MOLECULAR CHARACTERIZATION AND GENETIC DIVERSITY ANALYSIS OF BORO RICE GERMPLASM USING SSR MARKERS

A THESIS

BY

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CERTIFICATE

*This is to certify that the thesis entitled "**MOLECULAR CHARACTERIZATION AND GENETIC DIVERSITY ANALYSIS OF BORO RICE GERMPLASM USING SSR MARKERS**" 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 **FARHANA AFRIN VABNA,** Registration no.**11-04439** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

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

Dated: June, 2018

Place: Dhaka, Bangladesh

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DEDICATED TO

MY

BELOVED PARENTS

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ABSTRACT

An experiment was carried out with twenty four Boro rice germplasm at the laboratory of Genetic Resources and Seed Division (GRSD), Bangladesh Rice Research Institute (BRRI), Joydebpur, Gazipur, during the period of November 2017 to March 2018 to investigate molecular characterization and genetic diversity analysis. Rice genome specific twelve well known SSR primers were selected and synthesized for molecular diversity analysis. All of the twelve primers were able to produce polymorphic bands. The highest (8) number of bands was observed in SSR primers RM206. Fifty four alleles, ranged from 1 to 8 alleles per locus and an average of 4.5 were detected. Forty five polymorphic alleles were also identified. The Polymorphic Information Content (PIC) of SSR markers ranged from 0.08 (RM447) to 0.84 (RM206) with an average value of PIC = 0.49. Gene diversity ranges from 0.08 (RM447) to 0.86 (RM206) with an average value of 0.52. The RM206 marker can be considered as the best marker among the studied markers for 24 rice germplasms. Six primers showed high diversity, three primers showed moderate diversity and three primers showed low diversity on the basis of PIC value. The value of pair-wise comparisons of Nei's genetic distance (D) among 24 rice genotypes was computed from combined data for the 12 primers, ranged from 0.1667 to 0.9167 with an average of 0.5417. Dendrogram based on Nei's genetic distance using Unweighted Pair Group Method of Arithmetic Mean (UPGMA) indicated the segregation of 24 genotypes into three main clusters. This result can be conveniently used for molecular diversity analysis of rice genotypes to identify diverse parent for the development of high yielding variety in rice.

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ABBREVIATIONS AND ACRONYMS

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

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

CHAPTER I

INTRODUCTION

Rice (*Oryza sativa* L.) is a self-pollinated cereal crop belonging to the family Gramineae having chromosome number 2n=24 (Hooker, 1979). It is a monocot which is normally grown as an annual plant. It has been cultivated in Asia and Africa since ancient time. Rice (*Oryza sativa* L.) has been cultivated for an estimated 10,000 years (Liu *et al*., 2007). 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). It is assumed that there might have been two centres of origin of our cultivated rice; South-eastern Asia and West Africa.

At present, rice is the most widely consumed staple food for a large part of the world's human population. The major rice producing countries are China, India, Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Pakistan, Philippines, Korea and Japan. Asian farmers still account for production of 87% of the world's total rice. Now, 90 percent of world rice is produced in Asia on an area of almost 150 million hectares. Rice accounts for 50 % of agricultural income in Asia and supplies nutrition almost 80% of the planet. Rice being staple food constitutes over 90% of the food grain production in Bangladesh. About 75% of the total cropped land is covered by rice and more than 60% of the total agriculture labor force is employed in rice production, processing, marketing and distribution. 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).

The current world population of 7.6 billion is anticipated to reach 8.6 billion in 2030, 9.8 billion in 2050. A total of 83 million people is expected to be added in every year (UN DESA, 2017). World food production must increase to 70 percent more to feed this huge sum of the population (Hunter, 2017). The population of Bangladesh is also increasing at an alarming rate. With annual population growth of 1.8 million people, Bangladesh

requires approximately an additional 300,000 metric tons of rice every year. So, the highest priority has been given on rice production to meet up the demand.

Rice provides 20% of the world's dietary energy supply. Cooked, un-enriched, white, long-grained rice is composed of 68% water, 28% carbohydrates 3% protein and negligible fat. The health benefits of rice include its ability to provide instant energy, regulate and improve bowel movements, stabilize blood sugar levels and slow down the aging process. It also plays a role in providing vitamin B1 to the human body.

In Bangladesh, rice is regarded as the most important cereal as it is the staple food. In 2016, Bangladesh has ranked 4th position in rice production in the world (FAO, 2016). Despite the annual production shortfall of 2 to 4 million metric tons, rice provides more than 80% of the food requirements for the common people of Bangladesh (Jalaluddin *et al.*, 2007). Rice is grown in three overlapping seasons namely Aus, Aman and Boro. In 2016-2017, a total of 33804000 Metric tons rice was produced in 27,184000 acres of land. Of which 18,014000 metric tons was Boro rice grown in 11,060000 acres of land (BBS, 2017). Most of the requirement is being fulfilled by Boro varieties.

More than 1,27,000 rice accessions and wild relatives can be found in the world's largest gene bank for rice at International Rice Research Institute (IRRI) located in the Philippines (http://irri.org/our-work/research/genetic-diversity). Until now, Bangladesh Rice Research Institute (BRRI) has collected and preserved a Gene bank of about 8700 varieties/landraces/ cultivars/wild types from indigenous and exotic sources. Out of these, more than 8500 germplasm have been registered in BRRI Gene bank. Among these 24 Boro rice germplasm have been taken for molecular characterization.

Genetic diversity measured through morphological differences of quantitative important traits has some disadvantages in terms of time, space, cost involved and environmental factors. Molecular characterization by using DNA marker gives more precise, convenient and reliable results for genetic variability assessment. The use of DNA markers has been suggested for precise and reliable characterization and discrimination of rice genotypes (Karkousis *et al*., 2003). DNA markers are extensively used because of their advantages of other markers as they are technically simple, time saving, highly informative and

require small amount of DNA and independence from effects related to environmental conditions and the physiological stage of plant.

Among the PCR-based markers, the SSR markers have proved to be very effective tools in the study of genetic diversity and genetic relationships within and among the species. The SSR markers are highly polymorphic, highly transferable, abundant in eukaryotic organisms and well distributed throughout the genome. They are easily amplified by PCR reactions using DNA nucleotide primers, the unique sequences flanking the repeat motifs.

SSR markers have been widely applied in the genetic diversity analysis, genotypic identification and population structure estimation in several rice genetic studies (Salgotra *et al*., 2015). Genetic diversity in any crop is basically important for improving heterotic crop genotypes over the existing ones. Therefore, research emphasis has been given on genetic diversity for microsatellite DNA markers in local Boro rice landraces. The present study was designed with 24 Boro rice germplasm using molecular marker for characterization and diversity analysis.

OBJECTIVE(S)

With some consideration, the main objectives of this research were:

(1) To assess the polymorphism and molecular diversity of 24 Boro rice landraces using SSR markers.

(2) To establish dendrogram for classifying genotypes in different groups based on their genetic distances.

(3) To determine the genetic relationship among the Boro rice germplasm.

CHAPTER II

REVIEW OF LITERATURE

Rice is the main food for more than half of the world's population and is the staple food for the people of Bangladesh. Molecular characterization and genetic diversity analysis of rice is very important for hybrid variety development and other improvement program. Molecular markers are the powerful tools to detect genetic variation and genetic relationship within and among species. DNA markers are unmasking new genes for the improvement of crop varieties (Causse *et al.,* 1994). Studies on genetic variation and molecular characterization of different rice genotypes using Simple Sequence Repeats (SSR) markers have been carried out throughout the world. Therefore, the literature relevant to the present study on rice and their improved varieties of Bangladesh as well as in abroad are reviewed in this chapter under the following headings:

Aljumaili *et al*., (2018) carried out experiment on the genetic diversity among the 50 aromatic rice accessions from three regions (Peninsular Malaysia, Sabah, and Sarawak) with 3 released varieties as a control using the 32 simple sequence repeat (SSR) markers. Genetic diversity index among the three population ranged from 0.25 in control to 0.98 in Sabah population. The mean numbers of effective alleles and Shannon's information index were 0.36 and 64.90%, respectively. Nei's gene diversity index was 0.36. The dendrogram based on UPGMA and Nei's genetic distance classified the 53 rice accessions into 10 clusters. Analysis of molecular variance (ANOVA) revealed that 89% of the total variation observed in this germplasm came from within the populations, while 11% of the variation emanated among the populations. These results reflect the high genetic differentiation existing in this aromatic rice germplasm.

Islam *et al*., (2018) evaluated fifty red rice germ- plasm from the Bangladesh Rice Research Institute (BRRI) genebank. The genotypes were characterized both morphologically and genetically using fifty simple sequence repeat (SSR) markers.

Overall, 162 alleles were detected of which 22 were unique alleles. The highest and lowest polymorphic information content (PIC) indices were 0.75 and 0.04 found in markers RM282 and RM304 respectively. Genetic diversity was moderate, varying from 0.05 to 0.78 (average: 0.35). They suggested that the diverse germplasm and polymorphic trait-linked SSR markers of red rice are suitable for the detection of economically desirable trait loci/genes for use in future molecular breeding programs.

Chen *et al*., (2017) used 30 polymorphic SSR markers to assess the genetic diversity and molecular fingerprints of 53 rice genotypes of O. sativa, O. glaberrima, and NERICA. In total, 180 alleles were detected with average polymorphism information content and Shannon's information index were 0.638 and 1.390, respectively. Population structure and neighbor-joining phylogenetic tree revealed that 53 genotypes grouped into three distinct subpopulations conforming to the original three groups, except three varieties (IR66417, WAB450-4, MZCD74). NERICA showed a smaller genetic distance from *O. sativa* genotypes (0.774) than from *O. glaberrima* genotypes (0.889).

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 alleles per locus with mean Polymorphic Information Content (PIC) value of 0.29.

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

Ahmed *et. al*., (2016) evaluated a total of 350 alleles varied from 3 (RM277) to 14 (RM21) with an average of 7.8 per locus.45 microsatellite loci were detected across the

31 rice accessions. The gene diversity ranged from 0.48 to 0.90 with an average of 0.77.Again, the polymorphism information content values from 0.44 (RM133) to 0.89 (RM206) with an average of 0.74.

Siddique *et al*., (2016) evaluated 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. The allele frequency ranged from 8.33% (RM163) to 22.92% (RM60, RM125) with an average of 15.89. The UPGMA dendrogram based on Nei's genetic distance revealed seven distinct clusters with a similarity coefficient of 0.09.

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

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

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

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

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

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

A total of 24 SSR markers were used 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. The frequency of most common allele at each locus ranged from 41% (RM163, RM590, and RM413) to 91% (RM510) (Sajib *et al*., 2012)

The genetic diversity of 21 rice cultivars were evaluated using 34 microsatellite primer pairs. The number of alleles per locus ranged from 2 to 11 with an average of 4.18 alleles

across 34 loci. A total of 57 rare alleles were detected at 24 loci, whereas 42 unique alleles were identified at 20 loci. Polymorphic information content (PIC) values ranged from 0.157 to 0.838 with an average of 0.488 which revealed that much variation was present among the studied varieties. The PIC values revealed that RM401 might be the best marker for identification and diversity estimation of rice varieties. (Rahman *et al.* 2012).

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

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

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

Kibria *et al*., (2009) reported the genetic diversity among aromatic rice genotypes using simple sequence repeat (SSR) and randomly amplified polymorphic DNA (RAPD) markers through marker aided selection (MAS).Three SSR primers (RM223, RM342A and RM515) exhibited forty six bands among the genotypes and the average number of effective allele ranged from 1.78 to 2.49. The highest polymorphism (66.67%) was showed by the marker RM223.

A set of 48 simple sequence repeat (SSR) markers were used to assess the genetic diversity of 11 Venezuelan rice cultivars. The average genetic diversity (H) over all SSR

loci for the 18 genotypes was 0.524, ranging from 0.105 to 0.815. UPGMA cluster analysis based on genetic distance coefficients showed that the Venezuelan rice varieties are closely related (Herrera *et.*, 2008).

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

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

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

tropical japonica gene diversity of 0.56 and Fast of 0.38 between the two groups. All of the improved varieties sampled were indica and had an average gene diversity of 0.46.

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

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

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

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

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

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

CHAPTER III

MATERIALS AND METHODS

The chapter constitutes the materials and methods used in the experiment. The details of the methodology have been followed is described below:

3.1 Experimental site and time duration

To achieve the objectives, the experiment was conducted at the Genetic Resources and Seed Division (GRSD), Bangladesh Rice Research Institute (BRRI), Joydebpur, Gazipur -1701. The experiment was carried out during the period of November 2017 to March 2018.

