MORPHO-MOLECULAR CHARACTERIZATION OF AMAN RICE GERMPLASM

A THESIS

BY

ORPITA PODDER

DEPARTMENT OF BIOTECHNOLOGY

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BY

ORPITA PODDER

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Approved by:

Prof. Dr. Md. Ekramul Hoque
Department of Biotechnology
Sher-e- Bangla Agricultural University
Supervisor

Dr. Mohammad Khalequzzaman
Chief Scientific Officer and Head
Genetic Resources and Seed Division
Bangladesh Rice Research Institute (BRRI)
Gazipur
Co-Supervisor

Prof. Dr. Md. Ekramul Hoque
Chairman
Examination Committee
Department of Biotechnology
Sher-e- Bangla Agricultural University

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

CERTIFICATE

*This is to certify that the thesis entitled "**Morpho-molecular characterization of 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 **Orpita Podder**, Registration no.13-05690 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.

Date: June, 2020

Place: Dhaka, Bangladesh

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


DEDICATED TO
MY
BELOVED PARENTS

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ABSTRACT

An experiment was carried out to analysis the genetic diversity of 24 Bangladeshi T. Aman rice (*Oryza sativa* L.) germplasm using morphological traits and molecular markers. The experiment was conducted at Genetic Resources and Seed Division (GRSD), Bangladesh Rice Research Institute (BRRI), Gazipur, during the period of July to December 2019. Observed variables included qualitative and quantitative traits. A total of 10 qualitative and 10 quantitative characters were studied. Qualitative characters were descriptively analyzed and showed variation in different accessions. Quantitative data were also subjected to cluster analysis which showed total three cluster and many more sub-clusters. So the plant morphological traits exhibited more variation among the germplasm tested. A set of sixteen SSR markers was used for molecular diversity study which resulted 66 alleles with an average of allele number was 4.13. The polymorphic information content (PIC) values ranged from 0.08 (RM455) to 0.80 (RM206) with an average of 0.48. The highest PIC value (0.80) was obtained from RM206. The PIC value revealed that RM206 was the best marker for 24 genotypes tested. The value of pair-wise comparisons of Nei's genetic distance among 24 rice germplasm was computed from combined data for the 16 primers, ranged from 0.1250 to 0.9375 with an average of 0.5247. The cluster analysis based on UPGMA system grouped 24 germplasm into three main clusters and total six sub-cluster. Both the dendrogram showed diverse variation. Evaluation of morpho-molecular characters demonstrated that aman rice germplasm under the present study possessed a high genetic diversity which may be used for rice breeding program.

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LIST OF ABBREVIATIONS

ABBREVIATION	EINGLISH FULL NAME
BRRI	Bangladesh Rice Research Institute
Transplant aman	T.Aman
BBS	Bangladesh Bureau of Statistics
Base pair	Bp
CTAB	Cetyl Trimethyl Ammonium Bromide
Double Distilled Water	ddH_2O
Distilled Water	dH_2O
De-oxy ribonucleic Acid	DNA
EDTA	Ethylene Diamine Tetra Acetic Acid
et al.	And others (at elli)
etc.	Etcetera
FAO	Food and Agriculture Organization
g	Gram
CV	Coefficient of Variation
LSD	Least Significant Differences
G	Genotype
GD	Genetic Distance
g/l	Gram per Liter
Ml	Mili liter
NaCl	Sodium chloride
Na_2EDTA	Sodium salt of ferric Ethylene Diamine Tetra Acetic Acid
PCR	Polymerase Chain Reaction
pH	Negative Logarithm of Hydrogen ion concentration

ABBREVIATION	EINGLISH FULL NAME
PIC	Polymorphism Information Content
SSR	Simple Sequence Repeats
SNP	Single Nucleotide Polymorphism
SDS	Sodium Dodecyl Sulphate
Taq	*Thermophilus aquaticus*
TBE	Tris Boric Acid EDTA
TE	Tris-EDTA
T	Tons
UPGMA	Unweighted Pair Group of Arithmetic Mean
UV	Ultraviolet
V	Volt
Min	Minutes
Viz.	Namely

CHAPTER I
INTRODUCTION

Rice (*Oryza sativa* L.) is a self-pollinated cereal grain, a monocot annual plant. It belongs to the family Gramineae (synonym-Poaceae) having chromosome number, 2n=24 under the Order Cyperales and Class Monocotyledon (Hooker, 1979). Rice is the most widely consumed staple food for a larger part of the world's human population, especially in Asia. The Himalayan foothills including parts of Bangladesh are considered to be the secondary center of diversity of the genus *Oryza* (Morishima, 1984). There are mainly two *Oryza* species important for human nutrition: *Oryza sativa* (Asian rice), grown worldwide, and *Oryza glaberrima* (African rice), grown in parts of West Africa (Ricepedia, http://ricepedia.org/rice). *Indica, Japonica* and *Javanica* are the sub-species of *oryza sativa*.

Rice (*Oryza sativa* L.*)* is an important cereal crop grown exclusively for human consumption that is the staple food for about 50% of the global population (Garris *et al*., 2005; Ramkumar *et al*., 2010). China, India, Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Pakistan, Philippines, Korea, Japan and Africa are the major rice producing countries. In 2017-2018, a total of 13,993 metric tons aman rice was grown in 14,035 acres of land (BBS, Yearbook, 2018). Its contribution to agricultural GDP is about 70 percent while its share of national income is one-sixth (FAO, 2018). In many developing countries including Bangladesh, rice is the basis of food security and intimately associated with traditional culture and customs in local regions (Lu *et al*., 2005; Das., 2005). Bangladesh occupies third position as a producer and consumer of rice in the world. At present, most of the total food grains produced annually from rice in our country.

The population of Bangladesh is increasing at a terrible rate and the cultivable land of Bangladesh is reducing day by day due to urbanization and industrialization resulting in shortage of food. The nation is still adding about 2.3 million mouths every year to its total of 160 million people (Momin & Husain, 2009). In 2030 and 2050, world population is anticipated to reach 8.6 billion and 9.8 billion. A total of 83 million people is expected to be added in every year (UN DESA, 2017). World food production must increase to feed this huge sum of the population. An

additional 400,000 metric tons of rice are required every year. So, the highest priority should be given on rice production and cultivation.

It considers as a good source of B vitamins, thiamin, niacin, riboflavin, fiber, and iron. It is also an excellent source of manganese and magnesium. Enriched rice has vitamins and minerals added back after it is refined. It provides 75% of the calories and 55% of the proteins in the average daily diet for the people of the country (Bhuiyan *et al.*, 2002). Rice has ability to provide instant energy, regulate and improve bowel movements, stabilize blood sugar levels and slow down the aging process.

Rice is grown widely in three seasons, such as Aus, Aman and Boro. Among these seasons, total area under Aman crop has been estimated 1,38,92,398 acres 56,21,949 hectares this year (2018-2019) (BBS,Yearbook,2018)**.** During mid-June to mid-July, the seedlings of the Transplanted Aman (T. *Aman*) are raised. Transplanting starts from mid July and lasts up to October. During mid-October to mid-December harvesting is done. Highest varietal diversity is observed in the landraces of the T. *Aman* rice varieties. Photoperiod sensitivity, seed dormancy and adaptation to cold temperature at the reproductive phase are the major features of the T. Aman rice. T. Aman varieties are grown in medium high lands.

Asian farmers have isolated and maintained huge number of rice genotype over thousands of years. Scientists estimate that, more than 140,000 rice varieties have been developed/selected/isolated in Asia. More than 1,32,000 gemplasm can be found in the world's largest genebank for rice, the International Rice Research Institute (IRRI) located in the Philippines (Website of IRRI) (http://irri.org/our-work/research/genetic-diversity). Until now, BRRI has collected and preserved in the genebank about 8,700 varieties/land races/cultivars/wild from indigenous and exotic sources. Out of these, nearly 8000 varieties have been registered in the gene bank (Khalequzzaman *et al.,* 2012). BRRI Genebank is enriched with about 4,990 rice accessions for Aman.

There is wide genetic variability available within existing varieties of rice and wild relatives providing wide scope for further improvement (Chakravarthi and Naravaneni 2006; Rahman *et*

al. 2006, 2007a, b, c). For the development of effective breeding program to develop high yielding T. Aman rice varieties, morphological and molecular characterization are very important. Otherwise, genotypes of rice have already been lost from the farmer's field. Several genetic diversity studies have been successfully utilized in different crop species based on quantitative and qualitative traits in order to select genetically distant parents for hybridization (Bedoya *et al.*, 2017; Ahmed *et al*., 2016a; Islam *et al*., 2016; Malek *et al.*, 2014; Khodadadi *et al*., 2014).To avoid the varietal loss, recently advent molecular characterization along with morphological traits would be the best solution. Molecular markers can reveal abundant difference among genotypes at the DNA level, providing a more direct, reliable and efficient tool for germplasm characterization, screening and evaluation in contrast to morphological traits.

The use of DNA markers has been suggested for precise and reliable characterization and discrimination of rice genotypes (Karkousis *et al*., 2003). Many countries in the world have characterized their indigenous different crop landraces at both molecular and morphological level. This has been done for keeping their crop identity and for searching new genes for further crop improvement as well as breeding programme. Genetic diversity measured through morphological traits has some limitation. But it has some restrictions. It is time consuming, require much cost & space, depends on environmental factors & also misguides the scientist to recognize a particular genotype.To avoid these disadvantages DNA markers are extensively used. Markers are technically simple, distinct, time saving, highly informative, reliable, independence from effects related to environmental conditions of the plant and require small amount of DNA.

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

The SSR markers are particularly suitable for evaluating genetic diversity and relationships among plant species, populations, or individuals (Kostova *et al*., 2006 and Tu *et al*., 2007), studying rice germplasm for either conservation or utilization (Sharma *et al*., 2007); marker-assisted selection breeding (Perez-Sackett *et al*., 2011 and Rani and Adilakshmi 2011); cultivar identification; hybrid purity analysis and gene mapping studies (Weising *et al*., 1997; Altaf-Khan *et al*., 2006; Rajendrakumar *et al*., 2009 and Sarao *et al*., 2010).

Characterization as well as diversity analysis of germplasm will provide the information to plant breeder for helping them selecting the parent and best variety for varietal improvement program. Therefore, the research emphasis has been given on to characterize and to evaluate the genetic diversity of 24 Bangladeshi T.Aman rice germplasm using morphological traits and molecular markers.

OBJECTIVE(S)

Keeping above mentioned points in view, the present study has been formulated with following objectives:

(1) Morphological characterization of 24 T. Aman rice germplasm.
(2) Molecular diversity study of T. Aman rice germplasm.
(3) DNA profiling and polymorphism study among different T. Aman germplasm.
(4) Dendrogram establishment and genetic relationship study among the T. Aman germplasm.

CHAPTER II

REVIEW OF LITERATURE

Bangladesh has abundant diversified rice landraces, since rice plays an important role in the livelihood, cultures and socio-economic aspects of the people and is also the principal cereal food in Bangladesh. It occupies almost one-fifth of the total land area covered under cereals. Morpho-molecular characterization and genetic diversity analysis of rice is very important for variety development and germplasm management program. SSRs are useful for integrating the genetic, physical and sequence-based maps of rice as well as provide an efficient tool to link phenotypic and genotypic variation. Studies on morph-molecular characterization of different rice genotypes using Simple Sequence Repeats (SSR) markers have been carried out throughout the world as well as Bangladesh. Various researchers have performed research activities on rice genetic diversity and relationship and characterization through molecular markers. The most relevant literature about the present study has been reviewed and some of the relevant literatures are cited below:

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

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

Islam *et al*., (2018) studied about 36 similar named aromatic rice landraces of Bangladesh to assess the genetic variation for the agro-morphological traits.The landraces were grouped into four clusters. The inter-cluster distances were higher than intra-cluster distances indicating wider genetic diversity among the landraces of different clusters. The intra-cluster distances were lower in all the cases reflecting homogeneity of the landraces within the clusters.The principal component analysis showed that the first five components with vector values > 1 contributed 76.51% of the total variations. The highest number (13) of landraces was constellated in cluster III and the lowest (3) in cluster I. The intra- and inter-cluster distances were the maximum in cluster I (0.63) and between clusters I and IV (17.13), respectively and the minimum in cluster II (0.03) and between clusters III and IV (3.86), respectively. The maximum value of inter cluster distance indicated that the landraces belonging to cluster IV. Besides, the cluster mean revealed that the crosses between the genotypes of cluster I with those of clusters IV would exhibit high heterosis for maximum good characters. Hence, yield, grain breadth, days to maturity, culm diameter, ligule length had maximum contribution towards genetic divergence.

Okello *et al*., (2017) evaluated forty eight rice germplasm with 18 simple sequence repeat (SSR) markers to study their genetic diversity and phylogeny structure. Each primer showed 100% polymorphism. A total of 275 alleles were generated by 18 primers and each primer produced on an average 15.27 alleles of the size ranging from 172.22 bp (base pair) to 329.44 bp. The number of alleles amplified for each primer pair was ranged from 5 to 35. The markers pTA-248 generated a maximum number of alleles (35), while the primer RM-309 produced minimum number of alleles (5). The polymorphism information content (PIC) values of primers ranged from 0.58 (RM-206) to 0.85 (RM-140) with an average PIC value of 0.77. It was also observed that there was no correlation between percentage polymorphism and PIC value as SSR primer RM-206 showed minimum PIC value but were 100% polymorphic. The higher the PIC value, the more informative is the primer. Primers RM-140 and RM-122 were found to be more informative.

Mau *et al.,* (2017) studied a number of upland red and black rice accessions were collected from various locations of East Nusa Tenggara Province, Indonesia that was carried out in the Glasshouse involving 40 upland red and black rice accessions. Observed variables included qualitative and quantitative agro-morphological characters. A total of 26 qualitative and 16 quantitative characters were observed. Research results revealed a significant difference among rice accessions in both qualitative and quantitative characters. The tested rice accessions exhibited substantial differences in most of the observed qualitative and quantitative variables. 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. Evaluation of agro-morphological characters demonstrated that the rice germplasm under the present study possessed a high genetic diversity.

Ahmed *et al*., (2016) studied about 21 qualitative agro-morphological characters of 40 *Balam* rice were studied during T. Aman seasons at BRRI, Gazipur.. 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.

Islam *et al*., (2016) studied about genetic variability among 113 aromatic and fine local rice genotypes of which five were exotic in origin. The test genotypes were evaluated for 19 growth traits, yield components, and yield. All the quantitative traits varied significantly among the test genotypes. High heritability along with high genetic advance was observed for flag leaf area, secondary branches per panicle, filled grains per panicle, grain length, grain breadth, grain length breadth ratio, and 1000 grain weight. Grain yield was significantly and positively correlated with days to flowering, days to maturity, panicle length, filled grains per panicle, and 1000 grain weight. According to $D2$ cluster analysis, 113 test genotypes formed 10 clusters. Selection of parents from the clusters V and X followed by hybridization would possibly result in desirable heterosis for the development of heterotic rice hybrids.

