 74 p.

Huang, Y.P. and Lai, H.M. (2016). Bioactive compounds and antioxidative activity of colored rice bran. *J Food Drug Anal.,* (2016), DOI: 10.1016/j.jfda.2016.01.004.

Hyun, J.W. and Chung, H.S. (2004). Cyanidin and malvidin from Oryza sativa cv. Heugjinjubyeo mediate cytotoxicity against humanmonocytic leukemia cells by arrest of G2/M phase and induction of apoptosis. *J Agric Food Chem.,* **52**: 2213-2217.

Herrera, T.G., Duque, D.P., Ameida, I.P., Nunez, G.T., Pieters, A.J., Martinez, C.P. and Tohme, J.M. (2008). Assesment of genetic diversity in Venezuelan rice cultivars using simple sequence repeats markers. *Elect. J. Biotech.* **11**(5): 1-14.

Hooker, J. D. (1979). The flora of british india. vol. 2L. Reeve Co. Kent, England. p.25.

Ijaz, S. (2011). Microsatellite markers: An important fingerprinting tool for characterization of crop plants. *Afr. J. Biotech.*, **10**(40): 7723-7726.

Islam, M. Z., Siddique, M. A., Rashid, E. S. M. H., Ahmed, M. S. and Khalequzzaman, M. (2014). Genetic Diversity in Sadajira Rice (*Oryza sativa* L.) Germplasm. *The Agriculturists.* **12**(1): 26-32.

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*. (**2016)**, Article ID 2796720, http://dx.doi.org/10.1155/2016/2796720.

Islam, M.Z., Khalequzzaman, M., Siddique, A., Akter, N., Ahmed, M.S. and Chowdhury, M.A.Z. (2017). Phenotypic characterization of Jhum rice *(Oryza sativa* L.) landraces collected from Rangamati district in Bangladesh. *Bangladesh Rice J.* **21**(1): 47-57.

Islam, M.Z., Siddique, M.A., Akter, N., Prince, M.F.R.K., Islam, M.R., Anisuzzaman, M. and Mian, M.A.K. (2019). Morpho-molecular Divergence of Restorer Lines for Hybrid Rice (*Oryza sativa* L.) Development. Cereal Research Communications **47**(3): 531–540.

Islam, M.Z., Khalequzzaman, M., Bashar, k., Ivy, N.A., Haque, M.M., Mian, M.A.K. and Tomita, M. (2018a). Agro-morphological Characterization of Bangladeshi Aromatic Rice (*Oryza sativa* L.) Germplasm Based on Qualitative Traits. *Bangladesh Rice J.* **22** (2): 41-54.

Islam, M.Z., Khalequzzaman, M., Prince, M.F.R.K., Siddique, M.A., Rashid, E.S.M. H., Ahmed, M.S.U., Pittendrigh, B.R. and Ali M.P. (2018b). Diversity and population structure of red rice germplasm in Bangladesh. *J. Pl.* **13**(5): 1-20.

Islam, M. Z., Khalequzzaman, M., Bashar, M. K., Ivy, N.A., Mian, M.A. K., Pittendrigh, B. R., Haque, M. M. and Ali M. P., (2018c) Variability Assessment of Aromatic Rice Germplasm by Pheno-Genomic traits and Population Structure Analysis *8*:9911. DOI:10.1038/s41598-018-28001-z.

Islam, M.R., Singh, R.K., Salam, M.A., Hasan, L. and Gregorio, G.B. (2008). Molecular diversity of stress tolerant rice genotypes using SSR markers. SABRAO J. Breed. *Genet.* **40**(2): 127-139.

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.

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

Jayamani, P., Negrao, S., Martins, M., Macas, B. and Oliveira, M.M. (2007). Genetic relatedness of Portuguese rice accessions from diverse origin as assessed by microsatellite markers. *Crop Sci.,* **47**: 879 – 886.

Joshi, R.K., Subudhi, E., Kar, B. and Nayak, S. (2010). Comparative genetic analysis of lowland rice cultivars of India using microsatellite markers. *Biores Bull.,* **4**: 213-223.