3.2 Collection of materials

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

3.3 Collection of leaf samples

Leaf samples were collected from young green leaves from the rice plants at 10-15 DAT (Days after transplanting). About 3 cm long leaf tips were collected from the plants and kept inside 1.5 ml microfuge tubes. The ice buckets were carried to the transplanting field and the microfuge tubes containing leaf samples were immediately preserved in ice. The microfuge tubes containing the leaf samples were kept in poly bags and placed in the chamber of -80°C freezer. The leaf samples were crushed immediately for DNA extraction. Before collecting leaf samples, microfuge tubes were labeled properly.

Table 1: Name of the Boro rice germplasm with their accession number

Sl. No.	Name	Accession No.	Cropping Season	Collected from	
				Upazila/ Division	District
1.	Kalo Boro	7663	Boro	Sadar	Barguna
2.	Asami Boro	7667	Boro	Sadar	Satkhira
3.	Pabdafor	7668	Boro	Sadar	Priojpur
4.	Amania	7669	Boro	Sadar	B. Baria
5.	Khaiya Boro	7671	Boro	Sadar	Moulovibajar
6.	Rata	7676	Boro	Sadar	Sunamgonj
7.	Lakhai Boruni	7677	Boro	Sadar	Sylhet
8.	Sada Boro	7679	Boro	Sadar	Narayangonj
9.	Sona Biron	941	Boro	Sadar	Sylhet
10.	Rajkumar	8052	Boro	Potia	Chittagong
11.	Dol Boro	8053	Boro	Belabo	Narsingdi
12.	Boro Dhan (Indian)	8055	Boro	Kumarkhali	Kushtia
13.	Tepi Boro Dhan (Kalo)	8058	Boro	Kumarkhali	Kushtia
14.	Dhali Boro 7/2	2243	Boro	PBD	Gazipur
15.	Black Rice	8096	Boro	PBD	Gazipur
16.	Dhali Boro 74/3	2244	Boro	PBD	Gazipur
17.	Boro Habj1	8100	Boro	Sadar	Netrokona
18.	Vawailia	8103	Boro	Savar	Dhaka
19.	Kali Boro	8104	Boro	Savar	Dhaka
20.	Jira	NC	Boro	Sadar	Natore
21.	Dhali Boro 87/1	2245	Boro	PBD	Gazipur
22.	Dhali Boro 94	2246	Boro	PBD	Gazipur
23.	Dhali Boro 104/1	2247	Boro	PBD	Gazipur
24.	Dhali Boro 105/2	2248	Boro	PBD	Gazipur

3.4 Reagent preparation 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). 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 H_2O-DNA) to a working concentration of 50 ng/µl and stored at 4°C for PCR based marker analysis.The detailed laboratory procedures of this method including chemical preparation are described as given follow.

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

Tris is available in different forms *viz*. Tris HCL, Trisma base, etc. The formula weight (FW) of Trisma base is 121.14 with chemical formula $C_4H_{11}NO_3$. The required concentration for this chemical is 1 M with pH=8.0. The main functions of Tris are to maintain pH of the DNA solution and to provide buffering capacity. Tris performs the important operation by keeping pH steady at 8.0. However, for an example, 250 ml 1M Tris with pH 8.0 can be prepared as follows:

Here, We Know,

N= CV (N= mole number, C= Conc. in Molar, V= Volume in liter)

i.e. N =1 M X 250 ml= 1 M X 0.25 liter = 0.25

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

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

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

When dissolved in water Na_2EDTA makes the solution acidic. The formula weight (FW) of the chemical is 372.24 with the chemical formula $C_{10}H_{14}N_2Na_2O_8.2H_2O$. The required concentration is 0.5 M with pH=8.0. The Na_2EDTA acts as chelating agent which chelates inorganic or metal ion. It deactivates endonuclease enzyme by chelating with its

co-factor (e.g. Mg^{++}). However, for an example, 100 ml 0.5 M Na_2EDTA with pH 8.0 can be prepared as follows:

Here, We Know,

N= CV (N= mole number, C= Conc. in Molar, V= Volume in liter)

i.e. N =0.5 M X 100 ml= 0.5 M X 0.1 l = 0.05

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

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

3.4.3 NaCl (5M NaCl solution)

The formula weight (FW) of this chemical is 58.44 with the chemical formula NaCl. The required concentration is 5 M. NaCl digest cellular components and helps to burst out cell wall, cell membrane through generating osmotic pressure. However, for an example, 250 ml 5 M NaCl can be prepared as follows:

Here We Know,

N= CV (N= Mole number, C= Conc. in Molar, V= Volume in liter)

i.e. N =5 M X 250 ml= 5 M X 0.25 l = 1.25

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

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

3.4.4 SDS (Sodium Dodecyl Sulphate) solution

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

3.4.5 Chloroform

This chemical available as it is in the market is used in the extraction method. The function of this chemical is to disrupt two-dimensional structure of protein forming its precipitation. This chemical was used under fume hood and was not inhaled.

3.4.6 Ethanol

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

3.4.7 1X TE Buffer

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

1 M Tris pH 8.0	10 ml
0.5 M Na_2EDTA pH 8.0	200 µl

Finally water is added up to 100 ml. This chemical redissolves DNA into solution and acts as DNA preserving solution.

3.4.8 Extraction buffer (200ml)

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

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

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

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

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

3.4.11 3.5M NaCl (250 mL)

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

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

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

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

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

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

At first 5 mL phenol was taken in a 100mL volumetric flask. Then 91.2 mL Chloroform and 3.8mL Isoamyl alcohol was added and mixed well. The solution was stored at 4^0C.

3.4.15 10X TBE Buffer (1000mL)

108g Tris-HCL was taken in a volumetric flask (1000mL).Then, 9.3g of EDTA and 55g Boric acid was added. Sterilized dH_2O was added to make the volume 1000mL.

3.4.16 1X TBE buffer

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

3.4.17 1% PVP

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

3.4.18 70% ethanol (1000mL)

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

3.5 Sequential steps for DNA extraction from leaf sample of Boro rice

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

1. For genomic DNA extraction, young, vigorous, actively growing leaf tissues were collected from 24 different Boro rice germplasm.

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

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

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

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

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

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

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

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

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

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

3.6 Synthesis of SSR markers

Rice genome specific 12 well known SSR primers viz. RM1, RM 206, RM 207, RM 253, RM 304, RM 252, RM 1337, RM 320, RM 205, RM 447, RM 3646, RM 413 were selected and synthesized for molecular diversity analysis in 24 Boro rice germplasm. A list of the primer used is given below:

Table 2: List of SSR markers used for diversity analysis of Boro rice germplasm

Sl. No.	Primer Name	Location in Chromosome	Forward primer (5′–3′)	Reverse primer (5′–3′)	Annealing Temp.
1	**RM1**	1	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC	55
2	**RM206**	11	CCCATGCGTTTAACTATTCT	CGTTCCATCGATCCGTATGG	56
3	**RM207**	2	CCATTCGTGAGAAGATCTGA	CACCTCATCCTCGTAACGCC	55
4	**RM253**	6	TCCTTCAAGAGTGCAAAACC	GCATTGTCATGTCGAAGCC	54
5	**RM304**	10	TCAAACCGGCACATATAAGAC	GATAGGGAGCTGAAGGAGAG	54
6	**RM252**	4	TTCGCTGACGTGATAGGTTG	ATGACTTGATCCCGAGAACG	55
7	**RM1337**	12	GTGCAATGCTGAGGAGTATC	CTGAGAATCTGGAGTGCTTG	55
8	**RM320**	7	CAACGTGATCGAGGATAGATC	GGATTTGCTTACCACAGCTC	54
9	**RM205**	9	CTGGTTCTGTATGGGAGCAG	CTGGCCCTTCACGTTTCAGTG	56
10	**RM447**	8	CCCTTGTGCTGTCTCCTCTC	ACGGGCTTCTTCTCCTTCTC	56
11	**RM3646**	3	ACTAGAGCACCCTCGCTGAG	CTCAGCCACCCCATCAAC	55
12	**RM413**	5	GGCGATTCTTGGATGAAGAG	TCCCCACCAATCTTGTCTTC	55

3.7 Amplification of SSR markers by PCR

3.7.1 Principle of the amplification of SSR marker

Microsatellites or SSR are tandem repeats of 1-6 nucleotides. For example, $(A)_{n,}$ $(AT)_{n,}$ $(ATG)_n$,$(GATT)_n$, $(CTACG)_n$, $(TACGAC)_n$ and so on. They are abandoned in genomes of all organisms. The sequence of unique flanking regions of SSR can be used to design primers and carry out PCR to amplify SSR containing sequences. SSRs were first used as markers for use in genetic mapping bin humans (Litt and Luty, 1989).

3.8 Polymerase chain reaction (PCR) amplification

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

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

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

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

Table 4 : Temperature Profile of PCR (Easy shortcut new method)

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

3.9 Polyacrylamide Gel Electrophoresis (PAGE)

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

3.9.1 Assembling of Glass Plates

1. Two glass plates, two spacers and one comb were washed properly using laboratory detergent (bleaching powder based) and rinsed with water. Glass plates were also washed by 0.5 M NaOH solution. Glass plates were air dried and chosen inner surfaces of the plate was sprayed with 100% ethanol and wiped with lint-free tissue.
2. The short plate (round-bottom) was hold and the rubber gasket was attached starting from one side of the plate. The notches on the gasket were aligned on the corners. The circular portion of the gasket was exposed to the inner side of the plate.
3. The short plate was lain on the table with the inner side up. Then, the spacers were put along the inside edges of the gasket.
4. The other plate was put on top of the short plate.
5. The clamps were set on both sides of the plates for tightening and the plate assembly was laid flat on the table.

3.9.2 Preparation of Polyacrylamide Gel

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

Table 5: Composition and preparation of polyacrylamide gel

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

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

3.9.3 Polyacrylamide Gel Electrophoresis

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

1. Two µl of 10X loading dye was added to the each well containing 10 µl PCR product and the plates were centrifuged at a speed of 3000 rpm for 30 sec in a high speed refrigerated centrifuge machine. Around 2 µl of the mixer was loaded in the wells of PAGE gel with the help of 2-2.5 µl pipette. DNA size marker like 50 bp

DNA ladder was loaded for size determination. Thermo Scientific GeneRuler 1Kb Plus DNA Ladder was used from ThermoScientific company.

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

3.9.4 Staining and Visualization of the Gel

1. The power supply unit was turned off and the plates were removed from the tank. The glass plates were separated using a knife. The acrylamide gel was removed carefully and transferred in the SYBR Safe staining solution (0.5 mg/ml) for around 20 minutes.
2. The stained gels were put in the exposure cabinet of the gel documentation system (Molecular Imager Gel Doc XR System, BIO-RAD, Korea). The gel was viewed in the computer monitor by exposing it first to white light. The necessary adjustments were made by moving the gel inside the exposure box. The gel was exposed to UV light and photograph (gel image) was taken and saved as a JPEG file.

3.10 SSR data analysis

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

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

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

Shannon diversity indexwas quantified using following formula:

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

total of n alleles.

The individual fragments were assigned as alleles of the appropriate microsatellite loci. Genetic diversity also was assessed, and the phylogeny trees were drawn using MEGA 5.1 (Tamura *et al*., 2011 and Hall, 2013) based on Nei's (1983) genetic distance.