Siddique *et al*., (2016) analyzed about genetic diversity of 20 geographical indication (GI) rice landraces of Bangladesh for 30 loci using simple sequence repeat markers to characterize the varieties .The number of alleles per locus ranged from five (RM275) to 15 (RM180), with an average of 9.7. The results revealed unique alleles that could be used for identification and molecular characterization of five landraces. The polymorphism information content (PIC) values which ranged from 0.59 (RM275) to 0.90 (RM180), with an average of 0.815, revealed much variation among the studied cultivars. The frequency of the most common allele at each locus ranged from 15 (RM85 and RM180) to 55 % (RM275 and RM277). RM180 was the best marker for identification and diversity estimation of GI rice genotypes as revealed by PIC values. A dendrogram revealed six clusters with a similarity coefficient of 0.14. The findings of this

study are useful for varietal identification, thus assisting plant breeders in selecting the suitable genetically diverse parents for crossing programs.

Siddique *et al*., (2016) studied about genetic diversity of 96 Aman (rainfed, partially irrigated) rice landraces of Bangladesh using eight SSR markers to characterize the landraces and also to establish the sovereignty of Bangladeshi rice gene pool. A total of 159 alleles were detected. The number of alleles per locus ranged from 13 (RM60, RM237) to 34 (RM163), with an average of 19.88.The polymorphism information content (PIC) which ranged from 0.86 (RM237) to 0.95 (RM163) with an average of 0.90 revealed much variation among the studied landraces. RM163 was the best marker for identification and diversity estimation of Aman rice landraces as revealed by PIC values. The allele frequency ranged from 8.33% (RM163) to 22.92% (RM60, RM125) with an average of 15.89. The UPGMA dendrogram based on Nei's genetic distance revealed seven distinct clusters with a similarity coefficient of 0.09.

Halder *et al*., (2016) studied about the genetic diversity of 12 Bangladeshi local Boro rice (*Oryza sativa* L.) germplasm using morphological traits and molecular markers. Eight morphological traits (*viz.,* days to 50 percent flowering, growth duration, plant height, filled grain/panicle, 1000 grain weight and grain yield) and eight simple sequence repeat (SSR) markers were used for this analysis. The plant morphological traits presented more variation among the genotypes tested. A set of eight SSR primer pairs was used for molecular characterization resulting 49 alleles, where average of allele number was 6.13. The polymorphic information content (PIC) values ranged from 0.67 (RM1) to 0.86 (RM314) with an average of 0.76. The highest PIC value (0.86) was obtained for RM314 which also gave maximum alleles. The PIC value revealed that RM314 was the best marker for 12 genotypes tested. The cluster analysis based on UPGMA system grouped 12 genotypes into four clusters.

Singh *et al*., (2016) studied a set of 729 Indian rice varieties. The varieties were genotyped using 36 HvSSR markers to assess the genetic diversity and genetic relationship. A total of 112 alleles was amplified with an average of 3.11. Polymorphic information content (PIC) value of 0.29.

Sinha *et al.,* (2015) has studied 55 traditional rice varieties of West Bengal, and investigated for grain morphological characters. A wide variation of grain characters, like grain size and shape, anthocyanin colouration of lemma-palea and kernels, presence or absence of aroma, awning characteristics, were found among the studied varieties. Wide variation among the grain morphological characters indicated wide genetic variation present among these varieties, which may be utilized for the selection of the parents for the plant breeding and production of new improved variety.

Sajid *et al.,* (2015) has characterized 30 indigenous rice germplasm on the basis of 32 different agro-morphological traits (15 qualitative and 17 quantitative). Highly significant differences ($p<0.01$) were observed for the traits of flag leaf length, flag leaf breadth, culm length, days to 50% flowering, panicle length,length of primary branches panicle-1secondary branches panicle-1, grain length,grain width, awn length and percent leaf lesion while significant differences($p<0.05$) were observed for peduncle length and primary branches. The rice germplasm exhibited sufficient genetic variation for most of the qualitative and quantitative traits.

Ahmed *et al*., (2015) studied about 21 similar or duplicate named *Kartiksail* rice for 21 qualitative agro morphological characters at BRRI during T. Aman 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. Besides, the genotypes including BR4 and BR23 were grouped into two clusters by the UPGMA clustering method, where cluster II had the maximum genotypes (20).

Siddique *et al*., (2014) used five microsatellite markers across 24 rice genotypes for their characterization and discrimination. The number of alleles per locus ranged from 6 alleles (RM153) to 14 alleles (RM151), with an average of 9.6 alleles across the 5 loci obtained in the study. The polymorphism information content (PIC) values ranged from 0.65 (RM153) to 0.91 (RM151), were identified in all loci. RM151 was found the best marker for identification of 24 genotypes as revealed by PIC values. The frequency of the most common allele at each locus ranged from 4.17% (RM1, RM153 and RM335) to 50% (RM153), which is comparable with Thomson et al. (2007). The two and three dimensional principal coordinate analysis (PCoA) with 24 genotypes showed that the genotypes Chaulamaghi, Sackorkhana, Chinikanai, Chata Bazail, Dudshail, Paizra, Beto, Orgoja, Beti Chicon, Kasrail were found far away from centroid of the cluster and rest of the genotypes were placed more or less around the centroid.

Sarawgi *et al*., (2014) on the basis of frequency distribution for 18 qualitative traits of 408 rice germplasm accessions reported that majority of genotypes possessed green basal leaf sheath colour (87.25 %), green leaf blade colour (89.70 %), pubescent leaf (48.03 %), well panicle exsertion (57.10 %),white stigma colour (65.93 %), straw apiculus colour (78.18 %), compact panicle type (55.63 %), awnless (88.48 %), white seed coat (82.84 %), straw hull colour (70.34 %), intermediate thresh ability (47.30 %), erect flag leaf angle (57.59 %),medium leaf senescence (67.15 %) and straw sterile lemma (97.05 %).

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

Gupta *et al*., (2013) worked on characterization of 53 accessions of rice germplasm from IGKV, Raipur, Chhattisgarh germplasm. These germplasm accessions were evaluated for 14

morphological and seventeen agronomical characters. The specific genotypes S: 663, K: 1514, J: 311 were identified for agronomic characteristics. These may be used in hybridization programme to achieve desired segregants for higher yield.

Mamunur *et al*., (2012) elucidated thirty-four microsatellite markers across 21 types of rice to characterize and discriminate among different varieties. 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 detected at 20 loci. The results revealed that 14 rice varieties produced unique alleles that could be used for identification, molecular characterization, and DNA fingerprinting of these varieties. Polymorphic information content (PIC) values ranged from 0.157 to 0.838, with an average of 0.488, which revealed that much variation was present among the studied varieties. The PIC values revealed that RM401 might be the best marker for identification and diversity estimation of rice varieties, followed by RM566, RM3428, RM463, and RM8094 markers. The UPGMA cluster dendrogram created in this study identified five clusters with a similarity coefficient of 0.50. The findings of this study should be useful for varietal identification and could help in background selection in backcross breeding programs.

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

Ashfaq *et al.,* (2012) associated various morphological traits with yield, there was a strong association revealed between the plant yield and the other yield component traits namely panicle length, number of seeds per panicle, productive tillers per plant and seed weight per panicle. The yield component traits were associated with other traits that also had a great contribution to the

improvement of yield. For instance, panicle length was associated with flag leaf area, number of primary branches per panicle, number of spikelets per panicle, number of seeds per panicle and grain weight per panicle were directly or indirectly associated with the plant yield, leading to increased rice yield.

Seetharam *et al*., (2009), thirty rice genotypes comprising land races, pure lines, somaclones, breeding lines and varieties specifically adapted to coastal saline environments were characterized by SSR markers and morphological characters, when out of 35 primers of SSR markers, 28 were polymorphic.

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

rice cultivars.

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

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

Hossain *et al.*, (2007) used thirty microsatellite markers across 21 rice genotypes for their characterization and discrimination. The number of alleles per locus ranged from three (RM165,

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

According to Lapitan *et al*., (2007) studied twenty-four rice cultivars by using 164 SSR markers. A total of 890 alleles were detected by 151 polymorphic markers with an average of 5.89 per locus. Out of these markers, 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.

According to 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, 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.

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

CHAPTER III
MATERIALS AND METHODS

The chapter focused on the materials and methods used in this experiment. The details of the methodology under this study have been described below:

Two sub-experiments were conducted to fulfill the mentioned objectives. The titles of sub-experiments were as follows.

Sub-experiment- 01

Morphological characterization and genetic diversity analysis of T. Aman rice germplam.

Sub-experiment -02

Molecular diversity analysis and DNA profiling of some T. Aman rice germplasm.

The details methodology of each experiment is given below.

Sub-experiment- 01

Morphological characterization and genetic diversity analysis of T. Aman rice germplam.

3.1. Experimental site and time duration

The experiment was conducted at the Genetic Resources and Seed Division (GRSD), Bangladesh Rice Research Institute (BRRI), Gazipur -1701 in T. Aman season. The experiment was carried out during the period of July to December 2019**.** The place is located at about 24.00 ºN latitude and 90.25 ºE longitude with an elevation of 8.4 meters geographically from the sea level and is characterized by sub-tropical climate.

3.2. Experimental materials

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

3.3. Experimental design

The experiment was conducted following a randomized complete block design (RCBD) with three replicates for each treatment. Twenty five days old seedlings of each test germplasm were

transplanted on the 31July, 2019. 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 was used.

3.4. Intercultural operations

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.

3.5. Agro-morphological traits observation and data collection

Twenty-four (24) T. Aman rice germplasm were characterized using the standard 'Rice Germplasm Descriptors and Evaluation Form'. The observations on various agro-morphological traits including qualitative and quantitative traits were recorded. Ten qualitative traits namely blade colour, basal leaf sheath colour, ligule colour, ligule shape, awn distribution, apiculus colour, lemma and palea colour, seed coat (bran) colour, decorticated grain scent color and leaf senescence were recorded. Again, specifically ten plants from each entry were randomly selected for recording data on 10 quantitative traits namely, plant height (cm), effective tiller number, panicle length(cm), days to flowering, days to maturity, filled grains per panicle (no.), grain length (mm), grain breadth (mm) , 1000 grain weight (TGW) (g) and yield per hill (g).

3.6. Statistical analysis

Univariate analysis of the individual character (ANOVA) including the estimation of mean, maximum, minimum ,total, coefficient of variation (CV%), Standard deviation (SD), Standard Error (SE), LSD (5%) was conducted using Microsoft excel. Multivariate analysis was conducted using another statistical software package PAST 3.16.The observed qualitative data were subjected to descriptive analysis. The quantitative data were subjected to cluster analysis to further classify the tested accessions based on their genetic diversity. A dendrogram was drawn using software.

Sub-experiment -02

Molecular diversity analysis and DNA profiling of some T. Aman rice germplasm.

3.7. Collection of leaf samples for DNA extraction

Young, green, soft leaf samples were collected when the rice plants were at 15-20 DAT (Days after transplanting). About 3 cm long leaf tips were collected from the plants and store in eppendorf tube.The eppendorf tubes containing leaf samples were immediately preserved in ice buckets which was carried to the transplanting field. Then the leaf samples with microfuge tube were kept in polybags and placed in the chamber of -80°C freezer. The leaf samples were crushed immediately for DNA extraction.

Table 1. Name of 24 transplant aman (T.Aman) rice germplam used for experimental purpose

Sl. No.	Name	BRRI accession number	Upazila	District	Season
1.	Kali jira(3)	247	Fulbaria	Mymensingh	T. Aman
2.	Telot	248	Muktagacha	Mymensingh	T. Aman
3.	Bazail	249	Muktagacha	Mymensingh	T. Aman
4.	Joli amon	250	Muktagacha	Mymensingh	T. Aman
5.	Bazail	251	Muktagacha	Mymensingh	T. Aman
6.	Bazail	252	Muktagacha	Mymensingh	T. Aman
7.	Kancha noni	270	Mithapukur	Rangpur	T. Aman
8.	Naria bochi	275	Rangpur Sadar	Rangpur	T. Aman
9.	Khirsha bhog	276	Rangpur Sadar	Rangpur	T. Aman
10.	Sham rush	277	Pirgacha	Rangpur	T. Aman
11.	Dudh kalam	278	Sundarganj	Gaibandha	T. Aman
12.	Dudh kalam	279	Pirgacha	Rangpur	T. Aman
13.	Bora dudh kalam	280	Pirgacha	Rangpur	T. Aman
14.	Lal soru	281	Hatibandha	Lalmonirhat	T. Aman
15.	Gojol goria	282	Hatibandha	Lalmonirhat	T. Aman
16.	Sojoni	283	Hatibandha	Lalmonirhat	T. Aman
17.	Ganjia	284	Hatibandha	Lalmonirhat	T. Aman
18.	Bindi pakri	285	Sundarganj	Gaibandha	T. Aman
19.	Jhoshua	286	Sundarganj	Gaibandha	T. Aman
20.	Akand sail	287	Sundarganj	Gaibandha	T. Aman
21.	Lal dupa	289	Sundarganj	Gaibandha	T. Aman
22.	Jiga sail	290	Sundarganj	Gaibandha	T. Aman
23.	Cheng sail	291	Sundarganj	Gaibandha	T.Aman
24.	Shul kumor	292	Sundarganj	Gaibandha	T.Aman

3.8. Preparation of reagents for DNA extraction

DNA was extracted following modified method of Ferdous *et al.*, 2012. Quantification of DNA samples was done by using the Nanodrop (Origin, Germany) and the quality of the DNA was estimated by using 0.8% agarose gel electrophoresis. High concentration of DNA samples was further diluted in 10:1 (DD H2O-DNA) to make a working concentration .Then stored at 4°C for PCR based marker analysis. The detailed laboratory procedures of this method were given below-

3.8.1 Tris buffer (1 M Tris solution, pH=8.0)

Tris HCL, Trisma base are the different forms of Tris. The formula weight (FW) of Trisma base is 121.14 and the required concentration for this chemical is 1 M with pH=8.0. There are two main functions of Tris - to maintain pH of the DNA solution and to provide buffering capacity. By keeping pH steady at 8.0, tris is very important. An example, 250 ml 1M Tris with pH 8.0 can be prepared as follows:

i.e. N =1 M X 250 ml= 1 M X 0.25 liter = 0.25	N= CV
Again,	N= mole number,
Mass = N X FW = 0.25 X 121.14 = 30.29 g	C= Conc. in Molar,
	V= Volume in liter

So, 30.29 g Tris was dissolved in 180 ml of autoclaved distilled water and pH was adjusted in 8.0. The final volume is made to 250 ml in a graduated measuring cylinder by adding sterile H_2O and the solution is autoclaved.

3.8.2 NaCl (5M NaCl solution)

The chemical formula is NaCl with the formula weight (FW) of this chemical is 58.44. The required concentration is 5M. NaCl helps to 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,

i.e. N =5 M X 250 ml= 5 M X 0.25 l =1.25	N= CV
Again,	N= Mole number,
Mass = N X FW = 1.25 X 58.44 = 73.05g	C= Conc. in Molar,
	V= Volume in liter

So, 73.05 g NaCl was dissolved in 175 ml of autoclaved and distilled water. The final volume was made to 250 ml in a graduated measuring cylinder by adding sterile H_2O. The solution was finally autoclaved.