Juneja, S., Das, A., Joshi, S.V., Sharma, S., Vikal, Y., Patra, B.C., Bharaj, T.S., Sidhu, J.S. and Singh, K. (2006). *Oryza nivara* Sharma et Shastry, the progenitor of *Oryaz. sativa* L. subspecies *indica* harbours rich genetic diversity as measured by SSR markers. *Cur. Sci*., **91**:1079-1085.

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.

Kushwaha U.K.S. (2016). Black Rice. Springer International Publishing, Switzerland.

Kristamtini, Purwaningsih H. (2009). The potency of red rice development as germplasm of Yogyakarta. *Jurnal Litbang Pertanian* **28** (3): 88-95.

Kurlovich, B.S. (1998). Species and intra specific diversity of white, blue and yellow lupins. *Pl. Genet. Resour. Newsl*., **115**: 23-32.

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.

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

Lu, H., Redus, M.A., Coburn, J.R., Rutger, J.N., McCouch, S.R. and Tai, T.H. (2005). Population structure and breeding patterns of 145 US rice cultivars based on SSR marker analysis. *Crop Sci*. **45**: 66-76.

Mia, M.F., Begum, S.N., Islam, M.M., Manidas, A.C. and Halder, J. (2010). Identification and differentiation of aromatic rice genotypes using SSR markers. *Int. J. BioRes*., **2** (9): 07-12.

Mau, Y.S., Markus, J.E.R., Shirly, Oematan, S., Ndiwa, A.S.S., Handoko, D.D., Nasution, A. and Makbul, K. (2017). Genetic diversity of red and black upland rice accessions from East Nusa Tenggara, Indonesia as revealed by agro-morphological characters. *Biodiversitas* **18**: 197-211.

McCouch, S.R., Teytelman, L., Xu, Y., Lobos, K.B., Clare, K., Walton, M., Fu, B., Maghirang, R., Li, Z., Xing, Y., Zhang, Q., Kono, I., Yano, M., Fellstrom, R., DeClerck, G., Schneider, D., Cartinhour, S., Ware, D. and Stein, L. (2002). Development and mapping of 2240 new SSR 35 markers for rice (*Oryza sativa* L.). *DNA Res*., **9**: 199-207.

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.

Nam, S.H, Choi, S.P, Kang, M.Y., Koh, H.J., Kozukue, N. and Friedman, M. (2006). Antioxidative activities of bran from twenty one pigmented rice cultivars. *Food Chem* **94**: 613-620.

Noldin, J.A. (2000) Red rice status and management in Americas. In: Baki BB, Chin DV, Mortimer M (eds) Wild and weedy rice in rice ecosystems in Asia—a review. International Rice Research Institute, Los Ban˜os, pp 21–24.

Nei, M. and Takezaki, N. (1983). Estimation of genetic distances and phylogenetic trees from DNA analysis. Inst. of Mol. Evol. Genet. and Dept. of Biol. USA. **21**: 405- 412.

Pachauri, V., Taneja, N., Vikram, P., Singh, N.K. and Singh, N.K. (2013). Molecular and morphological characterization of Indian farmers rice varieties (*Oryza sativa* L.). *Aust. J. Crop Sci*., **7**(7): 923-932.

Palanog, A.D., Calayugan, M.I.C., Descalsota-Empleo, G.I., Amparado, A., Inabangan-Asilo, M.A., Arocena, E.C., Sta. Cruz, P.C., Borromeo, T.H., Lalusin, A., Hernandez, J.E., Acuin, C., Reinke, R., and Swamy, B.P.M. (2019) Zinc and iron nutrition status in the Philippines population and local soils. *Front Nutr.,* **6**: 81.

Rahman, M.M., Rasaul, 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. Crop Improv*., **26**: 244–257.

Rahman, L., Molla, M.R., Sultana, S., Islam, M.N., Ahmed, N.U., Rahman, M.S. and Nazim-ud-Dowla, M. (2008). Plant Varieties of Bangladesh: Morphological and

Molecular Characterization. Published by Seed Wing, Ministry of Agriculture, Government of Peoples Republic of Bangladesh, **2**: 300.