Some pictorial view of molecular work

Figure 1: Collection of juvenile leaf sample

Figure 2: Grinding of sample for DNA extraction

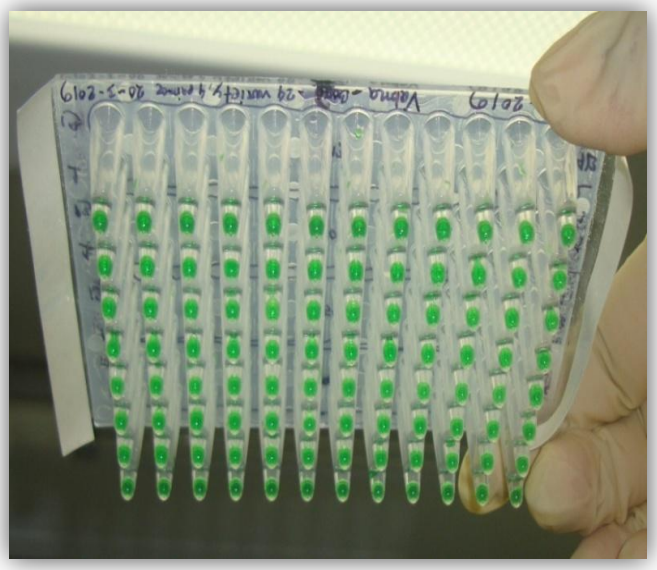

Figure 3: Samples after grinding placed in 96-well plate sample

Figure 4: Sample preparation for PCR amplification

Some pictorial view of molecular work (Continued)

Figure 5: Setting of PCR reaction for DNA amplification in PCR machine

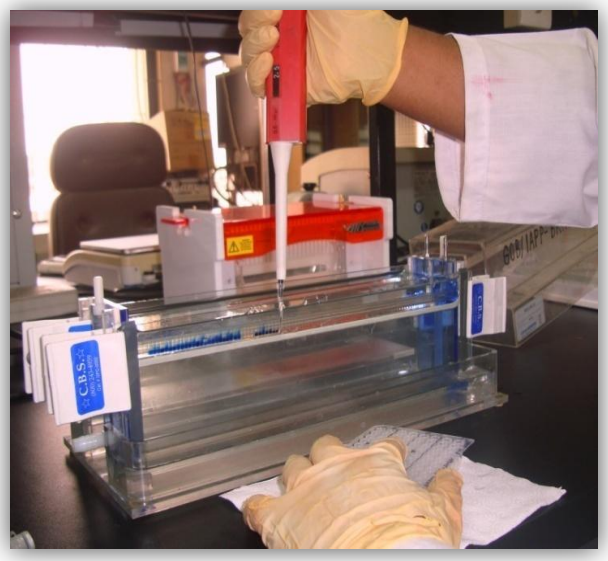

Figure 6: Loading DNA samples in gel electrophoresis unit

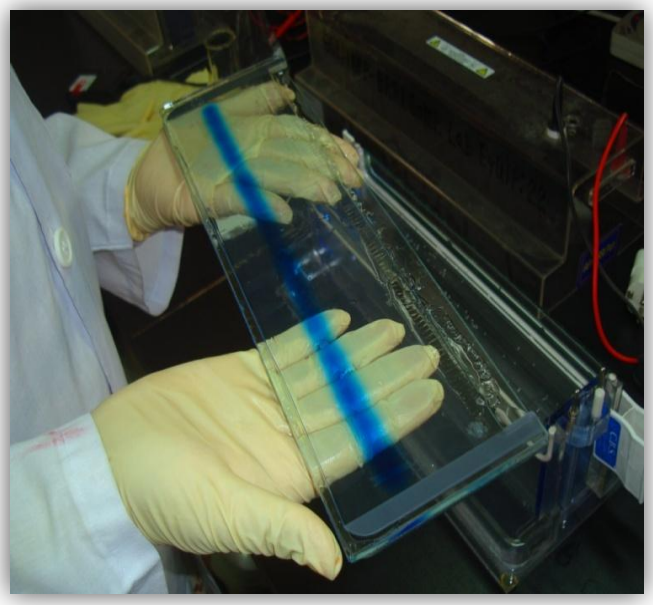

Figure 7: PAGE gel run after electrophoresis

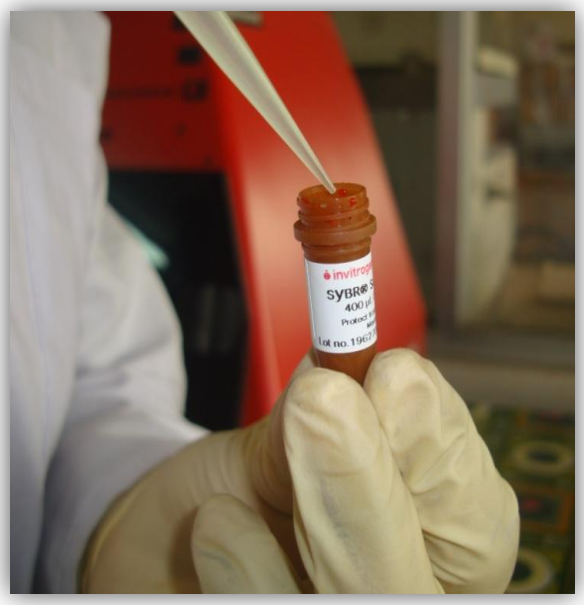

Figure 8: Gel staining with SYBR Safe dye

CHAPTER IV

RESULTS AND DISCUSSION

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

4.1 DNA Extraction

The genomic DNA extraction of 24 local Boro genotypes were completed by using CTAB method with minor modification. The extracted, purified DNA was stored at -20^0 freezer for further use.

4.2 DNA amplification by SSR markers and its polymorphism

Twelve SSR primers *viz*. RM1, RM206, RM207, RM253, RM304, RM252, RM1337, RM320, RM205, RM447, RM3646, RM413 produced different banding pattern separately with 24 rice genotypes. The amplification of each SSR primers are presented in Table 6 and Plate 1 to 12.

The SSR primer RM1 showed polymorphic band. Different DNA amplifications were obtained which were distributed throughout all the genotypes under study. The size of amplification ranged from 80 bp to 175 bp. The genotype Dol Boro showed amplification at 80 bp. Most of the genotypes have given amplification at 85 bp. Again, Sada Boro, Sona Biron, Rajkumar, Black Rice, Boro Habj 1 genotypes have given polymorphic band at 85bp, 100 bp, 125 bp, 150 bp and 175 bp. The amplification product is presented Plate 1.

Eight fragment of DNA amplification were noticed by SSR primer RM206. The size of the amplification ranged from 130 bp to 280 bp. Eight types of band indicate a highly polymorphic band. Whereas, band 130 bp was observed in Rata, Dol Boro, Vawailia, Dhali Boro 94. DNA fragment 150 bp was obtained from Pabdafor, Tepi Boro Dhan

(kalo), and Jira genotypes. Pabdafor, Amania, Sona Biron, Tepi Boro Dhan (kalo), Jira gave their amplification at 150 bp. Fragment 180 bp was produced by Lakhai Boruni, Rajkumar, Dhali Boro 7/2, Black Rice, Dhali Boro 74/3, Boro Habj 1, Dhali Boro 104/1 and Dhali Boro 105/2 genotypes. The 190 bp fragment was produced by Rajkumar, Boro Dhan (Indian), Dhali Boro 7/2, Dhali Boro 74/3, Boro Habj 1, Jira, Dhali Borol 87/1, Dhali Boro 104/1 and Dhali Boro 105/2 genotypes. On the other hand, the genotypes Khaiya Boro, Rajkumar, Black Rice, Dhali Boro 74/3, Boro Habj 1, Jira, Dhali Boro 104/1 and Dhali Boro 105/2 were able to produce 200 bp polymorphic band. The genotypes Rajkumar, Black Rice, Dhali Boro 74/3, Jira, Dhali Boro 104/1 and Dhali Boro 105/2 were able to produce 220 bp polymorphic band. The DNA fragment 250 bp was obtained from Dhali Boro 7/2, Dhali Boro 74/3, Boro Habj 1, Jira, Dhali Boro 87/1, Dhali Boro 104/1 and Dhali Boro 105/2 varieties. The highest fragment 280 bp was obtained from Dhali Boro 7/2, Dhali Boro 74/3, Kali Boro, Dhali Boro 87/1, Dhali Boro 105/2 genotypes (Plate 2).

The SSR primer RM207 produced six fragments of DNA amplification. The amplification of band ranged from 70 bp to 190 bp. All the genotypes produced 70 bp fragment which indicated a monomorphic band. Whereas, the genotypes Sona Biron, Dol Boro, Black Rice, Boro Habj 1 were able to produce 140 bp, 150 bp, 165 bp, 175 bp, 190 bp polymorphic bands (Plate 3).

The SSR primer RM253 was able to amplify seven fragments of DNA among all the individuals. The DNA product ranged from 125 bp to 200 bp. Among them 131 bp fragment was common in most of the genotypes. The germplasm Sada Boro, Sona Biron, Rajkumar, Dol Boro, Black Rice, Boro Habj 1- all of them showed amplification at 153 bp, 160 bp and 180 bp. Dol Boro, Black Rice, Boro Habj 1 amplified their fragment at 190 bp. Only Dol Boro was able to produce 200 bp unique band. The amplification product is presented in Plate 4.

Four fragments of DNA amplification were noticed by the SSR primer RM304.The approximate fragment size ranged from 140 bp to 200 bp. Pabdafor, Amania showed amplification at 140 bp. Asami Boro, Rata, Boro Dhan (Indian), Dhali Boro 74/3, Dhali Boro 94, Dhali Boro 105/2 genotypes produced polymorphic band at 160 bp. Asami Boro, Pabdafor, Khaiya Boro, Lakhai Boruni, Sada Boro, Rajkumar, Dol Boro, Dhali Boro 7/2, Black Rice, Kali Boro, Dhali Boro 87/1, Dhali Boro 104/1 genotypes produced

polymorphic band at 175 bp. Khaiya Boro, Dol Boro, Jira have produced their band at 200 bp. The amplification product is presented in Plate 5.

The SSR primer RM252 has the ability to amplify 2 fragment of DNA among all the experimental materials. The band size ranged from 200 bp to 240 bp. It was noticed that 200 bp fragment was common in all the genotypes and was monomorphic for all. Again, 240 bp fragment was produced by Sona Biron, Boro Habj 1 genotypes. (Plate 6).

The SSR primer RM1337 showed six different fragments of DNA amplification and obviously a polymorphic banding pattern. The fragments size varies from 180 bp to 300 bp. Most of the genotypes have given their amplification at 200 bp which is a monomorphic banding pattern. The other genotypes have given amplification of different size, *viz.* Kalo Boro, Pabdafor, Amania, Rata, Lakhai Boruni, Sada Boro, Rajkumar, Boro Dhan (Indian), Tepi Boro Dhan (Kalo), Dhali Boro 7/2, Dhali Boro 74/3, Vawailia, Kali Boro, Jira, Dhali Boro 87/1, Dhali Boro 94, Dhali Boro 104/1, Dhali Boro 105/2 at 225 bp; Kalo Boro, Asami Boro,Pabdafor, Amania, Rata, Lakhai Boruni, Sada Boro, Rajkumar, Boro Dhan (Indian), Tepi Boro Dhan (Kalo), Dhali Boro 7/2, Dhali Boro 74/3, Kali Boro, Jira, Dhali Boro 87/1, Dhali Boro 94 and Dhali Boro 104/1 at 250 bp; Kalo Boro, Asami Boro, Pabdafor, Amania, Rata, Lakhai Boruni, Sada Boro, Rajkumar, Boro dhan, Tepi Boro Dhan (Kalo), Dhali Boro 7/2, Dhali Boro 74/3, Kali Boro, Jira, Dhali Boro 87/1, Dhali Boro 94, Dhali Boro 104/1 at 300 bp. (Plate 7).

The SSR primer RM320 was able to amplify at 200 bp of DNA fragment for all the individuals which indicated a monomorphic band. The amplification product is presented in Plate 8.

The SSR primer RM205 produced seven DNA fragment among all the genotypes under study. The approximate fragment size varies from 175 bp to 300 bp. Most of the genotypes produced 200 bp fragment which indicated a monomorphic band. 175 bp band was produced by the variety Rajkumar. Band 180 bp was produced by Khaiya Boro, Sada Boro, Sona Biron, Rajkumar, Dol Boro, Black Rice, Boro Habj 1 and Vawailia genotypes.Again, 250 bp band was produced by Kalo Boro, Asami Boro, Pabdafor, Amania, Rata, Lakhai Boruni, Boro Dhan (Indian), Tepi Boro Dhan (Kalo), Dhali Boro 7/2, Dhali Boro 74/3, Dhali Boro 87/1, Dhali Boro 94, Dhali Boro 105/2 genotypes. 280 bp band was produced by Kalo Boro, Asami Boro, Pabdafor, Amania, Rata, Lakhai Boruni, Boro Dhan, Tepi Boro Dhan (Kalo), Dhali Boro 7/2, Dhali Boro 74/3, Boro Habj

1, Vawailia, Kali Boro, Jira, Dhali Boro 104/1 genotypes. Only two genotypes Asami Boro and Dhali Boro 87/1 amplified their fragment at 300 bp. The amplification product is presented in Plate 9.

Three fragments of DNA amplification were noticed by SSR primer RM447. The amplification product ranged from 110 bp to 180 bp. All the genotypes produced 110 bp fragment which indicated a monomorphic band. Whereas, only Sada Boro genotype was able to produce 125 bp polymorphic band and Dol Boro genotype was able to produce 180 bp polymorphic band. (Plate 10).