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

Na_2EDTA makes the solution acidic when it dissolved in water. The formula weight (FW) of the chemical is 372.24.The required concentration is 0.5 M with pH=8.0. The Na_2EDTA acts as chelating agent which chelates inorganic or metal ion and 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,

i.e. N =0.5 M X 100 ml= 0.5 M X 0.1 l = 0.05	N= CV
Again,	N= mole number,
Mass = N X FW = 0.05 X 372.24 = 18.61 g	C= Conc. in Molar,
	V= Volume in liter

Using 60 ml of autoclaved and distilled water, 18.61 g Na_2EDTA is dissolved. As Na_2EDTA is acidic in nature so NaOH pellets (or 5M NaOH) is added to adjust its pH. By using a graduated measuring cylinder the final volume is made to 100 ml by adding sterile H_2O.Then the solution that was made is autoclaved.

3.8.4 SDS (Sodium Dodecyl Sulphate) solution

The meaning of this chemical is Lauryl Sulphate with the formula weight (FW) 288.4 and chemical formula $C_{12}H_{25}O_4SNa$. About 10% concentration is required. SDS solution acts as a detergent agent. It helps in the digestion of protein by breaking disulphide bond (–S–S–). It also helps in the lysis of cell wall. However, at first to prepare 10% 250 ml SDS 25 g SDS was dissolved in 200 ml water. Then the final volume is made to 250 ml with the addition of sterile H_2O the final volume is made to 200ml. This chemical need not autoclaved. A mask was used while making this chemical.

3.8.5 Ethanol

Ethanol solutions are found in two forms such as. 70% and 100%.Two forms have two different functions.100% ethanol precipitates or coagulates DNA and the other one 70% ethanol functions as both in the precipitation of DNA and dissolving of salts. Ethanol (70%) also acts as a surface de-contaminating chemicals in the laboratory.

3.8.6 Chloroform

It is used in the extraction method. This chemical was used under fume hood and was not inhaled. The act of this chemical is to break-up two-dimensional structure of protein forming its precipitation.

3.8.7 1X TE Buffer

This is a secondary chemical and acts as DNA preserving solution. 100 ml 1X TE Buffer can be prepared by adding water finally as follows:

1 M Tris pH 8.0	10 ml
0.5 M Na2EDTA pH 8.0	200ul

3.8.8 Extraction buffer (200ml)

For the preparation of 200ml extraction buffer, 40mL of 1M Tris-HCL (pH 8) was mixed with 10 mL of 0.5M EDTA. Then added to 11.4mL 3.5M NaCl in a 200mL measuring cylinder. Finally, sterilized distilled water was added to make the volume up to the mark, then mixed well and autoclaved.

3.8.9 1M Tris HCL (pH= 8.0) (200mL)

Firstly, used 24.23g Tris-HCl dissolved in 100ml deionized water and pH 8.0 was adjusted using concentrated HCl. By using de-ionized water, then made top up the total volume to 200mL.

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

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

3.8.11 NaCl 3.5M (250 mL)

204.54 g NaCl was put on to 800 ml of de-ionized water and synthesized the final volume to 1 L with de-ionized water.

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

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

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

4 g CTAB+ 20mL Tris-HCL+ 8 mL EDTA (pH 8) + 2g PVP was dissolved into de-ionized water. All items should be added but NaCl should not added. Because NaCl does not dissolve if mixed together. About 80mL of NaCl was added afterwards.

3.8.14 Chloroform: Isoamyl Alcohol: Phenol= 24:1:5 (100mL)

To make this solution, 5 mL phenol was taken in a 100mL volumetric flask. Then 91.2 mL Chloroform + 3.8mL Isoamyl alcohol was used and mixed well. The solution was stored at 4^0C.

3.8.15 Other Chemicals

10X TBE Buffer (1000mL)

In a volumetric flask (1000mL) 108g Tris-HCL was taken. After that 9.3g of EDTA and 55g Boric acid was added. Sterilized dH2O (double distilled water) was added to make the volume 1000mL.

1X TBE buffer

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

1% PVP

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

70% ethanol (1000mL)

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

3.9 Genomic DNA isolation protocol from leaf sample of T.Aman rice

Full genomic DNA was isolated from the landraces of T.Aman rice. The protocol of using a quick modified CTAB method of DNA isolation was given below (Ferdous *et al.,* 2012).

1. Healthy, young, vigorous, actively growing leaf tissues were collected from 24 different T.Aman rice germplasm for whole genomic DNA extraction.

2. By running tap water these healthy rice leaves were washed properly. Then again it was washed in de-ionized water.

3. To remove dirts and other DNA material sources, ethanol was used for sterilization and tissue paper was used for dried.

4. About, one gram of leaf sample were cut into small pieces by scissors and then taken into morter. 600 µl of extraction buffer was added to it and grinded gently with the help of pestle. Then the grinding samples were poured into the 2 ml eppendorf tube.

5. Then 400µl of 2XCTAB solution was added to the same eppendorf tube and equal volume (400 µl) of Choloroform: Isoamyl Alcohol: Phenol (24:1:5 %) was added there . It was mixed well for 15 seconds in a vortex mixture.

6. This vortexed mixture was centrifuged at 8,400 rpm for 10 minutes.

7. The supernatant approximately, 800-900 µl was transferred into the new eppendorf tube and the lower layer was discarded.

8. Then two-third volume of the supernatant (465 µl) isopropanol was added to it and mixed gently by inverting the tubes.

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

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

11. Then the supernatant was discarded completely. By using 70% ethanol DNA pellet was wahed. These pellet was air dried for 1 hour.

12. After DNA pellet was re-suspended with 50 µl of TE Buffer. It was vortexed for 4-5 seconds. Then it was stored at 4^0 C refrigerator overnight.

13. Finally, DNA samples were stored at -20^0 C refrigerator until use.

3.10 SSR marker analysis

Simple sequence repeat (SSR) markers were used for molecular diversity analysis. Sixteen well-distributed SSRs primer were used for the diversity analysis. Most of these markers were obtained from a panel of fifty standard SSR markers, which has been proposed by CGIAR for rice diversity analysis (Roy *et al*, 2016; Islam *et al*, 2018).These 16 primers were *viz* RM1,RM5,RM207,RM 3646,RM273,RM26,RM190,RM253,RM455,RM447,RM205,RM228,RM206,RM277,RM224, RM411. List of primer and their DNA sequences were given below.

Table 2. 16 Microsatellite (SSR) markers used for molecular characterization in 24 accessions of T.Aman rice.

Sl. No	Primer Name	Location in Chromo some	Annealing Temp. ($_0$C)	Forward primer (5′–3′)	Reverse primer (5′–3′)
1	**RM1**	1	55	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC
2	**RM5**	1	55	TGCAACTTCTAGCTGCTCGA	GCATCCGATCTTGATGGG
3	**RM207**	2	55	CCATTCGTGAGAAGATCTGA	CACCTCATCCTCGTAACGCC
4	**RM3646**	3	55	ACTAGAGCACCCTCGCTGAG	CTCAGCCACCCCATCAAC
5	**RM273**	4	55	GAAGCCGTCGTGAAGTTACC	GTTTCCTACCTGATCGCGAC
6	**RM26**	5	55	GAGTCGACGAGCGGCAGA	CTGCGAGCGACGGTAACA
7	**RM190**	6	55	CTTTGTCTATCTCAAGACAC	TTGCAGATGTTCTTCCTGATG
8	**RM253**	6	55	TCCTTCAAGAGTGCAAAACC	GCATTGTCATGTCGAAGCC
9	**RM455**	7	55	AACAACCCACCACCTGTCTC	AGAAGGAAAAGGGCTCGATC
10	**RM447**	8	55	CCCTTGTGCTGTCTCCTCTC	ACGGGCTTCTTCTCCTTCTC
11	**RM205**	9	55	CTGGTTCTGTATGGGAGCAG	CTGGCCCTTCACGTTTCAGTG
12	**RM228**	10	55	CTGGCCATTAGTCCTTGG	GCTTGCGGCTCTGCTTAC
13	**RM206**	11	55	CCCATGCGTTTAACTATTCT	CGTTCCATCGATCCGTATGG
14	**RM277**	12	55	CGGTCAAATCATCACCTGAC	CAAGGCTTGCAAGGGAAG
15	**RM224**	11	55	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG
16	**RM411**	3	55	ACACCAACTCTTGCCTGCAT	TGAAGCAAAAACATGGCTAGG

3.11 PCR amplification

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

The ingredients of PCR reaction for SSR markers are as follows:

Table 3. Preparation of PCR Cocktail (master mix)

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

After initial denaturation at 94°C for five minutes, each cycle comprised of 30 sec denaturation at 95°C, 30 sec annealing at 55°C and 25 sec extension with a final extension for 5 min at 72°C at the end of 32 cycles.

Table 4. PCR Profile

Step	Temperature	Time
Initial denaturation	94^0C	2.0 min
35 cycles of the following steps:		
Denaturation	95^0C	30sec
Primer annelling	55^0C	30sec
Extension	72^0C	25sec
Final extension	72^0C	5min
Storage	4^0C	99:99 (overnight)

3.12 Polyacrylamide Gel Electrophoresis (PAGE)

The PCR products were separated using 8% polyacrylamide gel. Two glass plates, two spacers and one comb were used for gel preparation. These were washed properly using laboratory detergent (bleaching powder). 0.5 M NaOH solution was also used for glass plates washing. Glass plates were air dried and the inner surfaces of the plate was sprayed with 100% ethanol wiping with lint-free tissue. The gasket was attached on the round bottom plate and spacers were put along the inside edges of the gasket. Then the other plate was placed on top of the bottom assembly. On the both sides of the plates clamps were set and the plate assembly was laid flat on the table. Three microlitres of PCR products were run for 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.

Table 5. Composition and preparation of polyacrylamide gel

The gel solution was prepared in a beaker with a magnetic stirring bar. About 8% concentration of gels used for PAGE preparation.

Reagents	**Concentration**	**Volume for 8% PAGE gel**
Sterile nanopure H_2O	-	41.35ml
10X TBE buffer	5X	6.0ml
40% Acrylamide	8%	12ml
10% APS	0.1%	600ul
TEMED	1µl/ml	50ul
Total		60.00ml

The solution was stirred using magnetic stirrer for few seconds at a speed after adding TEMED. Then, the gel solution was poured into glass plate smoothly and continuously. Air bubbles also checked. For avoiding this situation, starting from one corner until it reached top portion of the short plate. The comb was inserted in the gel gently. Thirty minutes were allowed to polymerize the gel finally.

The gasket was removed starting from one corner of the plate assembly, after the gel was polymerized. Around 300 ml of 0.5X TBE buffer was added on top of the tank and around 500 ml of 0.5X TBE buffer was added in the base of the tank. Then the comb was removed gently. Loading dye 2 µl of 10X was added that containing 10 µl PCR product . After that the plates were centrifuged at a speed of 3000 rpm for 30 sec. Around 2 µl of the mixer was loaded in the wells of PAGE gel with the pipette. DNA ladder was loaded for size determination of DNA. The PCR products were analyzed by electrophoresis on 8% polyacrylamide gel with a 50 bp DNA ladder (Bio Basic, Canada) using mini vertical polyacrylamide gels (CBS Scientific Co. Inc., CA, USA). 2.5 µl of amplification products were resolved by running gel in 0.5X TBE buffer for 1.5-2.5 hrs depending upon the allele size at around 100volts and 500 mA current. After electrophoresis, gels were stained and visualized.

3.13 Staining and Visualization of the Gel

After electrophoresis the plates were removed from the tank. By using a knife, the glass plates were separated. The acrylamide gel was removed carefully and transferred into the SYBR Safe staining solution. The gels were stained in 5 µl SYBR Safe DNA gel stain (10,000X concentration in DMSO, USA) with 200 ml 0.5X TBE buffer for 15 min and exposed to UV light using a molecular imager gel documentation unit (XR System, Uvitec Cambridge, France) for visualization. The gel was viewed in the computer monitor and 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.

3.14 SSR data analysis

Molecular weight for each microsatellite products were estimated with AlphaEaseFC (Alpha Innotech Corporation) version 4.0 software. The summary statistics including the number of alleles per locus , major allele frequency, gene diversity and polymorphism information content (PIC) values were determined using POWERMAKER version 3.25 (Liu and Muse, 2005), a genetic marker data analysis software. Genetic diversity also was assessed based on Nei's (1983) genetic distance and software MEGA 5.1 was applied to construct the neighbor joining tree.