Rahman, L., Bashar, M.K., Mian, M.A.K., Siddeque, M.A., Rashid, E.S.M.H., Haque, N. and Islam, M.S. (2010). Plant Varieties of Bangladesh: Morphological and Molecular Characterization. Published by Seed Wing, Ministry of Agriculture, Government of Peoples Republic of Bangladesh, **4**:1-235.

Rahman, L., Molla, M.R., Sultana, S., Islam, M.N., Ahmed, N.U., Rahman, M.S. and Nazim-ud-Dowla, M. (2007). Plant Varieties of Bangladesh: Morphological and Molecular Characterization. Published by Seed Wing, Ministry of Agriculture, Government of Peoples Republic of Bangladesh, **1**: 486.

Rohlf, F. (2002). NTSYS-pc: Numerical taxonomy and multivariate analysis system, 2.2 edn. Department of Ecology and Evaluation, State university of NY, Stony Brook.

Ram, S.G., Thiruvengadam, V. and Vinod, K.K. (2007). Genetic diversity among cultivars, landraces and wild relatives of rice as revealed by microsatellite markers. *J. Appl. Genet.*, **48**(4): 337-45.

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

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.

Samyor, D., Das, A.B. and Deka, S.C. (2017) Pigmented rice a potential source of bioactive compounds: a review. *Int J Food Sci Technol.,* **52**:1073–1081.

Satue-Gracia, M., Heinonen, I.M. and Frankel, E.N. (1997). Anthocyanins as antioxidants on human low-density lipoprotein and lecithinliposome systems. *J Agric Food Chem* **45**: 3362-3367.

Singh, B.P., Singh, B., Mishra, S., Kumar, V. and Singh, N.K. (2016). Genetic diversity and population structure in Indian wild rice accessions. *AJCS*. **10**(2): 144-151.

Shao, Y., Zhang, G. and Bao, J. (2011). Total phenolic content and antioxidant capacity of rice grains with extremely small size. *Afr J Argic Res* **6** (10): 2289–2293.

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. *Bangladesh Rice J.* **20**(2): 1-11.

Siddique, M. A., Khalequzzaman, M., Islam, M. M., Kaniz Fatema and Latif, M. A. (2016). Molecular characterization and genetic diversity in geographical indication (GI) rice (*Oryza sativa* L.) cultivars of Bangladesh. *Braz. J. Bot*. DOI:10.1007/s40415-016-0271-1.

Sujatha, K., Upadhyay, R., Kaladhar, K., Rani, N.S. and Sarla, N. (2006). Genetic relationship among aromatic short grain and Basmati rice based on ISSR and SSR markers. *Rice Genet. Newsl.* **21**: 24-25.

Saini, N., N. Jain, S. Jain and R. K. Jain. (2004). Assessment of genetic diversity within and among Basmati and non-Basmati rice varieties using AFLP, ISSR and SSR markers. *Euphytica*, **140**: 133-146.

Santika, A. and Rozakurniati. (2010). Evaluation technique of rice quality of several glutanous and red upland rice. *Buletin Teknik Pertanian* **15**(1): 1-5. [Indonesian]

Suliartini, N.W.S., Sadimantara, G.R., Wijayanto, T. and Muhidin. (2011). Evaluation of anthocyanin content of upland red rice germplasm from Southeast Sulawesi. *Crop Agro* **4**(2): 43-48.

Sneath, P.H. and Sokal. (1973). Numerical taxonomy: The principles and practice of numerical classification. Freeman WH and Company, Sanfrancisco.USA.

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

Travis, A.J., Norton, G.J., Datta, S., Sarma, R., Dasgupta, T., Savio, F.L., Macaulay, M., Hedley, P.E., McNally, K.L., Islam, M.R. and Price, A.H. (2015). Assessing the genetic diversity of rice originating from Bangladesh, Assam and West Bengal. *Rice.* **8**: 35.

Tsuda, T., Horio, F. and Osawa, T. (2002). Cyanidin 3-O-â-glucoside suppresses nitric oxide production during a zymosan treatment in rats. *J Nutr Sci Vitaminol* **48**: 305-310.

Tilman, D., Reich, P., Knops, J., Wedin, D., and Mielke, T., (2001), Diversity and productivity in a long-term grassland experiment. *Science*, **294**: 843-845.