The SSR primer RM3646 was able to amplify three fragments of DNA among all the individuals. The size of amplification ranged from 150 bp to 162 bp. All of the individuals showed band at 155 bp. Three genotypes namely Sada Boro, Rajkumar, Boro Habj 1 produced 162 bp polymorphic band. The amplification of primer RM3646 is presented in Plate 11.

The SSR primer RM413 showed three different fragments of DNA amplification and obviously a polymorphic banding pattern. The fragments size varies from 70 bp to 110 bp. Most of the genotypes have given their amplification at 70 bp. The other genotypes have given amplification of different size, *viz.* Khaiya Boro, Sada Boro, Black Rice, Vawailia at 80 bp; Sona Biron, Rajkumar, Boro Habj 1 at 90 bp. Only Khaiya Boro showed amplification at 110 bp. The amplification of primer RM413 is presented in Plate 12.

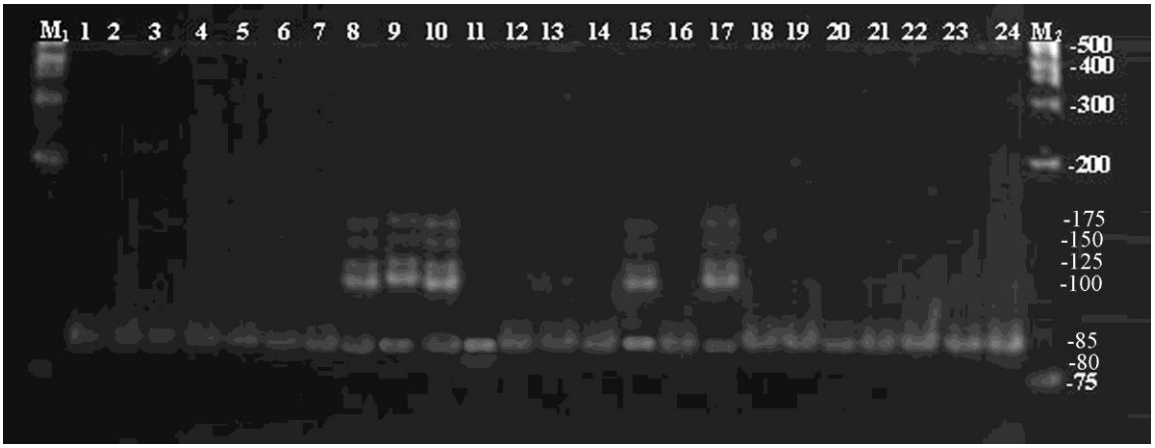

Plate 1. SSR profile of twenty four Boro rice genotypes using primer RM1. M_1 and M_2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane: 1. Kalo Boro, 2. Asami Boro, 3. Pabdafor, 4. Amania, 5. Khaiya Boro, 6. Rata, 7. Lakhai Boruni, 8. Sada Boro, 9. Sona Biron, 10. Rajkumar, 11. Dol Boro, 12. Boro Dhan (Indian), 13. Tepi Boro Dhan (Kalo), 14. Dhali Boro 7/2, 15. Black Rice, 16. Dhali Boro 74/3, 17. Boro Habj 1, 18. Vawailia, 19. Kali Boro, 20. Jira, 21. Dhali Boro 87/1, 22. Dhali Boro 94, 23. Dhali Boro 104/1, 24. Dhali Boro 105/2.

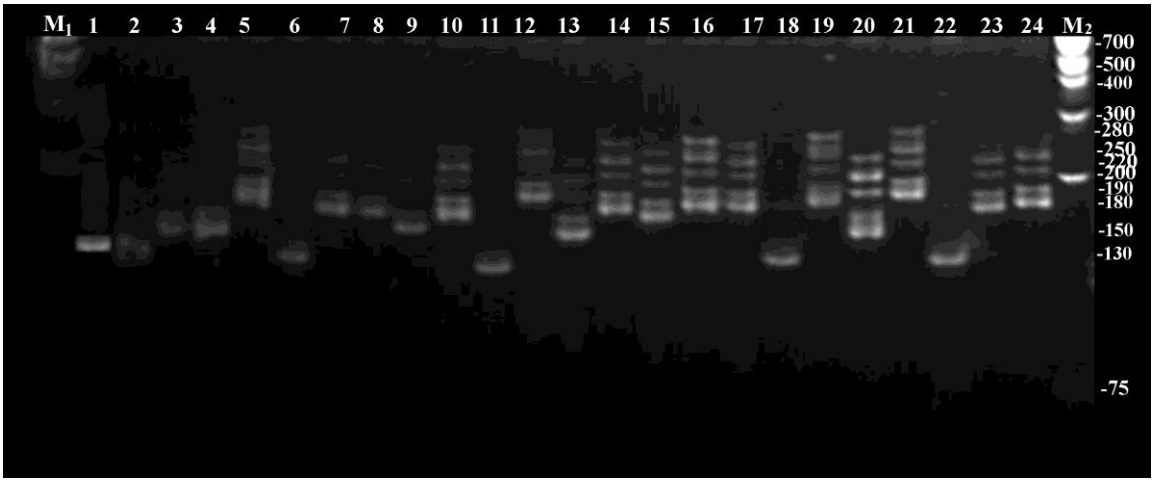

Plate 2. SSR profile of twenty four Boro rice genotypes using primer RM206. M_1 and M_2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane: 1. Kalo Boro, 2. Asami Boro, 3. Pabdafor, 4. Amania, 5. Khaiya Boro, 6. Rata, 7. Lakhai Boruni, 8. Sada Boro, 9. Sona Biron, 10. Rajkumar, 11. Dol Boro, 12. Boro Dhan (Indian), 13. Tepi Boro Dhan (Kalo), 14. Dhali Boro 7/2, 15. Black Rice, 16. Dhali Boro 74/3, 17. Boro Habj 1, 18. Vawailia, 19. Kali Boro, 20. Jira, 21. Dhali Boro 87/1, 22. Dhali Boro 94, 23. Dhali Boro 104/1, 24. Dhali Boro 105/2.

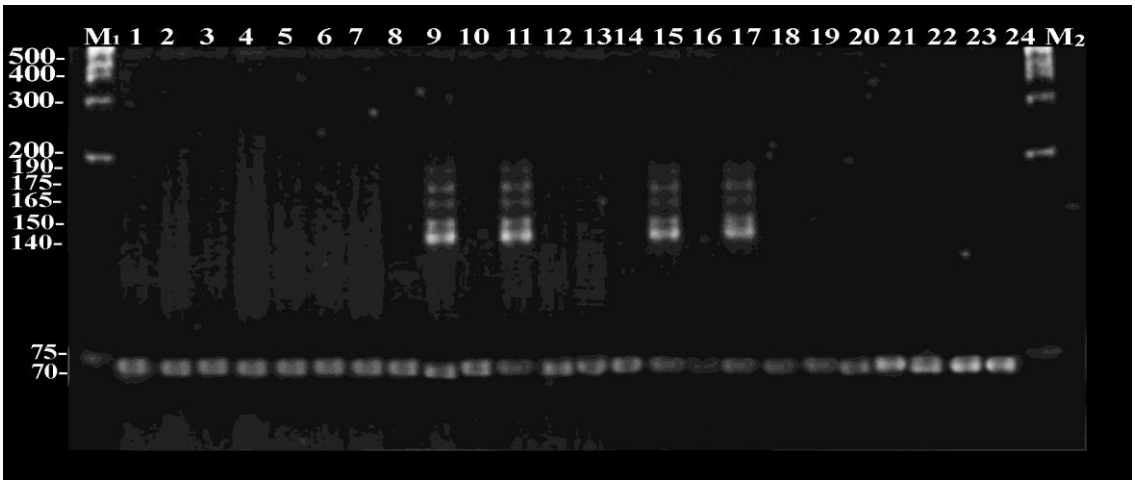

Plate 3. SSR profile of twenty four Boro rice genotypes using primer RM207. M_1 and M_2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane: 1. Kalo Boro, 2. Asami Boro, 3. Pabdafor, 4. Amania, 5. Khaiya Boro, 6. Rata, 7. Lakhai Boruni, 8. Sada Boro, 9. Sona Biron, 10. Rajkumar, 11. Dol Boro, 12. Boro Dhan (Indian), 13. Tepi Boro Dhan (Kalo), 14. Dhali Boro 7/2, 15. Black Rice, 16. Dhali Boro 74/3, 17. Boro Habj 1, 18. Vawailia, 19. Kali Boro, 20. Jira, 21. Dhali Boro 87/1, 22. Dhali Boro 94, 23. Dhali Boro 104/1, 24. Dhali Boro 105/2.

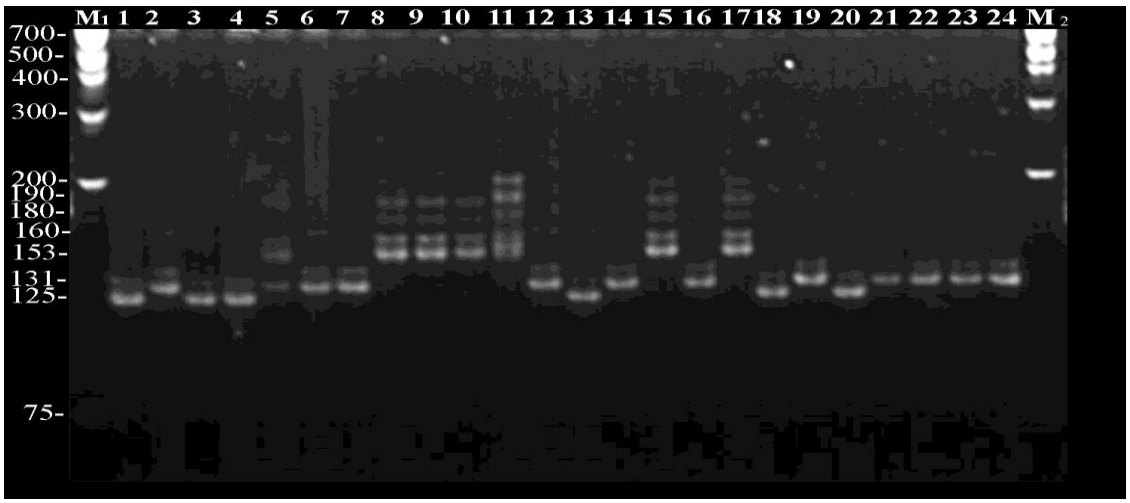

Plate 4. SSR profile of twenty four Boro rice genotypes using primer RM253. M_1 and M_2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane: 1. Kalo Boro, 2. Asami Boro, 3. Pabdafor, 4. Amania, 5. Khaiya Boro, 6. Rata,7. Lakhai Boruni, 8. Sada Boro, 9. Sona Biron, 10. Rajkumar, 11. Dol Boro, 12. Boro Dhan (Indian), 13. Tepi Boro Dhan (Kalo), 14. Dhali Boro 7/2, 15. Black Rice, 16. Dhali Boro 74/3, 17. Boro Habj 1, 18. Vawailia, 19. Kali Boro, 20. Jira, 21. Dhali Boro 87/1, 22. Dhali Boro 94, 23. Dhali Boro 104/1, 24. Dhali Boro 105/2.

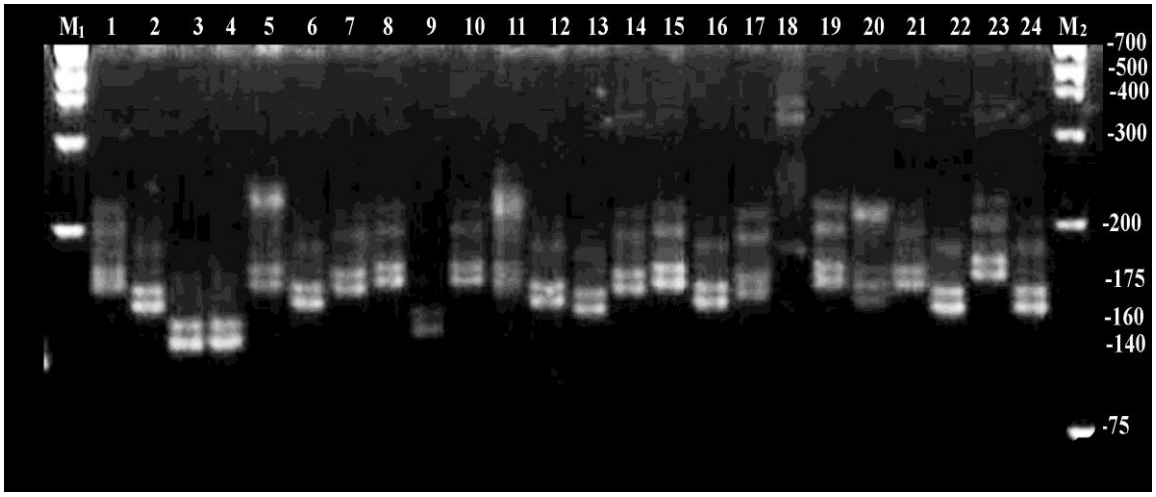

Plate 5. SSR profile of twenty four Boro rice genotypes using primer RM304. M_1 and M_2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane: 1. Kalo Boro, 2. Asami Boro, 3. Pabdafor, 4. Amania, 5. Khaiya Boro, 6. Rata, 7. Lakhai Boruni, 8. Sada Boro, 9. Sona Biron, 10. Rajkumar, 11. Dol Boro, 12. Boro Dhan (Indian), 13. Tepi Boro Dhan (Kalo), 14. Dhali Boro 7/2, 15. Black Rice, 16. Dhali Boro 74/3, 17. Boro Habj 1, 18. Vawailia, 19. Kali Boro, 20. Jira, 21. Dhali Boro 87/1, 22. Dhali Boro 94, 23. Dhali Boro 104/1, 24. Dhali Boro 105/2.