Some pictural view and demonstration of morpho-molecular work of the experiment

Figure 1. Young leaf sample collection from experimental field

Figure 2. Samples were arranged in ice buckets

Figure 3. Grinding of leaf sample with extraction buffer

Figure 4. After vortex solution was centrifuged

Some pictural view and demonstration of morpho-molecular work of the experiment

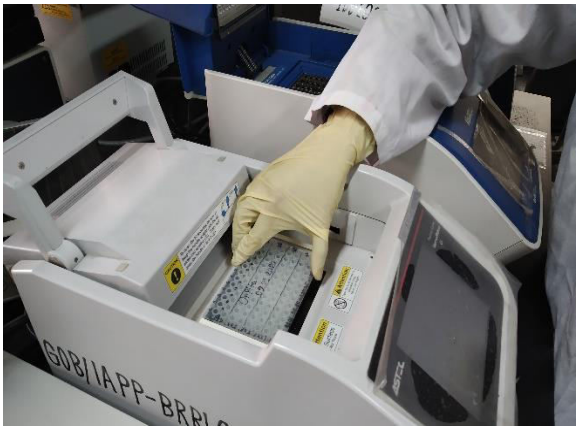


Figure 5. Plates were arranged in PCR machine for amplification

Figure 6. PAGE gel preparation

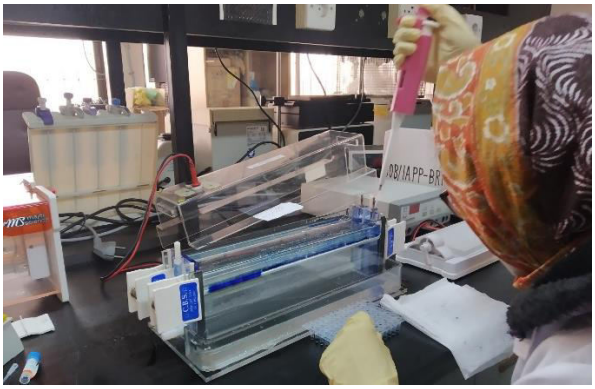


Figure 7. DNA samples were loading in gel electrophoresis unit

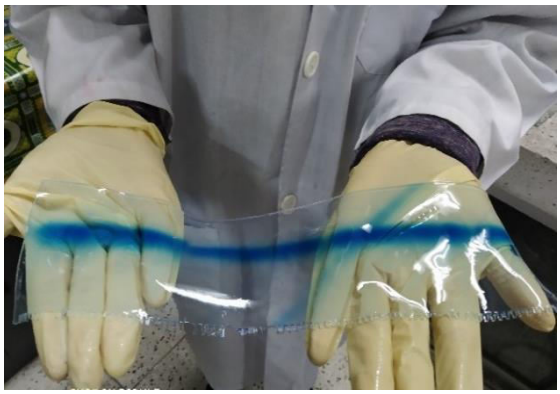


Figure 8. PAGE gel run after electrophoresis

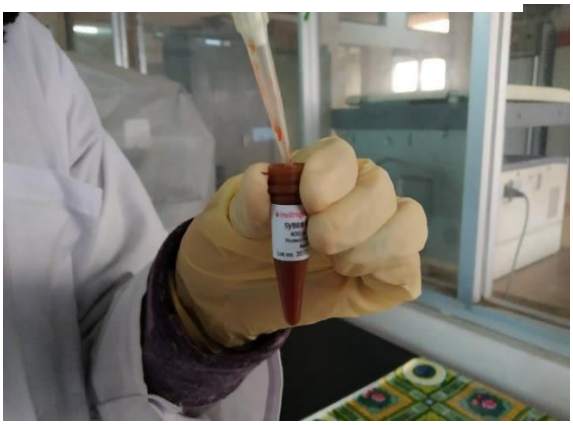


Figure 9. SYBR Safe dye was used for gel staining

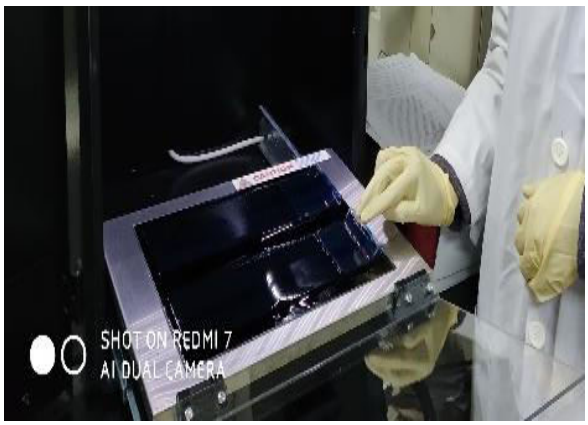


Figure 10. Stained gels were put in the exposure cabinet of the gel documentation system

CHAPTER IV
RESULTS AND DISCUSSION

The present study was conducted to characterize the morphological and molecular diversity of T.Aman rice germplasm. Results obtained from the study have been presented below

Sub-experiment- 01

Morphological characterization and genetic diversity analysis of T.Aman rice germplam.

4.1 Morphological characterization based on qualitative traits:

Qualitative characters are play an important role for plant description and mainly influenced by the consumers preference, socio-economic scenario and natural selection (Hien *et al.,* 2007). Agro-morphological characterization is an important function to evaluate the utilization of rice germplasm to improve yield. The diversity in crop varieties is essential for agricultural development. In this study frequency distribution for 10 qualitative traits is depicted in Table 6 and its graphical representation of frequency distribution showed in Figure 11.

The present study was aimed at identifying distinct qualitative traits variation among the tested T.aman rice landraces. Polymorphism was found in 7 of the 10 qualitative traits under studied. The non-polymorphic traits were the ligule colour, ligule shape and decorticated grain: Scent (aroma) (Table 6). Because all the 24 genotypes produced split or two-cleft type shape penultimate leaf ligule. Most of the morphological characters showed variation in different accessions. The major findings were given below.

4.1.1 Blade color

Out of 24 T.Aman rice germplasm, the trait was observed under two categories .Most of the evaluated rice accessions (20) exhibited green blade color (83.33%) while the rest of the accessions exhibited (4) purple tips (16.67%). Germplasm Bazail, Khirsha bhog, Sham rush and Shul kumor showed purple tips.

4.1.2 Basal leaf sheath colour

Out of 24 T. Aman rice germplasm, it was noticed that most of the tested rice accessions(18) possessed green basal leaf sheath color (75) and remaining (6) were light purple (25) basal leaf sheath color. Germplasm Bazail(acc.no 251),Bazail(acc.no.252), Khirsha bhog, Sham rush, Dudh kalam and Shul kumor showed light purple basal leaf sheath color.

4.1.3 Ligule color

All tested rice germplasm (100%) shared white ligule color.

4.1.4 Ligule shape

About (100%) 2-cleft ligule shape was present in all germplasm.

4.1.5 Awn distribution

Another most important trait is awn distribution. Among 24 T.aman rice germplasm, 15 rice germplasms were awnless (62.5%), tip only in 8 rice germplasms (33.33%) and upper quarter only in 1 germplasm (4.17%) (Bazail).

4.1.6 Apiculus color

Apiculus color showing huge variation in this study.The trait was observed under six categories. Most of the tested rice (13) showing straw (13) (54.17%), brown (tawny) (3) (12.5%), red apex(1) (4.17%), purple(5) (20.83%), purple apex(1) (4.17%) and black(1) (4.17%) apiculus color respectively.

4.1.7 Lemma and palea color

Data in Table 6 also demonstrate variation in lemma and palea color where the percentage of rice accessions grouped in six categories. These were straw(13), gold and gold furrows on straw(5), brown spots on straw(1), brown (2), reddish to light purple(1) and purple(2) respectively and percentage were (54.17%),(20.83%),(4.17),(8.33%),(4.17%) and (8.33%) respectively.

4.1.8 Seed coat (bran)

This trait was observed under four categories. Seed coat (bran) color was mostly white(12) (50.00%) and red(6) (25.00%), remaining tested rice accession were speckled brown(4) (16.67%) and brown(2) (8.33%).

4.1.9 Decorticated grain: Scent (aroma)

All the rice accession were non scented (100%)

4.1.10 Leaf senescence

The trait was observed under three categories. Among these germplam (58.33%) (14) showed intermediate leaf senescence, (33.33%)(8) late and slow leaf senescence and (8.33%)(2) had very late leaf senescence at the time of maturity.

Similar type of work was also reported by Bisne and Sarawgi (2008) and Moukoumbi *et al.* (2011).

Qualitative character showed morphological variation among rice genotypes. This implies that the T.aman rice accessions evaluated in the present study exhibited a high variability in most of the observed qualitative traits.

Table 6. Characterization of rice germplasm based on qualitative characters during T. Aman

Sl. no.	Characters	State of characters	No. of germplasm	Germplasm (Serial number in Table 1)	Frequency%
1	Blade colour	02. Green	20	1,2,3,4,5,7,8,11,12,13,14,15,16,17,18,19,20,21,22,23	83.33
		04. Purple tips	4	6,9,10,24	16.67
2	Basal leaf sheath colour	01. Green	18	1,2,3,4,7,8,12,13,14,15,16,17,18,19,20,21,22,23	75.00
		03. Light purple	6	5,6,9,10,11,24	25.00
3	Ligule colour	01.White	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
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)	15	1,3,4,5,8,12,14,16,17,18,20,21,22,23,24	62.5
		01.Tip only	8	2,7,9,10,11,13,15,19	33.33
		02.Upper quarter only	1	6	4.17
6.	Apiculus colour	02.Straw	13	2,3,4,7,8,12,13,16,17,18,19,20,23	54.17
		03. Brown(tawny)	3	14,21,22	12.5
		05.Red apex	1	15	4.17
		06.Purple	5	5,6,9,10,11	20.83
		07. Purple apex	1	24	4.17
		9.Black	1	1	4.17
7	Lemma and palea colour	0. Straw	13	2,3,5,6,9,10,11,12,13,16,17,20,23	54.17
		01. Gold and gold furrows on straw	5	1,4,7,19,22	20.83
		02. Brown spots on straw	1	18	4.17
		04.Brown	2	14,21	8.33
		05.Reddish to light purple	1	8	4.17
		08. Purple	2	15,24	8.33
8	Seed coat (bran) colour	01.White	12	8,9,10,13,14,15,16,17,18,22,23,24	50.00
		03. Speckled brown	4	2,3,7,20	16.67
		04. brown	2	12,19,	8.33
		05.Red	6	1,4,5,6,11,21	25.00
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	14	2,4,5,7,9,10,11,13,14,15,20,21,22,23	58.33
		07. Late and slow	8	3,6,8,12,16,17,18,19	33.33
		09.Very late	2	1,24	8.33

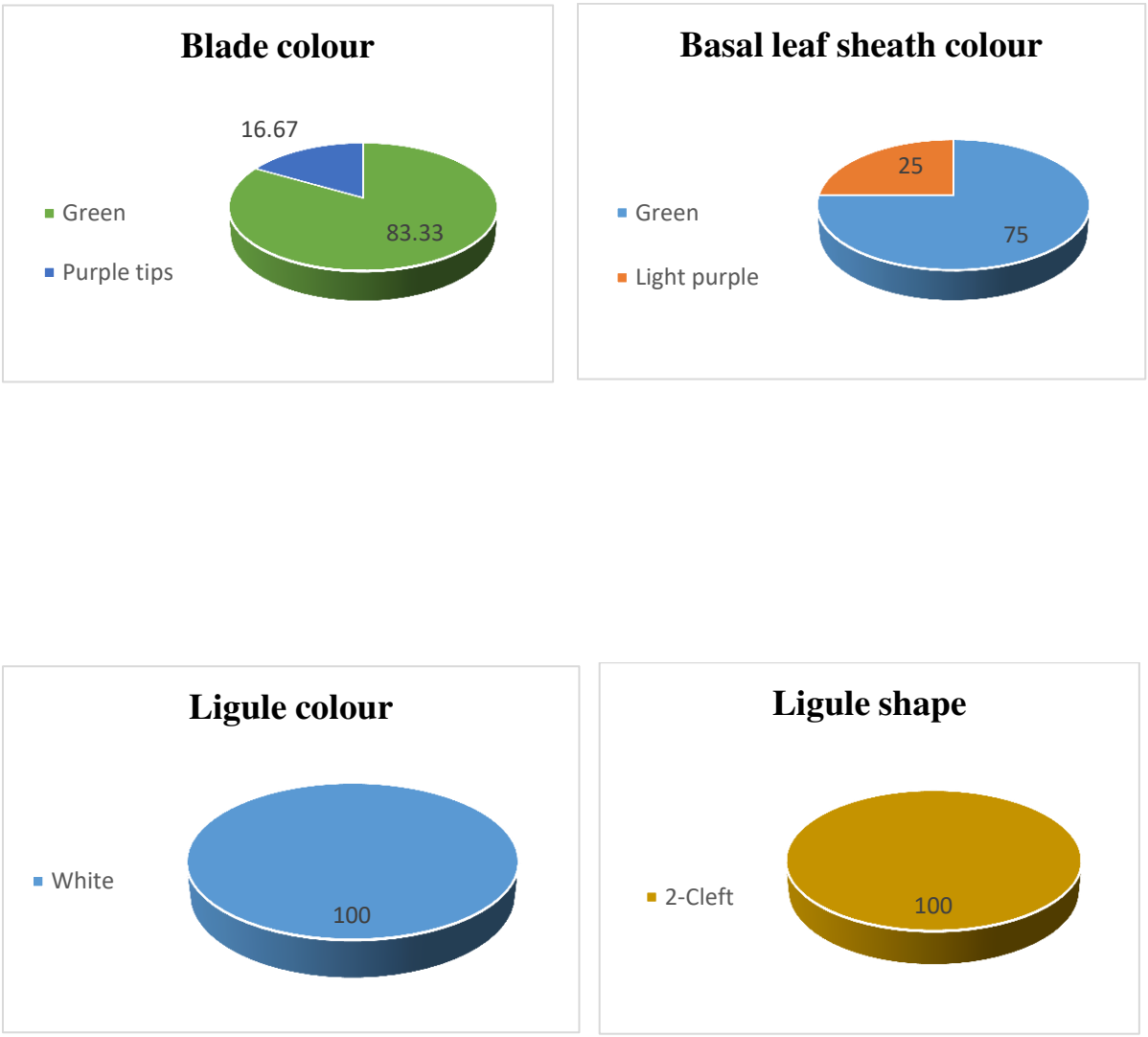
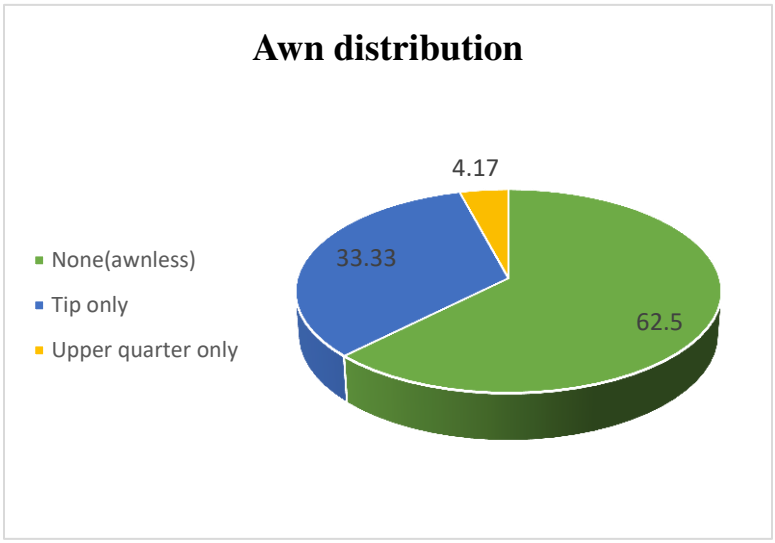
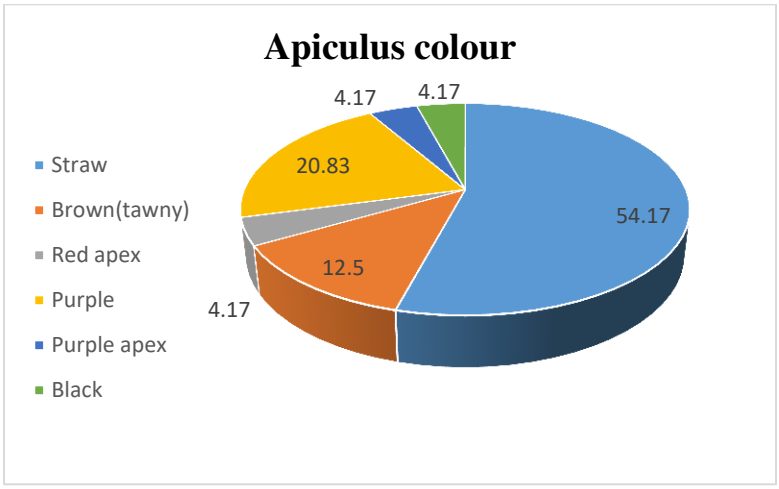
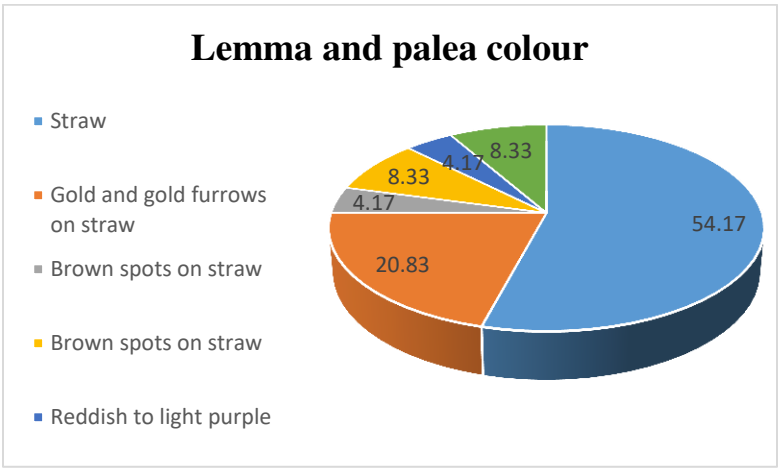