Temnykh, S., Park, W.D., Ayres, N., Cartinhour, S., Hauck, N., Lipovich, L., Cho, Y.G., Ishii, T. and McCouch, S.R. (2000) Mapping and genome organization of microsatellite sequences in rice (*Oryza sativa* L.). *Theor Appl Genet* **100**:697–712.

Thomson, M.J., Septininghih, E.M., Suwardio, F., Santoso, T.J., Silitonga, T.S. and McCouch, S.R. (2007). Genetic diversity analysis of traditional and improved Indonesian rice (*Oryza sativa* L.) germplasm using microsatellite markers. *Theor. Appl. Genet.,* **114**: 559-568.

Vabna, F. A. (2018). Molecular Characterization and Genetic Diversity Analysis of Boro Rice Germplasm Using SSR Markers. M.S. thesis, SAU, Dhaka-1207.

Venkatesan, K. and 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.

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.

Yadav, S., Singh, A., Singh, M.R., Goel, N., Vinod, K.K., Mohapatra, T. and Singh, A.K. (2013) Assessment of genetic diversity in Indian rice germplasm (Oryza sativa L.): use of random versus trait-linked microsatellite markers. *J Genet* **92**(3):545–557.

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$_2$EDTA (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): PCR cocktail for 96 samples

Reagent	Amount
DNA	288
Primer (F)	48
Primer (R)	48
Master mix	432
DDH$_2$O	144

Appendix II: Variability of Quantitative Data

Sl. No	FLA (cm²)	Culm dia (mm)	Total Tiller	Eff tiller	PL (cm)	PH (cm)	DF	DM	FG/P	1000 gwt.(g)	GL (mm)	GB (mm)	D L/B	Y/hill(g)
1	46.13	5.36	8	7	25.40	129.00	114	142	117	26.10	9.50	3.23	2.53	21.38
2	37.36	**5.76**	10	9	28.60	120.00	90	117	85	26.43	10.09	2.82	**3.00**	19.09
3	36.93	5.57	9	9	28.50	119.30	90	117	68	25.91	**10.30**	2.83	2.88	15.59
4	47.76	4.65	**5**	**5**	25.40	143.20	**117**	**146**	92	26.47	9.04	3.17	2.50	**11.18**
5	32.74	4.50	8	7	25.60	118.60	101	129	105	23.29	8.49	2.59	2.63	19.03
6	38.95	3.80	11	10	24.80	121.80	**80**	**107**	85	25.27	8.73	2.86	2.45	21.00
7	23.39	4.73	**5**	**5**	28.20	**158.20**	109	139	136	24.09	8.45	3.09	2.26	18.02
8	47.05	5.20	7	7	27.40	130.80	111	138	147	**17.00**	**8.08**	**2.36**	2.86	16.49
9	32.50	4.54	10	9	27.60	153.00	109	139	106	25.40	8.51	3.01	2.22	20.23
10	31.59	4.59	12	10	25.60	137.80	110	138	57	27.20	8.93	3.21	2.25	14.98
11	39.26	4.58	11	9	27.60	132.40	111	138	67	23.84	8.37	3.03	2.21	14.41
12	72.78	3.77	7	6	**21.40**	**87.20**	114	142	74	23.74	8.61	**3.35**	2.09	12.23
13	33.92	4.78	14	12	21.40	128.80	112	139	98	25.43	8.58	3.04	2.25	18.97
14	43.03	5.44	12	11	29.40	142.80	108	137	140	29.00	9.08	2.57	2.98	**22.57**
15	59.56	3.70	11	10	28.00	120.40	82	108	82	21.13	8.61	2.50	**2.03**	17.32
16	31.15	4.06	12	10	22.80	137.80	107	135	**39**	29.67	8.98	3.30	2.25	11.82
17	48.66	4.54	10	9	27.40	121.40	106	135	86	21.54	8.19	2.53	2.70	15.86
18	36.65	5.59	15	**13**	26.60	129.20	109	139	72	22.87	8.35	2.60	2.64	21.01
19	43.93	**3.16**	**16**	**13**	25.60	132.20	98	127	51	23.84	8.28	2.57	2.64	16.11
20	45.85	4.58	9	8	27.20	138.20	109	139	**153**	21.34	8.21	2.58	2.63	21.70
21	37.08	4.56	13	11	**30.20**	137.40	110	138	137	23.50	8.73	2.81	2.54	21.05
22	33.85	4.62	15	12	27.40	140.40	108	137	71	23.89	8.55	3.07	2.30	20.75
23	**17.65**	4.61	13	11	26.00	133.00	109	137	103	23.29	8.98	2.71	2.71	17.43
24	**73.66**	4.32	13	10	24.40	131.40	99	127	89	**34.25**	9.66	3.31	2.48	17.02
Min	17.65	3.16	5	5	21.40	87.20	80	107	39	17.00	8.08	2.36	2.03	11.18
Max	73.66	5.76	16	13	30.20	158.20	117	146	153	34.25	10.30	3.35	3.00	22.57
Mean	41.31	4.63	11	9	26.35	131.01	105	133	94	24.77	8.80	2.88	2.50	17.72
Std	13.15	0.64	2.98	2	2.27	13.87	9.96	10.57	31.13	3.34	0.58	0.30	0.28	3.26
CV	31.83	13.93	27.95	25.63	8.62	10.59	9.51	7.95	33.06	13.49	6.59	10.40	11.01	18.43
SE	6.50	2.84	5.71	5.23	1.76	2.16	1.94	1.62	6.75	2.75	1.35	2.12	2.25	3.76
LSD	12.73	5.57	11.18	10.26	3.45	4.24	3.81	3.18	13.23	5.40	2.64	4.16	4.41	7.37