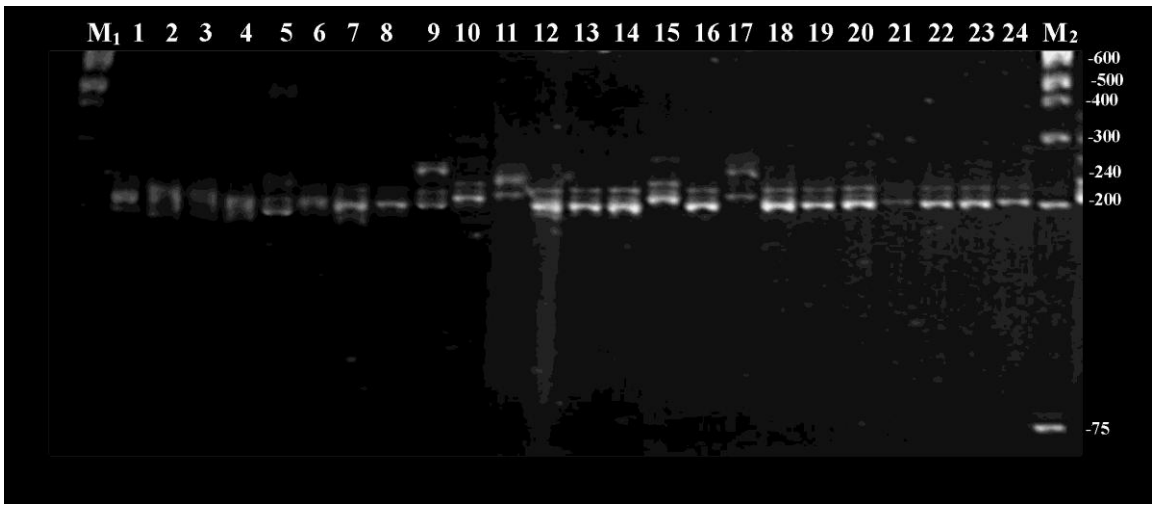

Plate 6. SSR profile of twenty four Boro rice genotypes using primer RM252. M_1 and M_2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane: 1. Kalo Boro, 2. Asami Boro, 3. Pabdafor, 4. Amania, 5. Khaiya Boro, 6. Rata, 7. Lakhai Boruni, 8. Sada Boro, 9. Sona Biron, 10. Rajkumar, 11. Dol Boro, 12. Boro Dhan (Indian), 13. Tepi Boro Dhan (Kalo), 14. Dhali Boro 7/2, 15. Black Rice, 16. Dhali Boro 74/3, 17. Boro Habj 1, 18. Vawailia, 19. Kali Boro, 20. Jira, 21. Dhali Boro 87/1, 22. Dhali Boro 94, 23. Dhali Boro 104/1, 24. Dhali Boro 105/2.

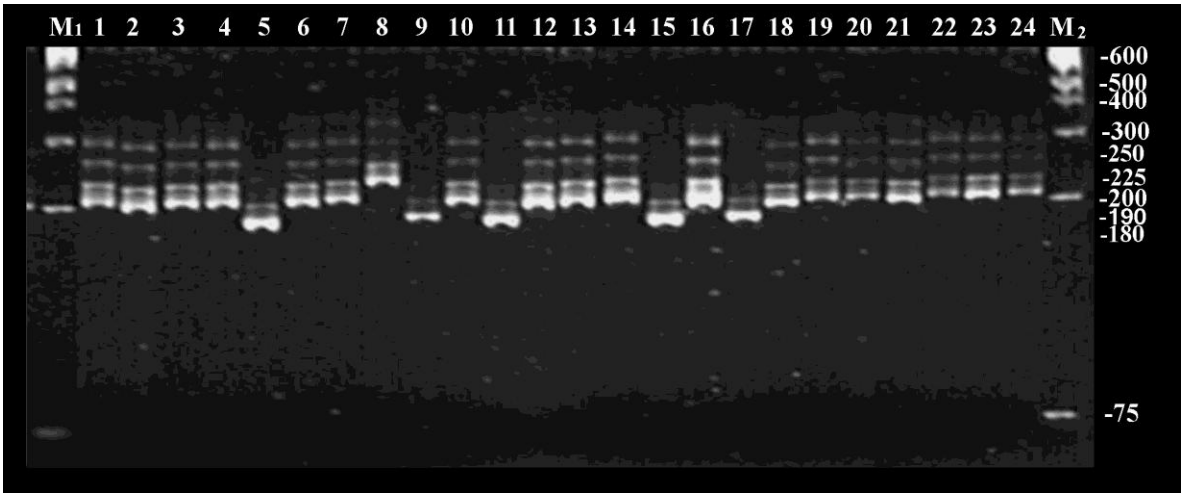

Plate 7. SSR profile of twenty four Boro rice genotypes using primer RM1337. M_1 and M_2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane: 1. Kalo Boro, 2. Asami Boro, 3. Pabdafor, 4. Amania, 5. Khaiya Boro, 6. Rata, 7. Lakhai Boruni, 8. Sada Boro, 9. Sona Biron, 10. Rajkumar, 11. Dol Boro, 12. Boro Dhan (Indian), 13. Tepi Boro Dhan (Kalo), 14. Dhali Boro 7/2, 15. Black Rice, 16. Dhali Boro 74/3, 17. Boro Habj 1, 18. Vawailia, 19. Kali Boro, 20. Jira, 21. Dhali Boro 87/1, 22. Dhali Boro 94, 23. Dhali Boro 104/1, 24. Dhali Boro 105/2.

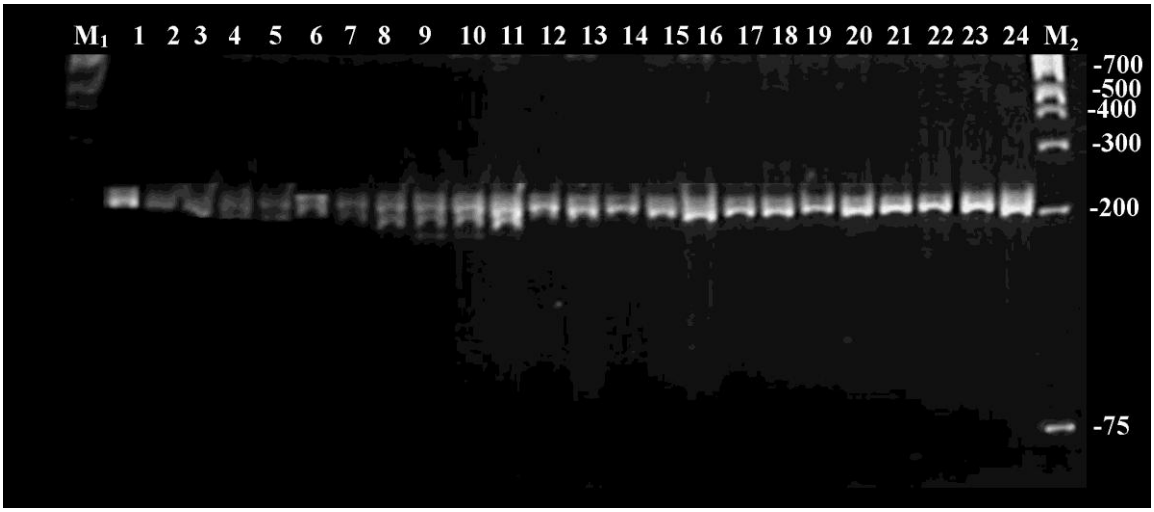

Plate 8. SSR profile of twenty four Boro rice genotypes using primer RM320. M_1 and M_2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane: 1. Kalo Boro, 2. Asami Boro, 3. Pabdafor, 4. Amania, 5. Khaiya Boro, 6. Rata, 7. Lakhai Boruni, 8. Sada Boro, 9. Sona Biron, 10. Rajkumar, 11. Dol Boro, 12. Boro Dhan (Indian), 13. Tepi Boro Dhan (Kalo), 14. Dhali Boro 7/2, 15. Black Rice, 16. Dhali Boro 74/3, 17. Boro Habj 1, 18. Vawailia, 19. Kali Boro, 20. Jira, 21. Dhali Boro 87/1, 22. Dhali Boro 94, 23. Dhali Boro 104/1, 24. Dhali Boro 105/2.

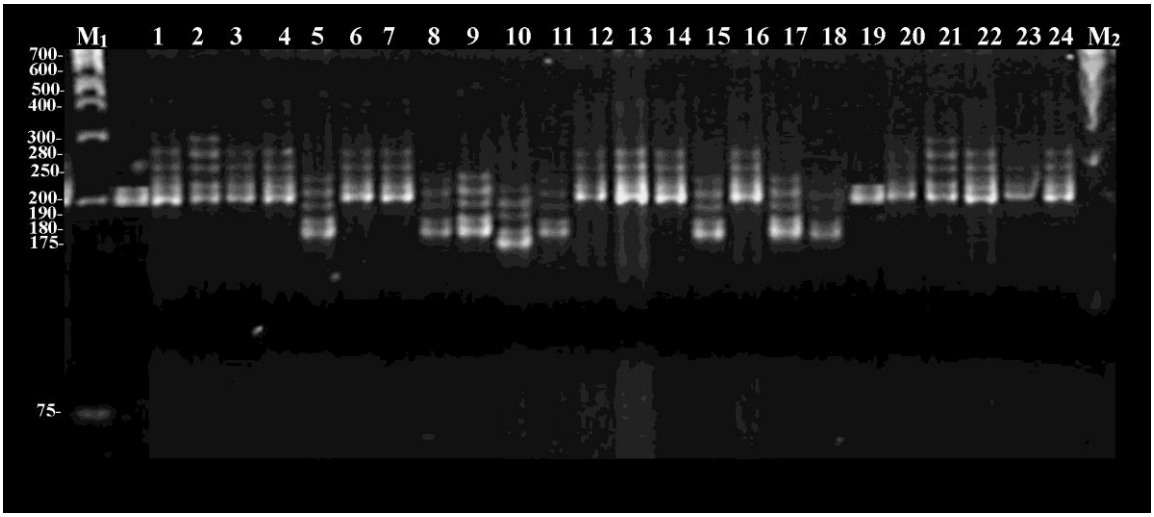

Plate 9. SSR profile of twenty four Boro rice genotypes using primer RM205. M_1 and M_2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane: 1. Kalo Boro, 2. Asami Boro, 3. Pabdafor, 4. Amania, 5. Khaiya Boro, 6. Rata, 7. Lakhai Boruni, 8. Sada Boro, 9. Sona Biron, 10. Rajkumar, 11. Dol Boro, 12. Boro Dhan (Indian), 13. Tepi Boro Dhan (Kalo), 14. Dhali Boro 7/2, 15. Black Rice, 16. Dhali Boro 74/3, 17. Boro Habj 1, 18. Vawailia, 19. Kali Boro, 20. Jira, 21. Dhali Boro 87/1, 22. Dhali Boro 94, 23. Dhali Boro 104/1, 24. Dhali Boro 105/2.