Figure 11. Frequency distribution of 10 qualitative morphological characters of 24 T.aman rice germplasm

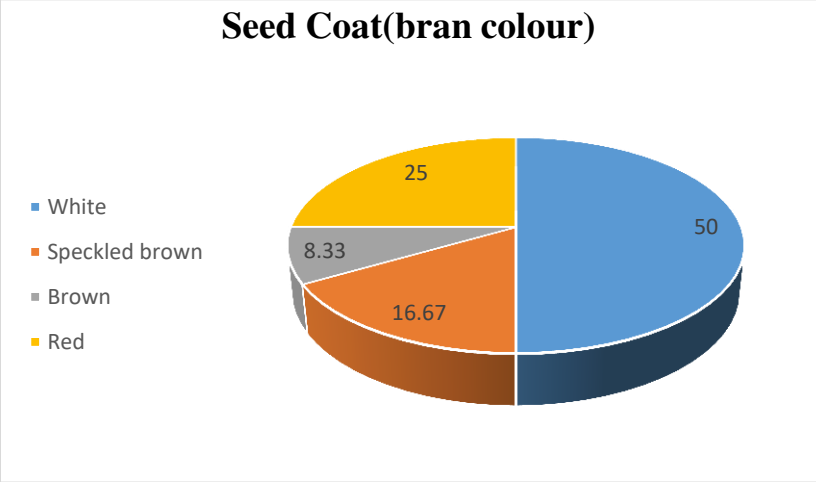
Con...

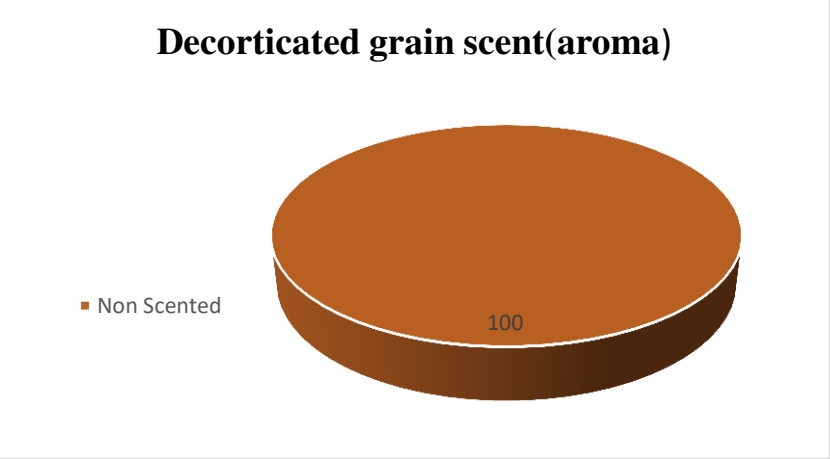

Awn distribution
None(awnless)
Tip only
Upper quarter only
4.17
33.33
62.5


Apiculus colour
Straw
Brown(tawny)
Red apex
Purple
Purple apex
Black
4.17
4.17
20.83
54.17
12.5
4.17


Lemma and palea colour
Straw
Gold and gold furrows on straw
Brown spots on straw
Brown spots on straw
Reddish to light purple
8.33
4.17
8.33
4.17
54.17
20.83

Con...


Seed Coat(bran colour)
White
Speckled brown
Brown
Red
25
50
8.33
16.67


Decorticated grain scent(aroma)
Non Scented
100

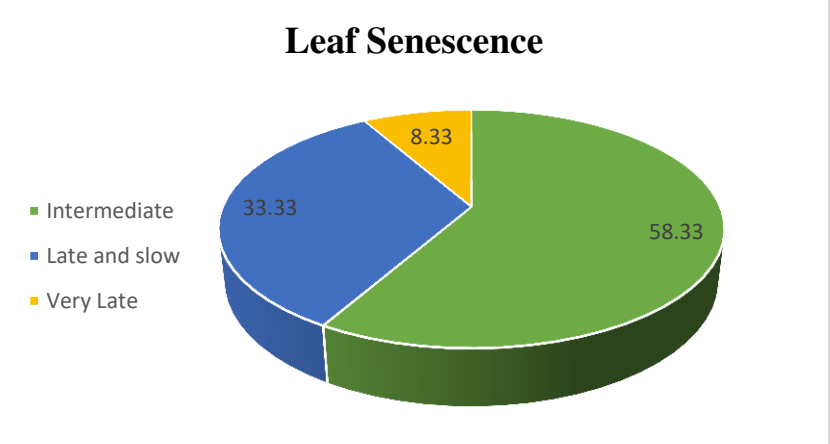
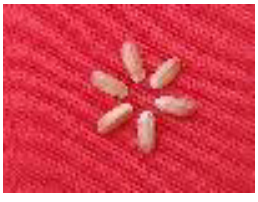

Leaf Senescence
Intermediate
Late and slow
Very Late
8.33
33.33
58.33

Table 7: Morphological variation in panicle, grain and kernel of aman rice germplasm

Name of germplasm	Accession No.	Panicle	Grain	Kernel
Kali jira(3)	247			
Telot	248			
Bazail	249			
Joli amon	250			
Bazail	251			

Bazail	252			
Kancha noni	270			
Naria bochi	275			
Khirsha bhog	276			
Sham rush	277			
Dudh kalam	278			

Dudh kalam	279			
Bora dudh kalam	280			
Lal soru	281			
Gojol goria	282			
Sojoni	283			
Ganjia	284			
Bindi pakri	285			

Jhoshua	286			
Akand sail	287			
Lal dupa	289			
Jiga sail	290			
Cheng sail	291			
Shul kumor	292			

4.2 Morphological characterization based on quantitative traits:

Quantitative trait variation among the 24 rice accessions also evaluated under the present study. Ten quantitative traits were observed in 24 T.aman rice germplasm. They were effective tiller number, panicle length , plant height, days of flowering, days of maturity, field grain per panicle, grain length, grain breadth,1000 grain weight, yield per hill. The variability in the quantitative characters was much higher than that of the qualitative characters.

4.2.1 Effective tiller number

Effective tiller number is an important character for rice. The effective tiller number range from 7 to 19. Maximum tiller number was 19 in the genotype Shul kumor and minimum tiller number was 7 in the genotype Telot and Naria bochi. Average effective tiller number was 11. Coefficient of variation was 25.42%.

4.2.2 Panicle length (cm)

Panicle length was ranged from 26.2 cm (Akand sail. Jiga sail, Cheng sail)) to 33.8 cm (Kancha noni) among the tested rice germplasm (Table 8). Average panicle length was 28.78 cm long and coefficient of variation was 6.35. It exhibited reasonable amount of variation with range values of (26.2-33.8) cm. shortest panicle length of 26.2 cm was recorded for (Akand sail. Jiga sail, Cheng sail) whereas the longest panicle length of 33.8 cm was recorded for (Kancha noni).

4.2.3 Plant height (cm)

Another important character was plant height. It had wider range (102-190.2 cm) of variation with a mean value of 153.98 cm. Plant height in rice is a complex character. Cheng sail (102cm) was the accession which falls under very dwarf group. Bazail (190.2cm) can be grouped as tall. Most of the plant height range from (140-170cm).Coefficient of variation was 11.11.

4.2.4 Days of 50% flowering

The time of flowering is also an important trait of rice germplasm. It has a direct influence on the success of cross pollination. The earliest flowering genotype was Cheng sail (97 days) however Sojoni and Ganjia took as long as 118 days. Here showing highest variation of days between two

rice accessions. Average date of flowering was 110 and coefficient of variation was maximum 55.35.

4.2.5 Days to maturity

It also exhibited high ranged from 119 to 150 days with an average of 139 days. The genotype Cheng sail had shorter maturity period (119 days) representing earliness. Minimum value for days to maturity represents that the variety has a benefit of early ripening. Maximum value for days to maturity represents the late variety. The germplasm Sojoni and Ganjia had higher maturity period (150days). Coefficient of variation was lower.

4.2.6 Field grains per panicle

The highest number of filled grains panicle recorded in the germplasm Lal soru (184) and lowest number of filled grain panicle recorded in Bazail (89). Average filled grain per panicle was 118.

4.2.7 Grain length (mm)

Grain length is an important quality parameter. It ranged from 6.06 to 10.09 mm. In the present study, Bora dudh kalam was recorded with maximum grain length (10.09 mm). Kali jira (3) was observed with minimum grain length. Coefficient of variation was 9.84.Average grain length was 8.22 mm.

4.2.8 Grain breadth (mm)

Minimum grain breadth was 1.9 mm and maximum grain breath was 3.41 mm. The genotype Lal soru showing minimum grain breadth and the cultivar Telot showing maximum grain breadth with average breadth was 2.75mm. Coefficient of variation was 16.17.

4.2.9 1000-grain weight (g)

Thousand grain weights was considered one of the major trait of yield component. Considering 1000-grain weight, the germplasm Bora dudh kalam had the maximum thousand grains weight (36.31 g) whereas it was least in the germplasm Kali jira (3) (12.3 g) with a higher coefficient variation was (25.13%) (Table 8).

4.2.10 Yield per hill (g)

The yield per hill ranged from 2.05 to 15.22 g with average yield per hill value was 9.83g. The highest yield per hill (15.22g) found in the genotype Dudh kalam and lowest yield per hill (2.05g) found in the germplasm Bazail. Coefficient of variation was the highest in yield per hill (30.23g). Average yield per hill was 9.83g.

Identifying germplasm accessions for different agronomical characters would help in breeding programs development. Results of this study revealed that the tested rice accessions exhibited significant variation in most of the observed quantitative traits. These traits were then used to assess the genetic diversity of the rice germplasm.

Table 8. Ten important quantitative traits of 24 T. Aman rice germplasm

Sl.No	Effective tiller number	Panicle length(cm)	Plant height(cm)	Days of flowering	Days of maturity	Field grain per panicle	Grain length (mm)	Grain Breadth (mm)	1000 grain weight (g)	Yield per hill (g)
1	9	30.4	160.6	108	138	168	6.06	2.19	12.3	10.71
2	7	29.4	151.8	115	141	163	8.55	3.41	26.63	9.76
3	10	30	163.8	117	143	115	8.6	3.33	27.3	7.08
4	9	30	163	114	141	129	8.3	3.32	26.94	10.18
5	8	29.6	190.2	103	133	89	8.29	3.03	25.23	4.7
6	9	28.4	171.8	115	141	102	8.5	3.06	26.17	2.05
7	14	33.8	168.2	108	138	92	8.5	3.01	25.32	6.55
8	7	28	135.8	114	141	144	6.88	3.05	21.67	8.55
9	10	29.8	150.8	107	138	119	8.71	2.52	25.55	11.89
10	8	28	148.8	108	138	117	9.07	2.51	23.27	6.46
11	8	28.2	179.4	115	141	114	8.15	3	27.7	10.06
12	10	27	142.8	117	143	96	7.84	2.34	15.76	15.22
13	10	30	164.8	114	140	128	10.09	3.13	36.31	12.03
14	9	28.4	161.4	112	138	184	8.22	1.9	13.63	8.81
15	13	26.6	145.2	111	138	95	8.03	2.58	20	12.41
16	9	30.8	163.2	118	150	99	7.48	2.24	17.93	11.05
17	12	28.4	156.8	118	150	109	7.46	3.33	16.71	10.11
18	11	28.2	145.6	104	138	109	8.11	2.17	16.01	9
19	10	31	146	116	141	93	9.04	2.67	22.6	9.87
20	11	26.2	146	117	144	111	7.33	2.31	15.38	13.17
21	8	29.2	145	107	138	130	7.96	3.22	24.97	11.46
22	11	26.2	153.2	100	135	103	8.97	2.59	21.72	11.42
23	10	26.2	102	97	119	106	8.62	2.32	19.59	9.14
24	19	27	139.4	104	135	107	8.58	2.73	22.16	14.19
Max	19	33.8	190.2	118	150	184	10.09	3.41	36.31	15.22
Min	7	26.2	102	97	119	89	6.06	1.9	12.3	2.05
Mean	11	28.78	153.98	110.79	139.25	118	8.22	2.75	22.12	9.83
SD	2.57	1.83	17.11	6.06	5.89	25.10	0.81	0.44	5.56	2.97
CV	25.42	6.35	11.11	5.47	4.23	21.35	9.84	16.17	25.13	30.23
SE	0.52	0.37	3.49	1.24	1.20	5.12	0.17	0.09	1.13	0.61
LSD(5%)	1.03	0.73	6.84	2.42	2.36	10.04	0.32	0.18	2.22	1.19

4.3 Quantitative trait based cluster analysis:

Quantitative traits were used for cluster analysis and established a dendrogram for identified genetic relationship among the studied germplasm. Agglomerative hierarchical clustering performed on the Euclidean (similarity index) distance matrix method and the resulting dendrogram is presented in (Fig. 12). Quantitative traits were used to construct the dendrogram (Table 9). Here 24 rice germplasm formed total three clusters. Distribution pattern indicated that minimum number (1) of germplasm was found in cluster I whereas maximum number found (18) in cluster II. Cluster III consisted of 5 germplasm.

In this study, cluster I contained 1 genotype named 12 (Dudh kalam). Maximum genotype showed in cluster II and Cluster III contained 5 genotypes.

Table 9. Morphological based dendrogram

Cluster I	Cluster II	Cluster III
12(Dudh kalam)	15(Gojol goria),19(Jhoshua), 16(Sojoni), 17(Ganjia),20(Akand sail), 18(Bindi pakri),24(Shul kumor), 3(Bazail), 22 (Jiga sail), 6(Bazail) (acc.no 252), 11(dudh kalam),7(Kancha noni), 4(Joli aman), 9(Khirsha bhog),10 (Sham rush), 13(Bora dudh kalam),21(Lal dupa), 5(Bazail) (acc.no.249)	14(Lal soru), 1(Kali jira (3)),2 (Telot), 8(Naria bochi), 23 (cheng sail).