Appendix III: Data of 24 Pigmented T. Aman Rice Germplasm DNA Against 16 SSR Markers

SL. No.	Marker	Chro. No.	Position (cM)	Motif*	Allele No.	Unique allele	Size range (bp)	Freq (%)	Gene diversity	PIC
1	RM1	1	29.7	(GA)26	8	1	84-180	33.33	0.82	0.80
2	RM5	1	94.9	(GA)14	5	-	113-124	50.00	0.64	0.58
3	RM125	7	24.8	(GCT)8	3	2	120-146	91.67	0.16	0.15
4	RM205	9	7.4	(CT)11	4	1	123-158	79.17	0.36	0.34
5	RM206	11	114.7	(CT)25	6	1	140-275	29.17	0.78	0.74
6	RM209	11	73.9	(CT)18	7	5	125-155	70.83	0.48	0.47
7	RM213	2	186.4	(CT)17	4	2	125-210	70.83	0.45	0.40
8	RM214	2	186.4	(CT)17	3	-	116-138	58.33	0.56	0.49
9	RM277	12	130.3	(CA)6(GA)36	3	-	140-175	75.00	0.41	0.37
10	RM304	10	73	(GT)2(AT)10(GT)33	4	1	152-167	70.83	0.47	0.44
11	RM307	4	0	(AT)14(GT)21	2	-	172-181	91.67	0.15	0.14
12	RM510	6	20.8	(GA)15	1	-	120	91.67	0.16	0.15
13	RM338	3	108.4	(CTT)6	4	1	114-172	62.50	0.50	0.41
14	RM342	8	78.4	(CAT)12	4	-	130-149	66.67	0.50	0.44
15	RM334	5	141.8	(CTT)20	6	-	154-170	50.00	0.61	0.54
16	RM1337	12	57.2	(AG)21	6	2	171-350	45.83	0.70	0.66
	Max.				8.00		171-350	91.67	0.82	0.80
	Min.				1.00			29.17	0.15	0.14
	Total				70	16			7.75	7.12
	Mean				4.38			64.84	0.4843	0.445