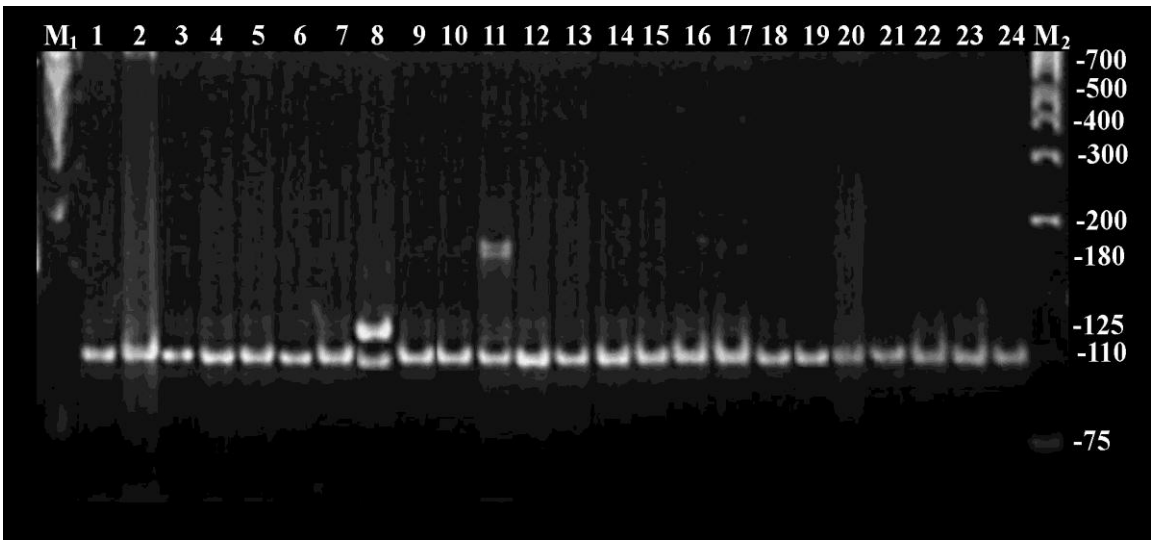

Plate 10. SSR profile of twenty four Boro rice genotypes using primer RM447. M_1 and M_2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane: 1. Kalo Boro, 2. Asami Boro, 3. Pabdafor, 4. Amania, 5. Khaiya Boro, 6. Rata, 7. Lakhai Boruni, 8. Sada Boro, 9. Sona Biron, 10. Rajkumar, 11. Dol Boro, 12. Boro Dhan (Indian), 13. Tepi Boro Dhan (Kalo), 14. Dhali Boro 7/2, 15. Black Rice, 16. Dhali Boro 74/3, 17. Boro Habj 1, 18. Vawailia, 19. Kali Boro, 20. Jira, 21. Dhali Boro 87/1, 22. Dhali Boro 94, 23. Dhali Boro 104/1, 24. Dhali Boro 105/2.

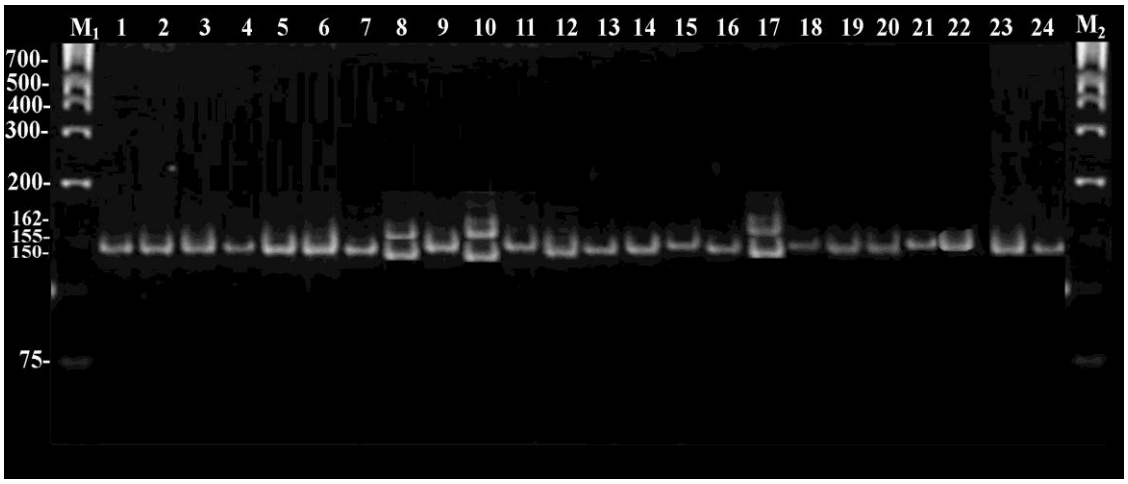

Plate 11. SSR profile of twenty four Boro rice genotypes using primer RM3646. M_1 and M_2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane: 1. Kalo Boro, 2. Asami Boro, 3. Pabdafor, 4. Amania, 5. Khaiya Boro, 6. Rata, 7. Lakhai Boruni, 8. Sada Boro, 9. Sona Biron, 10. Rajkumar, 11. Dol Boro, 12. Boro Dhan (Indian), 13. Tepi Boro Dhan (Kalo), 14. Dhali Boro 7/2, 15. Black Rice, 16. Dhali Boro 74/3, 17. Boro Habj 1, 18. Vawailia, 19. Kali Boro, 20. Jira, 21. Dhali Boro 87/1, 22. Dhali Boro 94, 23. Dhali Boro 104/1, 24. Dhali Boro 105/2.

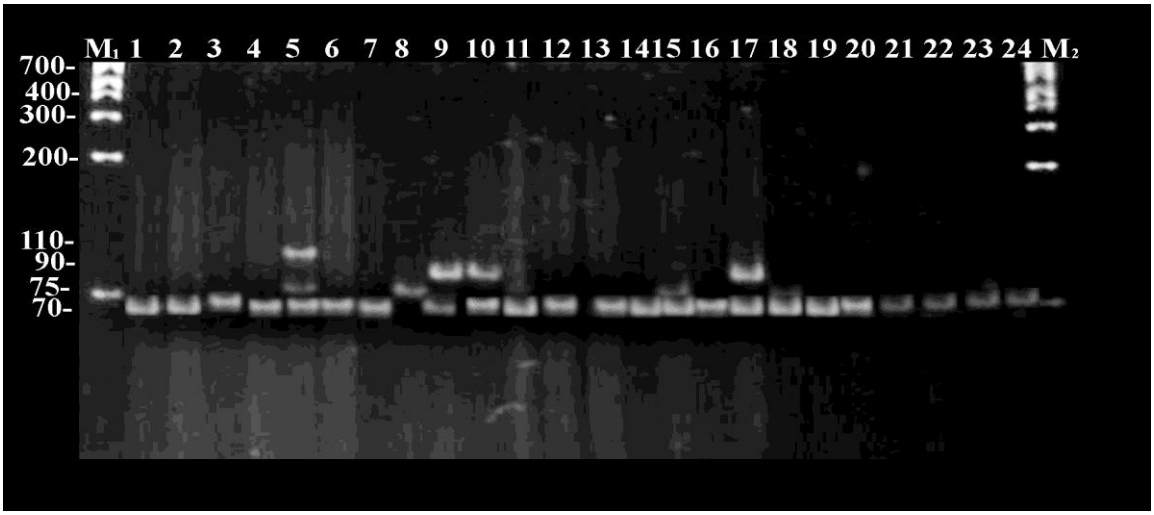

Plate 12. SSR profile of twenty four Boro rice genotypes using primer RM413. M_1 and M_2 = Molecular marker (Thermo Scientific GeneRuler 1 kb Plus DNA Ladder). Lane: 1. Kalo Boro, 2. Asami Boro, 3. Pabdafor, 4. Amania, 5. Khaiya Boro, 6. Rata, 7. Lakhai Boruni, 8. Sada Boro, 9. Sona Biron, 10. Rajkumar, 11. Dol Boro, 12. Boro Dhan (Indian), 13. Tepi Boro Dhan (Kalo), 14. Dhali Boro 7/2, 15. Black Rice, 16. Dhali Boro 74/3, 17. Boro Habj 1, 18. Vawailia, 19. Kali Boro, 20. Jira, 21. Dhali Boro 87/1, 22. Dhali Boro 94, 23. Dhali Boro 104/1, 24. Dhali Boro 105/2.

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

Number of alleles, range of DNA fragment, number of polymorphic alleles, allele frequency, gene diversity and polymorphism information content (PIC) found among experimental genotypes for 12 SSS markers are presented in Table 6. A total 54 alleles were detected for the 12 polymorphic SSR loci, with an average number of alleles/locus of 4.5 and a range between 1 to 8 no. of alleles (Table 6). A total 45 polymorphic alleles were detected among the experimental genotypes. RM1, RM253, RM205, RM447, RM413 all of them detected unique alleles. The length of the DNA fragments varies within a range of 70 bp to 300 bp. Primer RM207 and RM417 amplified 70 bp fragment whereas primer RM1337 amplified 300 bp fragment of DNA. The frequency of the major allele ranged between 16.67 (RM206) to 95.83 (RM447). Polymorphic Information Content (PIC) value for 12 SSR markers ranged from 0.08 (RM447) to 0.84 (RM206) and the average PIC value was 0.49. Gene diversity ranged between 0.08 to 0.86 with an average of 0.52. Primer RM206 showed highest gene diversity (0.86) followed by RM304 (0.82), RM1337 (0.81), RM413 (0.70), RM253 (0.64), RM252 (0.57), RM205 (0.47), RM3646 (0.45), RM1 (0.41), RM207 (0.28), RM320 (0.16) and RM447 showed the lowest gene diversity (0.08). Total gene diversity obtained 6.25 with an average 0.52. Polymorphic Information Content (PIC) value for 12 SSR markers ranged from 0.08 (RM447) to 0.84 (RM206) and the average PIC value was 0.49. The highest PIC value was obtained from RM206 (0.84) followed by RM304 (0.80), RM1337 (0.78), RM413 (0.66), RM253 (0.57), RM252 (0.53), RM3646 (0.41), RM205 (0.39), RM1 (0.37), RM207 (0.24), RM320 (0.15) respectively. The lowest PIC value 0.08 was showed by primer RM447. From the PIC value, it is clear that RM206 can be considered as the best marker for 24 rice germplasm followed by RM304, RM1337, RM413, RM253, RM252, RM3646, RM205, RM1, RM207, RM320 respectively. Again, RM447 can be considered as the least powerful marker due to its lowest PIC value. The results indicate that the 24 Boro rice landraces have very closeness among them. Again, they reveal a high degree of heterozygosity. They also reveal considerable intra-varietal group diversity and genetic differentiation and polymorphism exist in a certain degree which is really noticeable.

Table 6: Number of alleles, range of DNA fragment, no. of polymorphic alleles, allele frequency, gene diversity and Polymorphism information content (PIC) found among 24 Boro rice germplasm for 12 microsatellite markers

SL. No.	Marker	Chro. No.	Position (cM)	Motif	Total no. of allele	No. of polymor-phic allele	Range of DNA fragment (bp)	Frequency (%)	Gene diversity	PIC
1	RM1	1	29.7	(GA)26	6	5	80-175	75	0.41	0.37
2	RM206	11	102.9	(CT)21	8	8	130-280	16.67	0.86	0.84
3	RM207	2	191.2	(CT)25	6	5	70-190	83.33	0.28	0.24
4	RM253	6	37	(GA)25	7	7	125-200	45.83	0.64	0.57
5	RM304	10	73	(GT)2(AT)10GT)33	4	3	140-200	29.17	0.82	0.80
6	RM252	4	99	(CT)19	2	1	200-240	62.5	0.57	0.53
7	RM1337	12	0	(AG)21	6	4	180-300	33.33	0.81	0.78
8	RM320	7	62.4	(AT)11GTAT(GT)13	1	0	200	91.67	0.16	0.15
9	RM205	9	114.7	(CT)25	7	6	175-300	66.67	0.47	0.39
10	RM447	8	124.6	(CTT)8	3	2	110-180	95.83	0.08	0.08
11	RM3646	3	6.32	(GA)14	2	1	150-162	70.83	0.45	0.41
12	RM413	5	26.7	(AG)11	3	3	70-110	45.83	0.70	0.66
Total	-	-	-	-	54	45	-	-	6.25	5.82
Mean	-	-	-	-	4.5	--	-	--	0.52	0.49

4.3.1 Diversity revealed by different markers

The polymorphism information content (PIC) value is a reflection of allelic diversity and frequency among the varieties. The used twelve markers have different level of diversity on the basis of PIC value. Among them, RM207, RM320, RM447 showed low diversity. Again, RM1, RM205, RM3646 showed moderate diversity. Rest of the six markers showed highly diverse result. Kalo Boro, Asami Boro, Sada Boro, Sona Biron, Dol Boro, Rajkumar, Vawailia, Khaiya Boro produced unique alleles. The results revealed that the varieties produced unique alleles that could be used for molecular characterization and DNA fingerprinting of these Boro rice landraces. Because, these unique alleles may have some special characteristics which may be helpful in breeding as well as other research program concerning rice development. The result revealed to broaden the genetic base for wider adaptability.