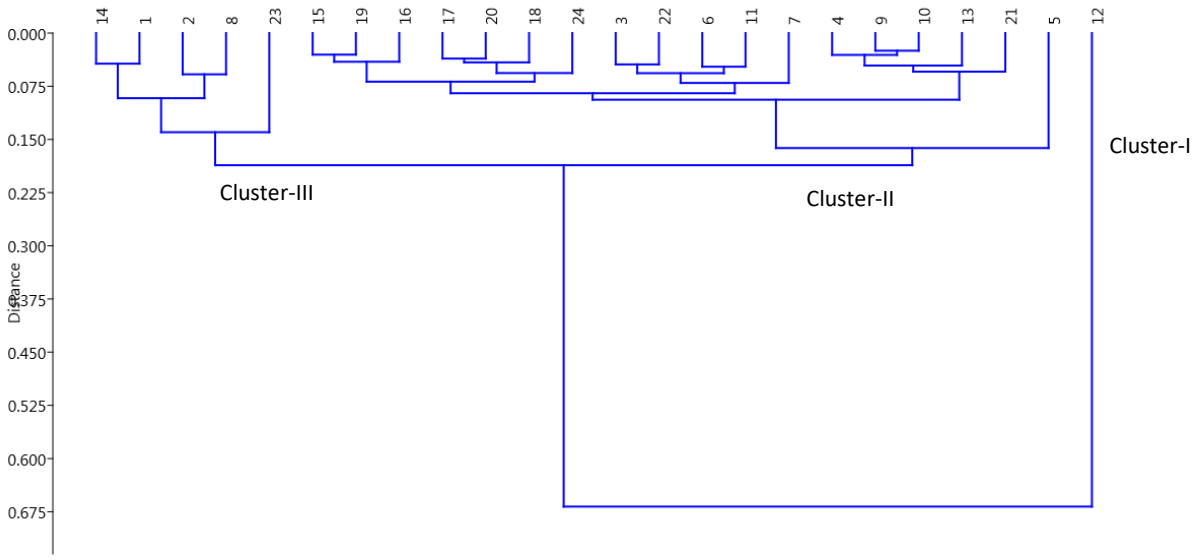


Figure 12. UPGMA(similarity index) using euclidean (1. Kali jira (3), 2.Telot, 3. Bazail, 4. Joli amon, 5. Bazail, 6.Bazail ,7.Kancha noni,8.Naria bochi, 9.Khirsha bhog,10.Sham rush,11.Dudh kalam,12.Dudh kalam,13.Bora dudh kalam, 14.Lal soru, 15.Gojol goria, 16.Sojoni, 17.Ganjia, 18.Bindi pakri,19.Jhoshua , 20.Akand sail, 21.Lal dupa, 22.Jiga sail,23.Cheng sail,24.Shul kumor)

Sub-experiment -02

Molecular diversity analysis and DNA profiling of some T. Aman rice genotypes.

4.4 Molecular Characterization

4.4.1. SSR marker analysis:

The DNA of 24 rice accessions were subjected to PCR. Sixteen simple sequence repeat (SSR) marker were used to ditect polymorphism which were presented in Table 10 and banding pattern of polymorphic primes are presented below.

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

Number of alleles, range of DNA fragment, number of unique alleles, allele frequency, gene diversity and polymorphism information content (PIC) were found among twenty four experimental genotypes. In this study, 16 SSR markers distributed over all chromosomes .The summary statistics of the 16 SSR markers are given in (Table 10). All the markers showed polymorphism. A total of 66 alleles were identified. The average number of allele per locus was 4.13 that was ranged from 2.00 (RM 411 and RM 455) to 8.00 (RM206). The primer RM1, RM 5, RM26, RM190,RM 205,RM 206,RM 207,RM 224,RM253,RM 277,RM 447,RM455,RM 3646 all of them detected as unique alleles. The size of the DNA fragments varies within a range of 77 bp to 201 bp. Primer RM1 amplified at 77 bp fragment whereas primer RM273 amplified at 201 bp fragment of DNA. The gene diversity ranged from 0.08 to 0.83 (maximum to minimum), with an average of 0.52. Total gene diversity is 8.40. Primer RM206 showed highest gene diversity (0.83) followed by RM1 and RM3646 (0.74), RM207 and RM 253(0.65), RM224(0.64), RM190(0.63), RM 273(0.60), RM 447(0.55), RM 5(0.46), RM 228(0.45), RM 277(0.41), RM 411(0.37),RM26 and RM 205(0.29) respectively and RM455 showed the lowest gene diversity (0.08).The polymorphism information content PIC value for 16 SSR markers ranged from 0.08 (RM455) minimum to 0.80 (RM206) maximum. The average PIC value is 0.48 and total PIC value is 7.76. The highest PIC value was obtained from RM206 (0.80) followed by RM 1 and RM 3646 (0.70), RM207 (0.62) , RM 253(0.61), RM224 and RM 190(0.57),RM

273(0.53), RM 447(0.50), RM5 (0.43), RM 228(0.41), RM 277(0.38),RM 411(0.30), RM205 (0.28) and RM 26(0.26) respectively. Primer RM206 had the highest PIC value (0.80) and the highest number of alleles (8). Therefore, RM206 was detected as the highest level of polymorphism and RM206 was supposed to be the best marker for characterizing the 24 T.aman rice germplasm. Moreover, RM455 considered as the least powerful marker. The frequency of the most common allele at each locus ranged from 25.00% (RM206) to 95.83% (RM455). On average, 61.20% of the 24 rice germplasm shared a common major allele at any given locus. The DNA profiles of 24 T.aman rice germplasm with RM206, RM277, RM455 were showed in plate 1, plate 2 and plate 3 respectively.

This result reveal that Aman rice germplasm showed genetic differentiation and a high degree of variability. They also indicate polymorphism that exist in a certain degree which is really remarkable. This result can be used for further genetic improvement programme.

Table 10. Data on Number of alleles, range of DNA fragment, number of unique alleles, allele frequency, gene diversity and Polymorphism information content (PIC) obtained among 24 rice genotypes.

SL. No.	Marker	Chro. No.	Position (cM)	Motif*	Allele No.	Unique allele	Size range (bp)	Size (bp)	Frequency (%)	Gene diversity	PIC
1	**RM 1**	1	29.7	(GA)26	5	1	77-125	125	37.50	0.74	0.70
2	**RM 5**	1	94.9	GA)14	4	1	110-128	128	70.83	0.46	0.43
3	**RM 26**	5	118.8	(GA)15	3	1	103-118	118	83.33	0.29	0.26
4	**RM 190**	6	7.4	(CT)11	4	1	110-128	110	50.00	0.63	0.57
5	**RM 205**	9	114.7	(CT)25	4	2	107-145	107	83.33	0.29	0.28
6	**RM 206**	11	102.9	(CT)21	8	3	129-174	129	25.00	0.83	0.80
7	**RM 207**	2	191.2	(CT)25	6	2	118-146	118	54.67	0.65	0.62
8	**RM 224**	11	120.1	(AAG)8(AG)13	4	1	135-157	157	50.00	0.64	0.57
9	**RM 228**	10	130.3	(CA)6(GA)36	3	_	109-134	109	70.83	0.45	0.41
10	**RM 253**	6	37	(GA)25	5	1	117-149	149	54.17	0.65	0.61
11	**RM 273**	4	94.4	(GA)11	3	_	182-201	182	54.17	0.60	0.53
12	**RM 277**	12	57.2	(GA)11	4	1	119-134	134	75.00	0.41	0.38
13	**RM 411**	3	127.9	(GTT)7	2	_	110-118	118	75.00	0.37	0.30
14	**RM 447**	8	124.6	(CTT)8	4	1	105-120	105	62.50	0.55	0.50
15	**RM 455**	7	65.7	(TTCT)5	2	1	131-136	131	95.83	0.08	0.08
16	**RM 3646**	3	6.32	(GA)14	5	1	130-152	152	37.50	0.74	0.70
	Max.				**8.00**			**157**	**95.83**	**0.83**	**0.80**
	Min.				**2.00**			**105**	**25.00**	**0.08**	**0.08**
	Total				**66**	**17**				**8.40**	**7.76**
	Mean				**4.13**				**61.20**	**0.52**	**0.48**

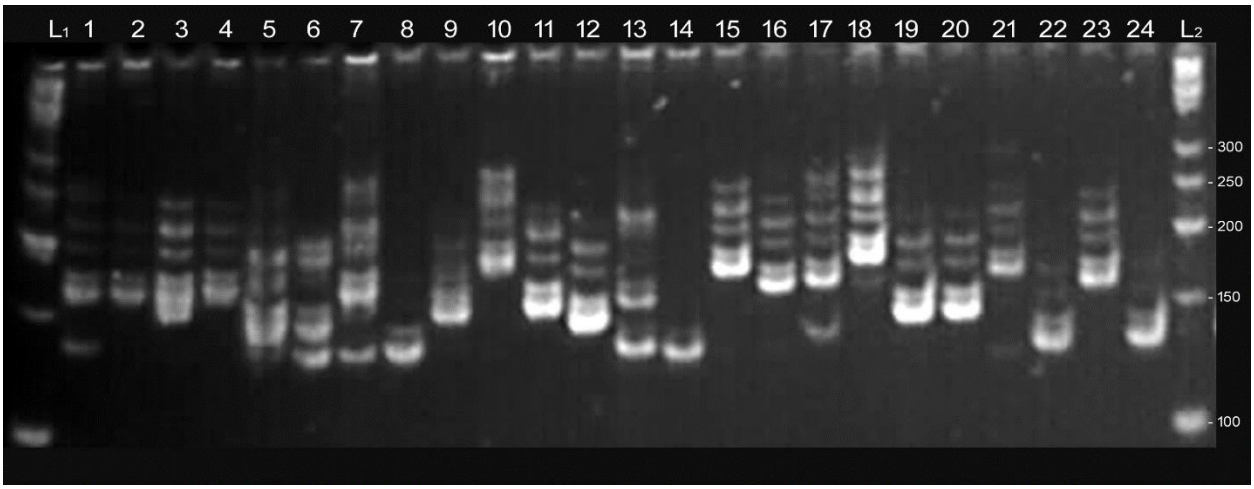


Plate 1: Banding pattern of 24 T.aman rice germplasm using primer RM 206 where Lane L: 50bp ladder; (1.Kali jira(3), 2.Telot ,3.Bazail,4. Joli amon,5.Bazail, 6.Bazail ,7.Kancha noni,8.Naria bochi, 9.Khirsha bhog,10.Sham rush,11.Dudh kalam,12.Dudh kalam,13.Bora dudh kalam, 14.Lal soru, 15.Gojol goria, 16.Sojoni, 17.Ganjia, 18.Bindi pakri,19.Jhoshua , 20.Akand sail, 21.Lal dupa, 22.Jiga sail,23.Cheng sail,24.Shul kumor)

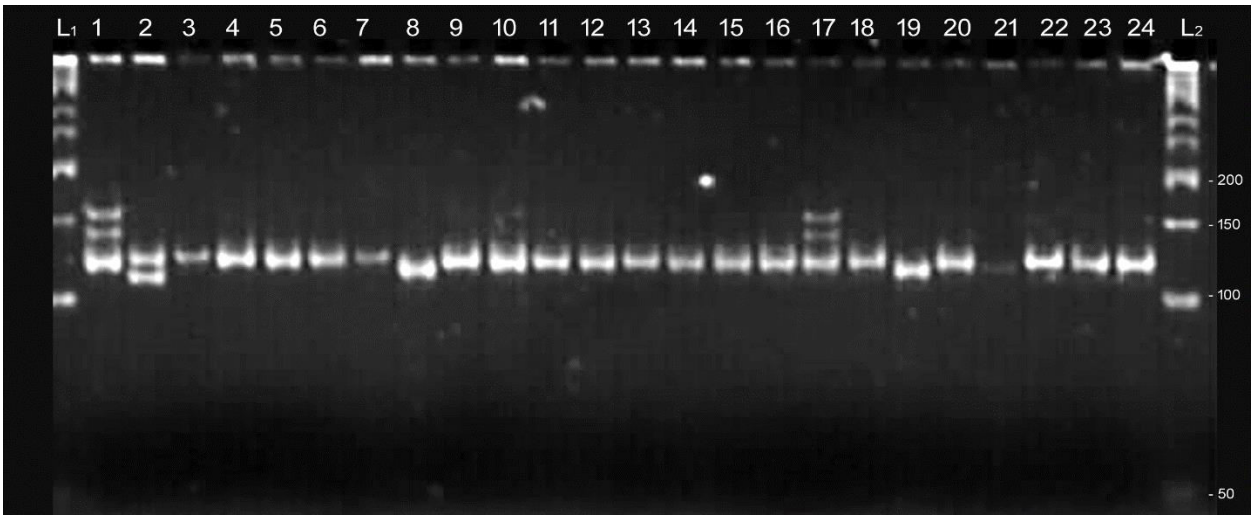


Plate 2: Banding pattern of 24 T.aman rice germplasm using primer RM 277 where Lane L: 50bp ladder; (1.Kali jira(3), 2.Telot ,3.Bazail,4. Joli amon,5.Bazail, 6.Bazail ,7.Kancha noni,8.Naria bochi, 9.Khirsha bhog,10.Sham rush,11.Dudh kalam,12.Dudh kalam,13.Bora dudh kalam,14.Lal soru, 15.Gojol goria, 16.Sojoni, 17.Ganjia, 18.Bindi pakri,19.Jhoshua , 20.Akand sail, 21.Lal dupa, 22.Jiga sail,23.Cheng sail,24.Shul kumor)

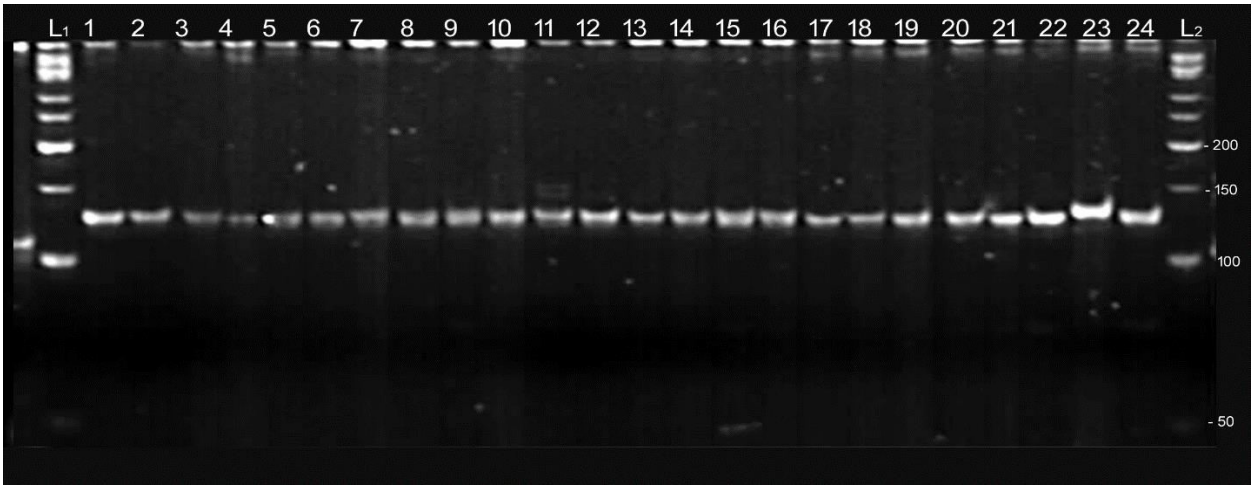


Plate 3: Banding pattern of 24 T.aman rice germplasm using primer RM 455 where Lane L: 50bp ladder; (1.Kali jira(3), 2.Telot ,3.Bazail,4. Joli amon,5.Bazail, 6.Bazail ,7.Kancha noni,8.Naria bochi, 9.Khirsha bhog,10.Sham rush,11.Dudh kalam,12.Dudh kalam,13.Bora dudh kalam,14.Lal soru, 15.Gojol goria, 16.Sojoni, 17.Ganjia, 18.Bindi pakri,19.Jhoshua , 20.Akand sail, 21.Lal dupa, 22.Jiga sail,23.Cheng sail,24.Shul kumor)

4.4.3 Unique Allele

Unique allele also called exclusive allele. Unique allele have some special characters as they act as an effectively indicator of particular landraces. These are also used for breeding purpose as well as molecular characterization of rice germplasm. The higher number of unique alleles in germplasm specifies as a source of novel alleles. A total of 17 unique alleles were detected at 12 microsattelite loci (Table 11).Thirteen of the rice germplasm had unique allels for at least one microsatellite locus. In our study, twelve microsatellite markers (eg., RM 1,RM 5,RM 26,RM 190, RM 205,RM 206,RM 207,RM 224,RM253,RM 277,RM 447,RM 455,RM 3646) were detected as unique alleles. Germplasm Telot, Bazail, Naria bochi, Khirsha bhog, Lal soru, Gojol goria, Ganjia, Bindi pakri, Jiga sail,cheng sail were revealed as unique alleles.