Appendix IV: Genetic Distance among 24 pigmented T. Aman rice germplasm

OUT	G1	G10	G11	G12	G13	G14	G1 5	G16	G17	G18	G19	G2
G1	0.0000											
G10	0.3750	0.0000										
G11	0.3750	0.1250	0.0000									
G12	0.4375	0.1875	0.0625	0.0000								
G13	0.5000	0.1250	0.1250	0.0625	0.0000							
G14	0.5625	0.4375	0.5000	0.5625	0.5000	0.0000						
G15	0.6250	0.6875	0.6250	0.5625	0.6250	0.5000	0.0000					
G16	0.6250	0.6250	0.5000	0.5625	0.6250	0.6875	0.5625	0.0000				
G17	0.6250	0.4375	0.3750	0.3750	0.4375	0.5625	0.5000	0.6250	0.0000			
G18	0.5000	0.3750	0.3750	0.4375	0.5000	0.4375	0.6250	0.6875	0.3750	0.0000		
G19	0.5000	0.4375	0.3750	0.4375	0.5000	0.5000	0.6250	0.6875	0.5000	0.3750	0.0000	
G2	0.3750	0.4375	0.5625	0.5625	0.5000	0.4375	0.6875	0.8125	0.6250	0.5625	0.6250	0.0000
G20	0.4375	0.4375	0.4375	0.5000	0.5625	0.5000	0.5000	0.5000	0.4375	0.5000	0.5625	0.5000
G21	0.3750	0.3750	0.3750	0.4375	0.5000	0.4375	0.5000	0.5625	0.5000	0.4375	0.5000	0.4375
G22	0.5000	0.3750	0.2500	0.3125	0.3750	0.5625	0.6250	0.5000	0.3750	0.4375	0.4375	0.6250
G23	0.4375	0.4375	0.5625	0.6250	0.5625	0.5000	0.6250	0.6875	0.6875	0.5625	0.6875	0.5625
G24	0.6250	0.4375	0.3750	0.4375	0.4375	0.5625	0.5625	0.6250	0.6250	0.5625	0.4375	0.6875
G3	0.3750	0.4375	0.5625	0.5625	0.5000	0.5000	0.7500	0.8125	0.6250	0.5625	0.6250	0.0625
G4	0.5625	0.3750	0.3750	0.4375	0.3750	0.4375	0.6250	0.5625	0.6250	0.6250	0.5625	0.4375
G5	0.4375	0.5625	0.5625	0.5625	0.6250	0.4375	0.6250	0.8125	0.5625	0.4375	0.5000	0.3750
G6	0.5625	0.5000	0.3750	0.4375	0.5000	0.6250	0.6250	0.6250	0.5625	0.5625	0.5625	0.6875
G7	0.3125	0.3750	0.3750	0.4375	0.5000	0.3750	0.3750	0.4375	0.5000	0.4375	0.5000	0.4375
G8	0.3750	0.4375	0.4375	0.5000	0.5625	0.5000	0.5000	0.4375	0.5625	0.5000	0.5625	0.5000
G9	0.5625	0.6250	0.7500	0.8125	0.7500	0.5625	0.6875	0.6875	0.8750	0.6250	0.7500	0.5625

Appendix IV Contd.: Genetic Distance among 24 pigmented T. Aman rice germplasm

OUT	G20	G21	G22	G23	G24	G3	G4	G5	G6	G7	G8	G9
G1												
G10												
G11												
G12												
G13												
G14												
G15												
G16												
G17												
G18												
G19												
G2												
G20	0.0000											
G21	0.0625	0.0000										
G22	0.3750	0.3125	0.0000									
G23	0.4375	0.4375	0.6250	0.0000								
G24	0.5000	0.5000	0.3750	0.6250	0.0000							
G3	0.5625	0.5000	0.6250	0.5625	0.7500	0.0000						
G4	0.4375	0.3750	0.4375	0.5625	0.5000	0.5000	0.0000					
G5	0.4375	0.3750	0.6250	0.6250	0.6250	0.4375	0.5000	0.0000				
G6	0.5625	0.5000	0.4375	0.7500	0.5625	0.6875	0.5000	0.6250	0.0000			
G7	0.2500	0.1875	0.3750	0.4375	0.5000	0.5000	0.4375	0.4375	0.5000	0.0000		
G8	0.2500	0.1875	0.4375	0.4375	0.5625	0.5625	0.4375	0.4375	0.5625	0.1875	0.0000	
G9	0.5625	0.5000	0.7500	0.5000	0.6875	0.6250	0.5625	0.5000	0.8125	0.5000	0.4375	0.0000