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

Sl. No.	PIC Value	Diversity level	Markers
1.	≤ 0.25	Low	RM207, RM320, RM447,
2.	0.25- 0.50	Moderate	RM1, RM205, RM3646
3.	0.50-1.0	High	RM206, RM304, RM1337, RM413, RM253, RM252

4.4 Nei's Genetic Distance

The value of pair-wise comparisons of Nei's (1983) genetic distance among 24 relatives of rice genotypes were computed from combined data for the 12 primers, ranged from 0.1667 to 0.9167 with an average of 0.5417(Table 8). Comparatively higher genetic distance (0.9167) was observed between a number of genotypes. Among them Sona Biron(G9) showed highest genetic dissimilarity with maximum number of genotypes. The highest Nei's genetic distance (0.9167) was observed in Kalo Boro vs. Lakhai Boruni vs. Sona Biron vs. Dhali Boro 104/1; Pabdafor vs. Boro Dhan (Indian); Lakhai Boruni vs. Boro Dhan (Indian); Sona Biron vs. Boro Dhan (Indian); Boro Dhan (Indian) vs. Dhali Boro 104/1. The highest genetic distance between them indicated that genetically they are dissimilar and also highly diverse. The difference between the highest and lowest genetic identity indicates the presence of variability among 24 germplasm of Boro rice. Those (genotypes) which have higher (0.9167) genetic distance is more dissimilar than those which have lower value. Again, many pairs have showed the lowest genetic distance (0.1667) which indicate their genetically much more closeness among them. The average genetic distance among the 24 genotypes was observed as 0.5417(Table 8).

Shah *et al*., (2013) evaluated the genetic diversity of 40 rice accessions using 24 microsatellite markers distributed over the whole rice genome and reported that a total of 66 alleles were detected. Polymorphism Information Content (PIC) value ranged from 0.0476 (RM315) to 0.5993 (RM252), with an average of 0.3785 per marker and average genetic diversity over all SSR loci was 0.4477 ranging from 0.0488 to 0.6638.

Table 8. : Summary of Nei's genetic distance (below diagonal) values among 24 Boro rice genotypes

Genotypes	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
G1	0.0000											
G2	0.7500	0.0000										
G3	0.8333	0.6667	0.0000									
G4	0.5833	0.6667	0.8333	0.0000								
G5	0.5000	0.6667	0.8333	0.2500	0.0000							
G6	0.4167	0.6667	0.7500	0.2500	0.3333	0.0000						
G7	0.9167	0.5000	0.4167	0.8333	0.8333	0.8333	0.0000					
G8	0.5833	0.7500	0.7500	0.3333	0.5000	0.3333	0.7500	0.0000				
G9	0.9167	0.5000	0.5000	0.8333	0.8333	0.8333	0.5000	0.7500	0.0000			
G10	0.6667	0.7500	0.6667	0.5833	0.5000	0.5833	0.5833	0.4167	0.7500	0.0000		
G11	0.5000	0.6667	0.7500	0.1667	0.2500	0.1667	0.8333	0.3333	0.7500	0.5833	0.0000	
G12	0.5000	0.7500	0.9167	0.3333	0.5000	0.5000	0.9167	0.4167	0.9167	0.5833	0.4167	0.0000
G13	0.5000	0.7500	0.8333	0.2500	0.1667	0.4167	0.8333	0.4167	0.8333	0.5000	0.3333	0.4167
G14	0.5833	0.6667	0.8333	0.0833	0.2500	0.2500	0.8333	0.4167	0.7500	0.5833	0.1667	0.4167
G15	0.5833	0.7500	0.8333	0.2500	0.2500	0.3333	0.8333	0.4167	0.8333	0.5000	0.2500	0.3333
G16	0.5000	0.5833	0.7500	0.1667	0.2500	0.1667	0.7500	0.4167	0.8333	0.5833	0.0833	0.4167
G17	0.5000	0.7500	0.8333	0.3333	0.3333	0.1667	0.8333	0.3333	0.8333	0.5833	0.3333	0.5000
G18	0.3333	0.6667	0.8333	0.4167	0.2500	0.5000	0.8333	0.5833	0.8333	0.5833	0.4167	0.4167
G19	0.4167	0.7500	0.8333	0.3333	0.1667	0.4167	0.8333	0.5000	0.8333	0.5000	0.3333	0.5000
G20	0.5833	0.6667	0.5000	0.4167	0.5000	0.4167	0.5833	0.5833	0.6667	0.5000	0.4167	0.6667
G21	0.5000	0.7500	0.8333	0.2500	0.4167	0.4167	0.7500	0.4167	0.8333	0.5833	0.3333	0.2500
G22	0.4167	0.6667	0.7500	0.2500	0.3333	0.0000	0.8333	0.3333	0.8333	0.5833	0.1667	0.5000
G23	0.9167	0.4167	0.7500	0.7500	0.7500	0.7500	0.4167	0.7500	0.5833	0.5833	0.7500	0.9167
G24	0.7500	0.6667	0.5000	0.7500	0.7500	0.7500	0.5833	0.8333	0.3333	0.7500	0.7500	0.8333

Legend: G1 (Kalo Boro), G2 (Asami Boro), G3 (Pabdafor), G4 (Amania), G5 (Khaiya Boro), G6 (Rata), G7 (Lakhai Boruni), G8 (Sada Boro), G9 (Sona Biron), G10 (Rajkumar), G11 (Dol Boro), G12 (Boro Dhan, Indian), G13 (Tepi Boro Dhan, Kalo), G14 (Dhali Boro 7/2), G15 (Black Rice), G16 (Dhali Boro 74/3), G17 (Boro Habj 1), G18 (Vawailia), G19 (Kali Boro), G20 (Jira), G21 (Dhali Boro 87/1), G22 (Dhali Boro 94), G23 (Dhali Boro 104/1), G24 (Dhali Boro 105/2).

Table 8: (Continued) Summary of Nei's genetic distance (below diagonal) values among 24 Boro rice genotypes

Genotypes	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
G1												
G2												
G3												
G4												
G5												
G6												
G7												
G8												
G9												
G10												
G11												
G12												
G13	0.0000											
G14	0.3333	0.0000										
G15	0.2500	0.2500	0.0000									
G16	0.3333	0.1667	0.2500	0.0000								
G17	0.3333	0.3333	0.1667	0.3333	0.0000							
G18	0.2500	0.4167	0.4167	0.4167	0.5000	0.0000						
G19	0.1667	0.3333	0.3333	0.3333	0.4167	0.0833	0.0000					
G20	0.5000	0.4167	0.5000	0.4167	0.5000	0.5833	0.5000	0.0000				
G21	0.3333	0.3333	0.2500	0.3333	0.4167	0.3333	0.3333	0.5833	0.0000			
G22	0.4167	0.2500	0.3333	0.1667	0.1667	0.5000	0.4167	0.4167	0.4167	0.0000		
G23	0.7500	0.7500	0.7500	0.6667	0.7500	0.8333	0.7500	0.5833	0.8333	0.7500	0.0000	
G24	0.7500	0.7500	0.7500	0.7500	0.7500	0.7500	0.7500	0.5833	0.7500	0.7500	0.7500	0.0000

Legend: G1 (Kalo Boro), G2 (Asami Boro), G3 (Pabdafor), G4 (Amania), G5 (Khaiya Boro), G6 (Rata), G7 (Lakhai Boruni), G8 (Sada Boro), G9 (Sona Biron), G10 (Rajkumar), G11 (Dol Boro), G12 (Boro Dhan, Indian), G13 (Tepi Boro Dhan, Kalo), G14 (Dhali Boro 7/2), G15 (Black Rice), G16 (Dhali Boro 74/3), G17 (Boro Habj 1), G18 (Vawailia), G19 (Kali Boro), G20 (Jira), G21 (Dhali Boro 87/1), G22 (Dhali Boro 94), G23 (Dhali Boro 104/1), G24 (Dhali Boro 105/2).

4.5 UPGMA dendrogram

On the basis of the Nei's genetic distance calculation of 24 Boro rice germplasm , a dendrogram was calculated. Unweighted Pair Group Method of Arithmetic Mean (UPGMA) indicated the segregation of 24 rice genotypes into three main clusters: G12, G19, G21, G23 were grouped in cluster I (Figure 9); G5, G7, G8, G9, G10, G11, G14, G15, G16, G17, G18, G24 were grouped in cluster II and G1, G2, G3, G4, G6, G13, G20, G22 were grouped in cluster III. Here, "G" indicated the Boro rice genotypes.

In cluster I, G19 (Kali Boro), G23 (Dhali Boro 104/1) formed sub cluster 1; again, G12 (Boro Dhan, Indian), G21 (Dhali Boro 87/1) grouped together in sub cluster 2. Here, G7 (Lakhai Boruni), G14 (Dhali Boro 7/2), G24 (Dhali Boro 105/2) formed sub cluster 1, G5 (Khaiya Boro), G8(Sada Boro), G9 (Sona Biron), G10 (Rajkumar), G11 (Dol Boro), G15 (Black Rice), G16 (Dhali Boro 74/3), G17 (Boro Habj 1), G18 (Vawailia) formed sub cluster 2 of cluster II. Again, G2 (Asami Boro), G6 (Rata), G22 (Dhali Boro 94) formed sub cluster 1 and G1 (Kalo Boro), G3 (pabdafor), G4 (Amania), G13 (Tepi Boro Dhan, Kalo), G20 (Jira) formed sub cluster 2 of cluster III. In this dendrogram, rice genotypes of more genetic similarity are placed in same sub cluster. The dendrogram revealed that the genotypes that derivatives of genetically similar type clustered together.

The genotypes grouped in the same cluster due to lower genetic distance and the other genotypes having more genetic dissimilarity grouped in another cluster due to higher genetic distance. It is clear that G12 (Boro Dhan, Indian), G21 (Dhali Boro 87/1), G19 (Kali Boro), G23 (Dhali Boro 104/1) are more different from most of the genotypes. They can be easily used for rice improvement program.

Yasmin *et al., (*2012) reported that genetic diversity of 24 rice (*Oryza sativa*) genotypes comprising five salt tolerant, one tidal submergence tolerant, 9 high yielding inbred and 10 hybrid rice varieties with one hundred SSR markers. The highest number of alleles were produced for RM1024 followed by RM6959 (21), RM154 (20), RM540 (19) and RM2518 (19) .The polymorphism information content (PIC) value ranged from 0.588 (RM38) to 0.927 (RM1024), with an average of 0.852. UPGMA-cluster-analysis based on genetic distance coefficients clearly separated all the genotypes into five main distinct clusters.