SL. No.	Marker	Chromosome No.	Unique Allele (bp)	Grmplasm
1	RM 1	1	77	9
2	RM206	11	129	14
3	RM206	11	161	17
4	RM206	11	174	18
5	RM5	1	110	8
6	RM26	5	103	8
7	RM190	6	128	8
8	RM 207	2	138	22
9	RM207	2	146	15
10	RM224	11	140	9
11	RM253	6	122	6
12	RM447	8	115	8
13	RM 3646	3	130	18
14	RM205	9	145	8
15	RM205	9	107	22
16	RM277	12	119	2
17	RM455	7	136	23

Table 11: List of unique allele in this study

4.4.4 Diversity level based on PIC value showed by SSR markers

Polymorphism information content (PIC) provides an estimate of determining power of a marker based on the number of alleles at a locus. Lower PIC value may be the result of closely related genotypes whereas higher PIC values might be the result of diverse genotypes. PIC value is a reflection of allelic diversity as well as frequency among the varieties. The used 16 markers have different level of diversity on the basis of PIC value. Among them, RM455 showed low diversity. Again, RM26, RM205, RM5, RM447, RM411, RM277, RM228 showed moderate diversity. Rest of the eight markers showed highly diverse result. They are RM1, RM190, RM206, RM207, RM224, RM273, RM455, RM3646.Botstein *et al*. (1980) reported that PIC can be used to estimate the level of gene variation, when PIC>0.5, the locus was of high diversity; when PIC<0.25, the locus was of low diversity and the locus was of moderate diversity, when PIC between 0.25 and 0.5.

4.5 Nei's Genetic Distance

Nei's (1983) Genetic Distance showed pair-wise comparisons of genetic distance among 24 T.aman rice genotypes. This distance ranged from 0.1250 to 0.9375 with an average of 0.5247 (Table 12). Comparatively higher genetic distance (0.9375) was observed between a number of genotypes. Among them Naria bochi (G8) showed highest genetic dissimilarity. The highest Nei's genetic distance (0.9375) was observed in Naria bochi vs. Cheng sail. The highest genetic distance between them indicated that they are genetically dissimilar and also highly diverse. The lowest Nei's genetic distance (0.1250) was observed in Ganjia (G17) vs.Sojoni (G16). These pair showed lowest dissimilarity or much more closeness among them. Lowest dissimilarity showed lower diversity. Moreover, many more pair show higher genetic distance above 0.80. Pair wise distributiom among them were Jiga sail vs. Kali jira (3), Bazail (Acc.no.251) vs.Kali jira (3),Naria bochi vs. dudh kalam vs. Gojol goria vs. Sojoni vs.Ganjia vs.Bazail (Acc.no.252), Khirsha bhog vs. jiga sail vs.Naira bochi. About 0.75 genetic distance was observed in a lot of pair wise distribution. The difference between the highest and lowest genetic distance indicates the presence of variability among 24 T.aman rice germplasm.

Table 12. Genetic distance among the 24 T.aman rice germplasm of Bangladesh

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

Legend: G1(Kali jira (3)), G2 (Telot), G3 (Bazail), G4 (Joli amon), G5 (Bazail), G6 (Bazail), G7 (Kancha noni), G8 (Naria bochi), G9 (Khirsha bhog), G10 (Sham rush), G11(Dudh kalam), G12 (Dudh kalam), G13 (Bora dudh kalam), G14(Lal soru), G15(Gojol goria),G16(Sojoni), G17(Ganjia), G18(Bindi pakri), G19(Jhoshua), G20(Akand sail),G21(Lal dupa), G22(Jiga sail), G23(Cheng sail), G24(Shul kumor)

(Continued)

Germplasms	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	0.2500	0.0000										
G22	0.6250	0.5000	0.0000									
G23	0.3750	0.4375	0.7500	0.0000								
G24	0.2500	0.3750	0.5625	0.4375	0.0000							
G3	0.6250	0.5000	0.6875	0.7500	0.5625	0.0000						
G4	0.4375	0.3750	0.6875	0.6250	0.5000	0.4375	0.0000					
G5	0.4375	0.5625	0.5625	0.5625	0.5000	0.6250	0.5000	0.0000				
G6	0.5000	0.6250	0.6875	0.5625	0.6875	0.7500	0.5000	0.2500	0.0000			
G7	0.5625	0.5625	0.6875	0.5625	0.6250	0.6250	0.5000	0.5625	0.6875	0.0000		
G8	0.7500	0.6875	0.6875	0.9375	0.6875	0.7500	0.6250	0.7500	0.8750	0.7500	0.0000	
G9	0.5625	0.5625	0.8125	0.5625	0.6250	0.6875	0.5625	0.6250	0.6250	0.5000	0.8750	0.0000

Legend:G1(Kali jira(3)), G2(Telot), G3(Bazail), G4(Joli amon), G5(Bazail), G6(Bazail), G7(Kancha noni), G8(Naria bochi), G9(Khirsha bhog),G10(Sham rush), G11(Dudh kalam),G12 (Dudh kalam),G13 (Bora dudh kalam),G14(Lal soru), G15(Gojol goria),G16(Sojoni), G17(Ganjia), G18(Bindi pakri), G19(Jhoshua), G20(Akand sail),G21(Lal dupa), G22(Jiga sail), G23(Cheng sail), G24(Shul kumor)

4.6 UPGMA dendrogram and cluster analysis

Cluster analysis was done using UPGMA method based upon genotypic data. The genetic similarity analysis was done using UPGMA (Unweighed Pair Group Method with Arithmetic averages) clustering in 24 Aman rice germplasm. Cluster analysis showed significant genetic variation among the germplasm tested**.** On the basis of the Nei's genetic distance, a dendrogram was established with 24 T.Aman rice germplasm. Here "G" indicated germplasm. Unweighted pair group method of arithmetic mean (UPGMA) indicated the dividation of 24 rice genotypes into three main clusters: G1,G10, G11, G12, G13,G14,G15,G16 were grouped in cluster I (Figure 13); 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 total subcluster are 6 in number.

Cluster I contains eight genotype; G1 (Kali jira (3)), G10 (Sham rush), G11 (Dudh Kalam),G12(Dudh kalam) formed sub cluster 1. Moreover, G13 (Boro Dudh Kalam), G14, (Lal Soru), G15 (Gojol Goria), G16 (Sojoni) grouped together in sub cluster 2. Again, G17 (Ganjia), G18 (Bindi pakri), G19 (Jhoshua),G2(Telot) formed sub cluster 1; G20(Akand sail),G21(Lal dupa),G22(Jiga sail),G23(Cheng sail) formed sub cluster 2 of cluster II. Again, cluster III contains eight genotypes. They are G24 (Shul kumor) , G3 (Bazail), G4(Joli amon), G5(Bazail), formed sub cluster 1 and G6 (Bazail), G7 (Kancha noni), G8 (Naria bochi), G9 (Khirsha bhog) formed sub cluster 2 of cluster III. In this dendrogram, rice genotypes of more genetic similarity are placed in same sub cluster and group. The dendrogram revealed that the genotypes that originate from genetically similar type clustered together.

The genotypes grouped in the same cluster show closeness or lower genetic distance, they are genetically similar such as G1,G10,G11,G12,G13,G14,G15,G16. The other genotypes showing more genetic dissimilarity grouped in another cluster due to higher genetic distance. These can be easily used for rice improvement program.

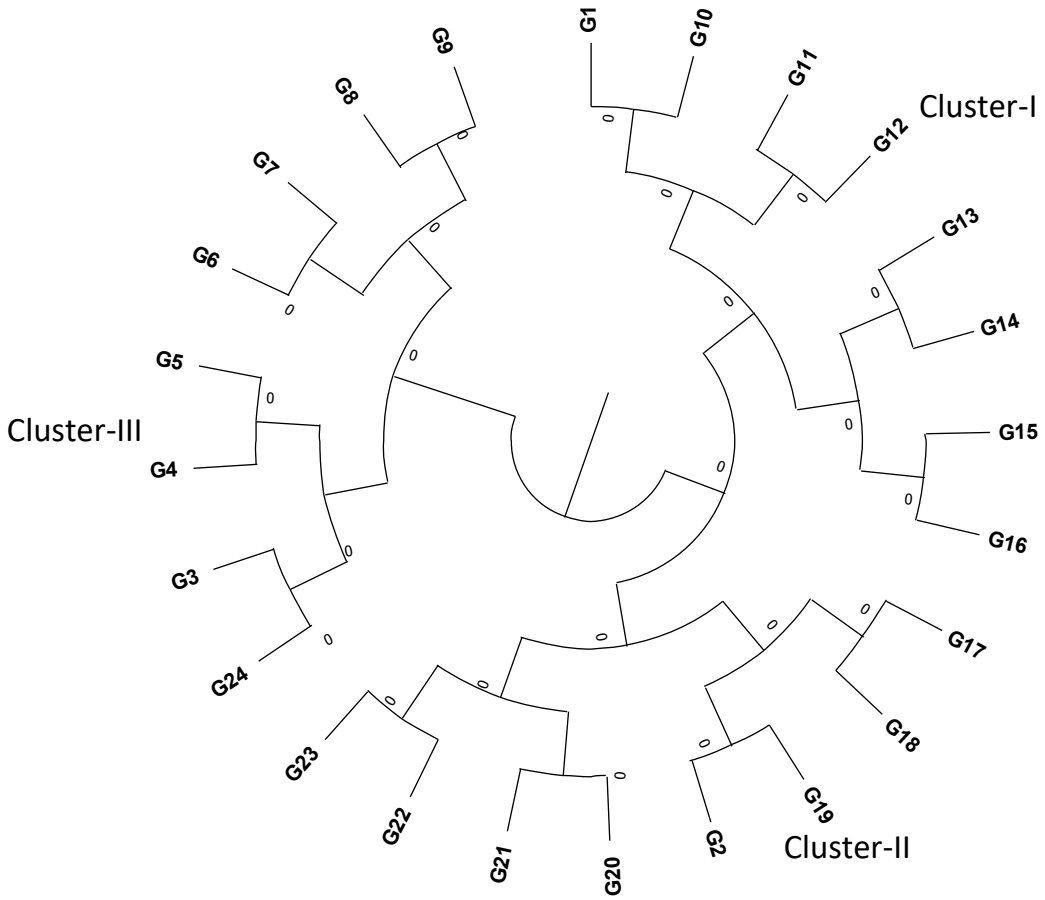


Figure 13. UPGMA based molecular dendogram based on Nei's (1983) genetic distance, showing 24 Aman rice genotypes using MEGA 5.1 version

Table 13. List of germplasm with their cluster based on UPGMA dendrogram

Cluster	Sub cluster	Germplasm
I	1	G1 (Kali jira (3)), G10 (Sham rush),G11 (Dudh Kalam),G12(Dudh kalam)
	2	G13 (Boro Dudh Kalam), G14, (Lal Soru), G15(Gojol Goria),G16(Sojoni)
II	1	G17(Ganjia),G18(Bindi pakri),G19(Jhoshua),G2(Telot)
	2	G20(Akand sail),G21(Lal dupa),G22(Jiga sail),G23(Cheng sail)
III	1	G24(Shul kumor) , G3(Bazail), G4(Joli amon), G5(Bazail
	2	G6(Bazail), G7(Kancha noni), G8(Naria bochi), G9 (Khirsha bhog)

CHAPTER V
SUMMARY AND CONCLUSION

The present study, morpho-molecular characterization of aman rice germplasm was carried out by using 24 rice germplasm with the objective of their morphological characterization at qualitative and quantitative level. Apart from morphological descriptors analysis, molecular markers were also applied in order to characterize these rice germplasm. The experiment was conducted in the Genetic Resources and Seed Division (GRSD), Bangladesh Rice Research Institute (BRRI), Gazipur.

Qualitative characters are considered as morphological markers in the identification of germplasm accessions of rice. In the present investigation, among the ten qualitative characters observed, blade color, basal leaf sheath color, awn distribution, apiculus color, lemma and palea color, seed coat color, leaf senescence recorded highest variation among accessions. Out of 10 quantitative traits observed, effective tiller number, panicle length (cm), plant height (cm), days of flowering, days of maturity, field grains per panicle, grain length (mm), grain breadth(mm),1000 grain weight (g),yield per hill(g) showed most variation among the genotypes. For establishing morphological based dendrogram quantitative traits were used. Clustering performed on the Euclidean similarity index method. Here 24 rice genotypes formed total three clusters. Distribution pattern indicated minimum number (1) of genotypes were included in cluster I and the maximum (18) in cluster II. Cluster III consisted of 5 genotypes.

Twenty four aman rice germplasm were used for PCR amplification. Sixteen SSR markers were applied for molecular diversity study. A total of 66 alleles were identified by the 16 SSR primer. The average number of allele per locus was 4.13 that was ranged from 2.00 (RM 411 and RM 455) to 8.00 (RM206). A total of 17 unique alleles were detected. The gene diversity ranged from 0.08 to 0.83(maximum to minimum), with an average of 0.52. Total gene diversity is 8.40. The frequency of the most common allele at each locus ranged from 25.00% (RM206) to 95.83% (RM455). On average, 61.20% of the 24 rice germplasm shared a common major allele. The polymorphism information content PIC value for 16 SSR marker ranged from 0.08 (RM455) minimum to 0.80 (RM206) maximum. The average PIC value is 0.48 and total PIC value is 7.76.