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

Cluster	Sub cluster	Genotypes
I	1	G19 (Kali Boro), G23 (Dhali Boro 104/1)
	2	G12 (Boro Dhan, Indian), G21 (Dhali Boro 87/1)
II	1	G7 (Lakhai Boruni), G14 (Dhali Boro 7/2), G24 (Dhali Boro 105/2)
	2	G5 (Khaiya Boro), G8 (Sada Boro), G9 (Sona Biron), G10 (Rajkumar), G11 (Dol Boro), G15 (Black Rice), G16 (Dhali Boro 74/3), G17(Boro Habj1), G18 (Vawailia)
III	1	G2 (Asami Boro),G6(Rata), G22 (Dhali Boro 94)
	2	G1 (Kalo Boro), G3 (Pabdafor), G4 (Amania), G13 (Tepi Boro Dhan, Kalo), G20 (Jira)

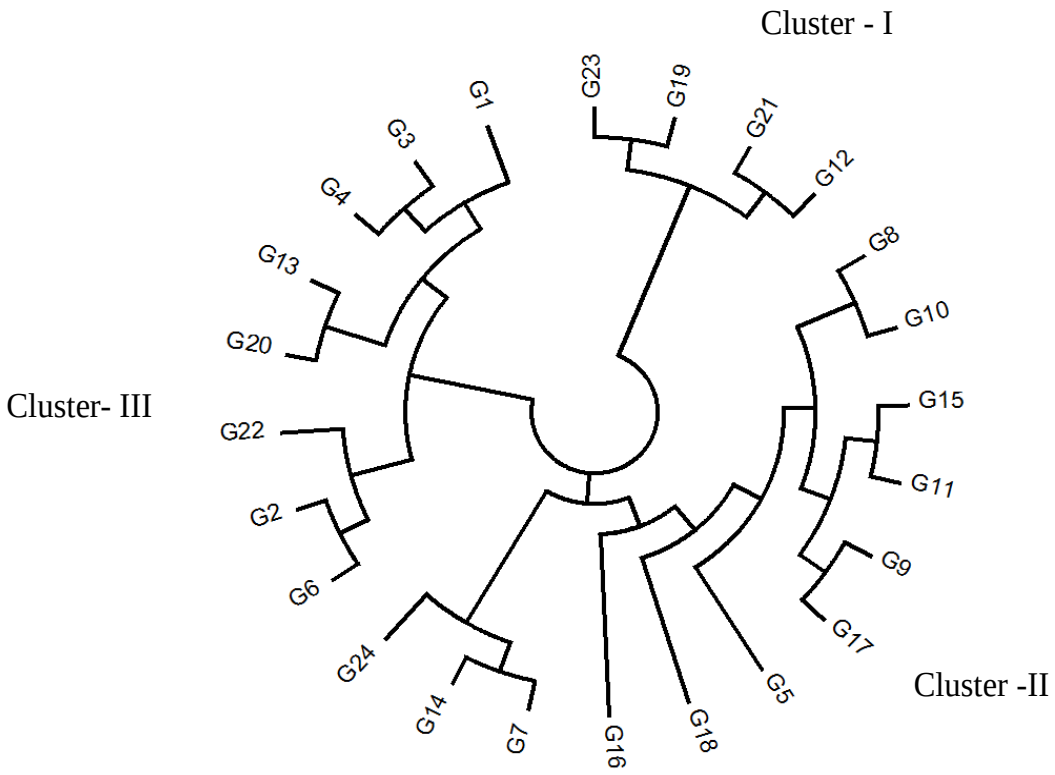

Figure 9. Unweighted pair group method of arithmetic mean (UPGMA) dendrogram based on Nei's (1983) genetic distance, summarizing the data on differentiation among 24 Boro rice genotypes using MEGA 5.1 version.

CHAPTER V

SUMMARY AND CONCLUSION

In Bangladesh, rice is central crop to agriculture and the national economy. Boro rice is the most important and single largest crop in Bangladesh in respect of volume of production. It has been persistently contributing to higher rice production in last successive years. The experiment was conducted to assess the genetic variation and diversity and genetic relatedness among 24 Boro rice landraces by using twelve highly polymorphic SSR markers. The experiment was carried out in the Genetic Resources and Seed Division (GRSD), Bangladesh Rice Research Institute (BRRI), Joydebpur, Gazipur-1701.

Twenty four Boro rice genotypes were used for PCR amplification. Twelve SSR primers were applied for polymorphism study. The primers were RM1, RM206, RM207, RM253, RM304, RM252, RM1337, RM320, RM205, RM447, RM3646, RM413 showed reproducible and distinct polymorphic amplification.

The primers were produced fifty four alleles, ranged from 1 to 8 alleles per locus and an average of 4.5 were detected. Forty five polymorphic alleles were also identified (Table 6). The frequency of the major allele ranged between 16.67 (RM206) to 95.83 (RM447). Polymorphic Information Content (PIC) value for 12 SSR markers ranged from 0.08 (RM447) to 0.84 (RM206) and the average PIC value was 0.49. Gene diversity ranged between 0.08 to 0.86 with an average of 0.52. Primer RM206 showed highest gene diversity (0.86) and primer RM447 showed the lowest gene diversity (0.08). PIC value revealed that RM206 was considered as the best marker for 24 rice germplasm followed by others and RM447 was considered as the least powerful marker for those genotypes.

The value of pair-wise comparisons of Nei's (1983) genetic distance among 24 relatives of rice genotypes were computed from combined data for the 12 primers. Genetic distance obtained with a range between 0.1667 to 0.9167 with an average of 0.5417. Though higher genetic distance (0.9167) was observed between a number of genotypes, Sona Biron showed highest genetic dissimilarity with maximum number of genotypes.

It can be concluded that SSR markers are the powerful tools to detect genetic variation and genetic relationship within and among different rice genotypes. These markers are unmasking new genes for the improvement of crop varieties assessment of genetic diversity, genome mapping, fingerprinting, determine the genetic structure, gene tagging and for marker-assisted selection (MAS). The study result can become a guideline for further research on genetic variation and diversity analysis of rice landraces of Bangladesh and other countries.

RECOMMENDATIONS

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

- A large number of SSR markers along with other markers like RAPD, ISSR etc could be used for obtaining more precise and diverse result.
- A large number of germplasm could be included for getting more diverse result.
- To get more precise result high throughput molecular markers such as Single Nucleotide Polymorphism (SNP) could be used for genome-wide coverage of rice germplasm.

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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 H_2O	118.6mL

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

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

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

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

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

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

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

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

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

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

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

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

I(h): Primer dilution

Primer was centrifuged at 13000 rpm for 5 min.

↓

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

↓

Kept overnight at 4^0 C

↓

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

↓

Stored at -20^0 C freezer

I(i): PCR cocktail for 96 samples

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

Appendix II: Similarity and distance indices below data

	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16	G17
	G18	G19	G20	G21	G22	G23	G24										
G1	0		26.962938		34.263683		35.693137		64.606501		28.124722		22.293497		62.417946		
	98.620485		62.721607		92.585096		33.867388		23.280893		22.293497		89.403579		28.600699		
	97.25739		48.815981		26.400758		22.693611		32.41913		33.837849		19		29.342802		
G2	26.962938		0		28.757608		30.446675		71.25307		9.2736185		42.237424		69.562921		95.577194
	67.446275		84.882271		51.672043		24.879711		42.237424		91.760558		44.034078		100.82658		
	49.315312		46.411206		27.820855		52.019227		21.771541		35.91657		43.220366				
G3	34.263683		28.757608		0		10		71.119618		28.124722		40.261644		71.83314		94.625578
	71.40028		95.163018		40.459857		19.79899		40.261644		96.213305		35.888717		100.51368		
	68.490875		42.673177		26.172505		43.874822		28.722813		36.945906		31				
G4	35.693137		30.446675		10		0		70.41306		28.124722		39		71.133677		98.051007
	72.097157		97.549987		39.204592		17.088007		39		96.213305		34.467376		103.74488		
	67.756918		41.484937		24.186773		42.720019		26.925824		35.566838		29.342802				
G5	64.606501		71.25307		71.119618		70.413067		0		71.812255		57.801384		64		95.215545
	49.071377		94.910484		54.616847		66.648331		57.801384		82.127949		54.405882		87.85784		
	68.22756		53.953684		68.051451		53.730811		76.439519		56.771472		62.617889				
G6	28.124722		9.2736185		28.124722		28.124722		71.812255		0		39.698866		66.355105		97.58586
	66.640828		86.977008		49.618545		20.273135		39.698866		92.46621		43.30127		102.73266		
	48.6621		44.11349		22.226111		49.979996		13.928388		32.893768		39.849718				
G7	22.293497		42.237424		40.261644		39		57.801384		39.698866		0		55.830099		101.93625
	59.858166		99.744674		17.029386		26.776856		0		88.487287		17.748239		97.590983		56.674509
	7.0710678		24.859606		13.928388		41.06093		8.6023253		17.088007						

Appendix II (Cont'd): Similarity and distance indices below data

G8	62.417946	69.562921	71.83314	71.133677	64	66.355105	55.830099	0	95.947903
	28.94823	102.44999	59.791304	62.193247	55.830099	84.208076	62.513998	90.846024	
	64.707032	55.919585	59.084685	58.472216	64.08588	54.616847	57.801384		
G9	98.620485	95.577194	94.625578	98.051007	95.215545	97.58586	101.93625	95.947903	0
	85.358069	56.586217	101.50369	98.96464	101.93625	46.808119	99.478641	32.449961	
	106.9065	101.88719	100.75217	102.44511	101.0198	100.22475	100.65287		
G10	62.721607	67.446275	71.40028	72.097157	49.071377	66.640828	59.858166	28.94823	
	85.358069	0	92.455395	62.777384	65.069194	59.858166	76.177424	62.593929	79.580148
	58.779248	59.101607	63.914005	61.522354	68.563839	57.870545	63.52165		
G11	92.585096	84.882271	95.163018	97.549987	94.910484	86.977008	99.744674	102.44999	
	56.586217	92.455395	0	105.17129	94.86833	99.744674	56.347138	100.33942	61.733297
	86.446515	101.08907	95.859272	104.7139	92.363413	95.973955	102.61092		
G12	33.867388	51.672043	40.459857	39.204592	54.616847	49.618545	17.029386	59.791304	
	101.50369	62.777384	105.17129	0	32.939338	17.029386	91.038453	15.588457	96.81942
	69.656299	13.038405	33.105891	6	49.618545	21.954498	15.165751		
G13	23.280893	24.879711	19.79899	17.088007	66.648331	20.273135	26.776856	62.193247	
	98.96464	65.069194	94.86833	32.939338	0	26.776856	92.633687	29.664794	101.6612
	55.668663	30.282008	7.8102497	34.365681	19	21.470911	22.293497		
G14	22.293497	42.237424	40.261644	39	57.801384	39.698866	0	55.830099	101.93625
	59.858166	99.744674	17.029386	26.776856	0	88.487287	17.748239	97.590983	56.674509
	7.0710678	24.859606	13.928388	41.06093	8.6023253	17.088007			
G15	89.403579	91.760558	96.213305	96.213305	82.127949	92.46621	88.487287	84.208076	
	46.808119	76.177424	56.347138	91.038453	92.633687	88.487287	0	87.743946	39.874804
	89.319651	88.543774	93.38094	90.177603	96.622979	87.726849	92.574294		

Appendix II (Cont'd): Similarity and distance indices below data

G16	28.600699	44.034078	35.888717	34.467376	54.405882	43.30127	17.748239	62.513998	
	99.478641	62.593929	100.33942	15.588457	29.664794	17.748239	87.743946	0	95.325757
	64.272856	17.748239	32.171416	16.703293	46.508064	21.424285	19.974984		
G17	97.25739	100.82658	100.51368	103.74488	87.85784	102.73266	97.590983	90.846024	
	32.449961	79.580148	61.733297	96.81942	101.6612	97.590983	39.874804	95.325757	0
	104.37433	96.509067	102.47927	96.633328	106.89247	97.252249	99.357939		
G18	48.815981	49.315312	68.490875	67.756918	68.22756	48.6621	56.674509	64.707032	
	106.9065	58.779248	86.446515	69.656299	55.668663	56.674509	89.319651	64.272856	
	104.37433	0	59.34644	53.702886	66.663333	55.00909	51.555795	66.932802	
G19	26.400758	46.411206	42.673177	41.484937	53.953684	44.11349	7.0710678	55.919585	
	101.88719	59.101607	101.08907	13.038405	30.282008	7.0710678	88.543774	17.748239	
	96.509067	59.34644	0	28.600699	8.6023253	45.343136	12	18.493242	
G20	22.693611	27.820855	26.172505	24.186773	68.051451	22.226111	24.859606	59.084685	
	100.75217	63.914005	95.859272	33.105891	7.8102497	24.859606	93.38094	32.171416	
	102.47927	53.702886	28.600699	0	33.645208	18.708287	19.026298	22.045408	
G21	32.41913	52.019227	43.874822	42.720019	53.730811	49.979996	13.928388	58.472216	
	102.44511	61.522354	104.7139	6	34.365681	13.928388	90.177603	16.703293	96.633328
	66.663333	8.6023253	33.645208	0	50.576674	19.646883	18.05547		
G22	33.837849	21.771541	28.722813	26.925824	76.439519	13.928388	41.06093	64.08588	
	101.0198	68.563839	92.363413	49.618545	19	41.06093	96.622979	46.508064	106.89247
	55.00909	45.343136	18.708287	50.576674	0	34.525353	37.336309		

Appendix II (Cont'd): Similarity and distance indices below data

G23	19	35.91657	36.945906	35.566838	56.771472	32.893768	8.6023253	54.616847	100.22475
	57.870545	95.973955	21.954498	21.470911	8.6023253	87.726849	21.424285	97.252249	
	51.555795	12	19.026298	19.646883	34.525353	0	19.131126		
G24	29.342802	43.220366	31	29.342802	62.617889	39.849718	17.088007	57.801384	
	100.65287	63.52165	102.61092	15.165751	22.293497	17.088007	92.574294	19.974984	
	99.357939	66.932802	18.493242	22.045408	18.05547	37.336309	19.131126	0	