The highest PIC value was obtained from RM206 (0.80). So primer RM206 is supposed to be the best marker for characterizing the 24 T.aman rice germplasm due to show highest diversity

Nei's (1983) Genetic Distance showed pair-wise comparisons of among 24 T.aman rice genotypes. Genetic distance ranged from 0.1250 to 0.9375. Among them Naria bochi (G8) vs (G23) showed highest genetic dissimilarity. The lowest Nei's genetic distance (0.1250) was observed in Ganjia (G17) vs.Sojoni.

Molecular based UPGMA dendrogram was established with 24 T.Aman rice germplasm. (UPGMA) indicated the segregation of 24 rice genotypes into three main clusters: G1,G10, G11, G12,G13,G14,G15,G16 were grouped in cluster I; 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.

Both morphological and molecular based dendrogram showed considerable differences. At morphological based dendrogram three cluster was identified which has many more sub-cluster. Cluster II showed highest number of germplasm (18). These were genetically similar in group. At molecular analysis three cluster was observed with a total six sub-cluster

The use of SSR markers has played an increasing role in rice breeding and genetics. SSR markers are the powerful tools to detect genetic variation and genetic relationship within and among different rice genotypes. For the detection of genetic diversity, genome mapping, DNA fingerprinting, determine the genetic structure SSR markers are used thoroughly. The study result can become a guideline for further research on breeding programme, variety improvement and genetic variation analysis of rice in Bangladesh.

RECOMMENDATIONS

The results obtained from this study on morphological and molecular characterization provided some important information. It helps in establishment of sovereignty of Bangladeshi Aman rice gene pool. The results revealed that there was a high level of genetic diversity among accessions of Aman rice. It is clear that SSR markers were effective tool in the detection of polymorphism. The following points might be considered for sustaining the genetic qualities of Aman rice in Bangladesh:

i. The newly developed data from morphological and molecular characterization would be useful for selecting source materials in future breeding program.
ii. Further work to be carried out on molecular basis by using SNP markers to carry out fingerprinting study.

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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 H2O	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
DDH2O	92mL

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

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

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

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

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

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

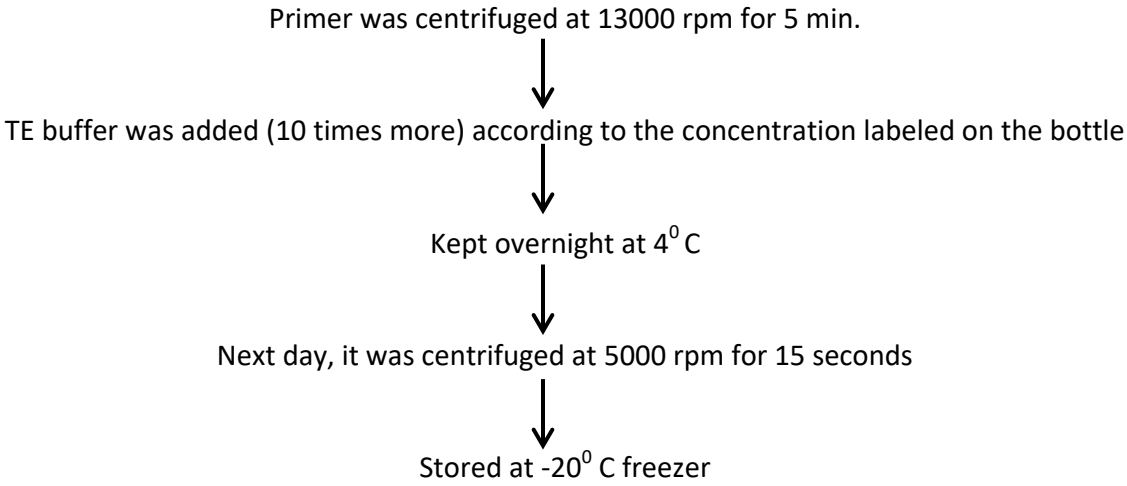
I(f): Composition and preparation of the 1X TBE buffer

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

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

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

I(h): Primer dilution



I(i): PCR cocktail for 96 samples

Reagent	**Amount**
DNA	288
Primer (F)	48
Primer (R)	48
Master mix	432
DDH2 O	144

Appendix II: SSR marker analysis related data

Marker	Major.Allele. Frquency	%	Genotype No	Sample Size	No. of obs.	Allele No	Availability	Gene Diversity	Heterozygosity	PIC
RM1	0.3750	37.5000	5.0000	24.0000	24.0000	5.0000	1.0000	0.7396	0.0000	0.6970
RM5	0.7083	70.8333	4.0000	24.0000	24.0000	4.0000	1.0000	0.4653	0.0000	0.4316
RM26	0.8333	83.3333	3.0000	24.0000	24.0000	3.0000	1.0000	0.2882	0.0000	0.2640
RM190	0.5000	50.0000	4.0000	24.0000	24.0000	4.0000	1.0000	0.6354	0.0000	0.5730
RM206	0.2500	25.0000	8.0000	24.0000	24.0000	8.0000	1.0000	0.8299	0.0000	0.8080
RM207	0.5417	54.1667	6.0000	24.0000	24.0000	6.0000	1.0000	0.6528	0.0000	0.6194
RM224	0.5000	50.0000	4.0000	24.0000	24.0000	4.0000	1.0000	0.6354	0.0000	0.5730
RM228	0.7083	70.8333	3.0000	24.0000	24.0000	3.0000	1.0000	0.4549	0.0000	0.4104
RM253	0.5417	54.1667	5.0000	24.0000	24.0000	5.0000	1.0000	0.6458	0.0000	0.6077
RM447	0.6250	62.5000	4.0000	24.0000	24.0000	4.0000	1.0000	0.5486	0.0000	0.4996
RM455	0.9583	95.8333	2.0000	24.0000	24.0000	2.0000	1.0000	0.0799	0.0000	0.0767
RM3646	0.3750	37.5000	5.0000	24.0000	24.0000	5.0000	1.0000	0.7361	0.0000	0.6923
RM205	0.8333	83.3333	4.0000	24.0000	24.0000	4.0000	1.0000	0.2951	0.0000	0.2806
RM277	0.7500	75.0000	4.0000	24.0000	24.0000	4.0000	1.0000	0.4132	0.0000	0.3856
RM411	0.7500	75.0000	2.0000	24.0000	24.0000	2.0000	1.0000	0.3750	0.0000	0.3047
RM273	0.5417	54.1667	3.0000	24.0000	24.0000	3.0000	1.0000	0.6007	0.0000	0.5331
Mean	0.6120	61.1979	4.1250	24.0000	24.0000	4.1250	1.0000	0.5247	0.0000	0.4848
Max	0.9583	95.8333	8.0000	24.0000	24.0000	8.0000	1.0000	0.8299	0.0000	0.8080
Min	0.2500	25.0000	2.0000	24.0000	24.0000	2.0000	1.0000	0.0799	0.0000	0.0767
Total	9.7917	979.1667	66.0000	384.0000	384.0000	66.0000	16.0000	8.3958	0.0000	7.7567

Appendix II: Similarity and distance indices below data

OTU	G1	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G2
	G20	G21	G22	G23	G24	G3	G4	G5	G6	G7	G8	G9
G1	0.0	0.6875	0.6875	0.7500	0.6875	0.7500	0.6875	0.6250	0.6250	0.5625	0.6250	0.3750
	0.5625	0.5625	0.8125	0.7500	0.6875	0.5625	0.5000	0.8750	0.6875	0.6875	0.6250	0.6875
G10	0.6875	0.0	0.5000	0.3750	0.4375	0.5000	0.3750	0.3750	0.3750	0.3750	0.5625	0.6250
	0.3125	0.1875	0.5625	0.5000	0.3750	0.6250	0.4375	0.4375	0.5000	0.5625	0.6875	0.5625
G11	0.6875	0.5000	0.0	0.5000	0.1875	0.3750	0.5625	0.5000	0.5000	0.5000	0.4375	0.5000
	0.4375	0.5000	0.5625	0.5000	0.5625	0.5625	0.4375	0.5000	0.6250	0.6250	0.6250	0.5000
G12	0.7500	0.3750	0.5000	0.0	0.5625	0.6250	0.3750	0.3750	0.3750	0.5000	0.6250	0.5625
	0.4375	0.5000	0.6250	0.5625	0.5625	0.5000	0.3125	0.2500	0.3125	0.5625	0.8125	0.4375
G13	0.6875	0.4375	0.1875	0.5625	0.0	0.3750	0.5000	0.5625	0.5625	0.4375	0.5625	0.5625
	0.5000	0.5000	0.5000	0.5000	0.4375	0.6250	0.5000	0.6250	0.7500	0.6875	0.6250	0.5000
G14	0.7500	0.5000	0.3750	0.6250	0.3750	0.0	0.5000	0.5000	0.5625	0.4375	0.6250	0.6875
	0.4375	0.5000	0.5000	0.5625	0.3750	0.5625	0.6250	0.6250	0.7500	0.7500	0.6875	0.7500
G15	0.6875	0.3750	0.5625	0.3750	0.5000	0.5000	0.0	0.4375	0.3750	0.2500	0.6875	0.6875
	0.4375	0.4375	0.5625	0.3750	0.3750	0.7500	0.5000	0.4375	0.4375	0.6875	0.8125	0.6250
G16	0.6250	0.3750	0.5000	0.3750	0.5625	0.5000	0.4375	0.0	0.1250	0.3750	0.3750	0.4375
	0.1875	0.3750	0.6875	0.4375	0.3125	0.6250	0.3750	0.4375	0.5000	0.4375	0.8125	0.5000
G17	0.6250	0.3750	0.5000	0.3750	0.5625	0.5625	0.3750	0.1250	0.0	0.3125	0.4375	0.5000
	0.2500	0.3750	0.6875	0.3750	0.3750	0.6250	0.4375	0.4375	0.4375	0.5000	0.8125	0.5000
G18	0.5625	0.3750	0.5000	0.5000	0.4375	0.4375	0.2500	0.3750	0.3125	0.0	0.6250	0.6250
	0.3125	0.3750	0.6250	0.3750	0.2500	0.6250	0.4375	0.5625	0.5625	0.6875	0.7500	0.6250
G19	0.6250	0.5625	0.4375	0.6250	0.5625	0.6250	0.6875	0.3750	0.4375	0.6250	0.0	0.5625
	0.4375	0.5625	0.6875	0.5625	0.6250	0.6875	0.6250	0.6250	0.6250	0.5625	0.7500	0.6875
G2	0.3750	0.6250	0.5000	0.5625	0.5625	0.6875	0.6875	0.4375	0.5000	0.6250	0.5625	0.0
	0.6250	0.5625	0.6875	0.7500	0.6875	0.3750	0.3750	0.6875	0.6250	0.6250	0.7500	0.6250
G20	0.5625	0.3125	0.4375	0.4375	0.5000	0.4375	0.4375	0.1875	0.2500	0.3125	0.4375	0.6250
	0.0	0.2500	0.6250	0.3750	0.2500	0.6250	0.4375	0.4375	0.5000	0.5625	0.7500	0.5625
G21	0.5625	0.1875	0.5000	0.5000	0.5000	0.5000	0.4375	0.3750	0.3750	0.3750	0.5625	0.5625
	0.2500	0.0	0.5000	0.4375	0.3750	0.5000	0.3750	0.5625	0.6250	0.5625	0.6875	0.5625

CON……

G22	0.8125	0.5625	0.5625	0.6250	0.5000	0.5000	0.5625	0.6875	0.6875	0.6250	0.6875	0.6875
	0.6250	0.5000	0.0	0.7500	0.5625	0.6875	0.6875	0.5625	0.6875	0.6875	0.6875	0.8125
G23	0.7500	0.5000	0.5000	0.5625	0.5000	0.5625	0.3750	0.4375	0.3750	0.3750	0.5625	0.7500
	0.3750	0.4375	0.7500	0.0	0.4375	0.7500	0.6250	0.5625	0.5625	0.5625	0.9375	0.5625
G24	0.6875	0.3750	0.5625	0.5625	0.4375	0.3750	0.3750	0.3125	0.3750	0.2500	0.6250	0.6875
	0.2500	0.3750	0.5625	0.4375	0.0	0.5625	0.5000	0.5000	0.6875	0.6250	0.6875	0.6250
G3	0.5625	0.6250	0.5625	0.5000	0.6250	0.5625	0.7500	0.6250	0.6250	0.6250	0.6875	0.3750
	0.6250	0.5000	0.6875	0.7500	0.5625	0.0	0.4375	0.6250	0.7500	0.6250	0.7500	0.6875
G4	0.5000	0.4375	0.4375	0.3125	0.5000	0.6250	0.5000	0.3750	0.4375	0.4375	0.6250	0.3750
	0.4375	0.3750	0.6875	0.6250	0.5000	0.4375	0.0	0.5000	0.5000	0.5000	0.6250	0.5625
G5	0.8750	0.4375	0.5000	0.2500	0.6250	0.6250	0.4375	0.4375	0.4375	0.5625	0.6250	0.6875
	0.4375	0.5625	0.5625	0.5625	0.5000	0.6250	0.5000	0.0	0.2500	0.5625	0.7500	0.6250
G6	0.6875	0.5000	0.6250	0.3125	0.7500	0.7500	0.4375	0.5000	0.4375	0.5625	0.6250	0.6250
	0.5000	0.6250	0.6875	0.5625	0.6875	0.7500	0.5000	0.2500	0.0	0.6875	0.8750	0.6250
G7	0.6875	0.5625	0.6250	0.5625	0.6875	0.7500	0.6875	0.4375	0.5000	0.6875	0.5625	0.6250
	0.5625	0.5625	0.6875	0.5625	0.6250	0.6250	0.5000	0.5625	0.6875	0.0	0.7500	0.5000
G8	0.6250	0.6875	0.6250	0.8125	0.6250	0.6875	0.8125	0.8125	0.8125	0.7500	0.7500	0.7500
	0.7500	0.6875	0.6875	0.9375	0.6875	0.7500	0.6250	0.7500	0.8750	0.7500	0.0	0.8750
G9	0.6875	0.5625	0.5000	0.4375	0.5000	0.7500	0.6250	0.5000	0.5000	0.6250	0.6875	0.6250
	0.5625	0.5625	0.8125	0.5625	0.6250	0.6875	0.5625	0.6250	0.6250	0.5000	0.8750	0.0
MAX	0.8750	0.6875	0.6875	0.8125	0.7500	0.7500	0.8125	0.8125	0.8125	0.7500	0.7500	0.7500
	0.7500	0.6875	0.8125	0.9375	0.6875	0.7500	0.6875	0.8750	0.8750	0.7500	0.9375	0.8750
AVR	0.6276	0.4557	0.4870	0.4792	0.5078	0.5443	0.4948	0.4323	0.4401	0.4661	0.5651	0.5625
	0.4297	0.4531	0.6146	0.5339	0.4766	0.5885	0.4714	0.5182	0.5651	0.5833	0.7135	0.5833
	0.5247											