POLYMORPHISM STUDY AND MOLECULAR DIVERSITY ANALYSIS OF DIFFERENT HYBRID VARIETIES OF RICE (*Oryza sativa* L.) THROUGH RAPD AND SSR MARKERS

ABSANA ISLAM

DEPARTMENT OF BIOTECHNOLOGY

SHER-E-BANGLA AGRICULTURAL UNIVERSITY

DHAKA-1207

June, 2018

POLYMORPHISM STUDY AND MOLECULAR DIVERSITY ANALYSIS OF DIFFERENT HYBRID VARIETIES OF RICE (*Oryza sativa* L.) THROUGH RAPD AND SSR MARKERS

BY

ABSANA ISLAM

Registration No: 17-08290

A Thesis Submitted to
The Department of Biotechnology, Faculty of Agriculture
Sher-e-Bangla Agricultural University, Dhaka-1207
In partial fulfillment of the requirements
For the degree of

MASTER OF SCIENCE (MS)
IN
BIOTECHNOLOGY

SEMESTER: JANUARY-JUNE, 2018

Approved by

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

Homayra Huq
Associate Professor
Department of Biotechnology
Sher-e-Bangla Agricultural University
Dhaka-1207
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
Sher-e-Bangla Nagar, Dhaka-1207

CERTIFICATE

This is to certify that the thesis entitled ***"POLYMORPHISM STUDY AND MOLECULAR DIVERSITY ANALYSIS OF DIFFERENT HYBRID VARIETIES OF RICE (Oryza sativa L.) THROUGH RAPD AND SSR MARKERS"*** *submitted to the Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of* ***MASTER OF SCIENCE (MS) in BIOTECHNOLOGY****, embodies the result of a piece of bona fide research work carried out by* ***ABSANA ISLAM,*** *Registration No.* ***17-08290*** *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 during the course of this investigation has been duly acknowledged and style of this thesis have been approved and recommended for submission.

Dated: June, 2018
Place: Dhaka, Bangladesh

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

ABBREVIATIONS

FULL WORD	ABBREVIATION
Agricultural	Agril.
Agriculture	Agric
American	Am.
Amplified Fragment Length Polymorphism	AFLP
And others (at elli)	*et al.*
As for example	e.g.
Base pair	bp
Biology	Biol.
Biotechnology	Biotech
Botany	Bot.
Continued	Cont"d
Degree Celsius	^{o}C
Deoxyribonucleic acid	DNA
Distilled deionized water	ddH_20
Etcetera	etc
Ethidim Bromide	Et-Br
Ethylene Diamine Tetra Acetic Acid	EDTA
Genetics	*Genet.*
Government	Govt.
Gram	g
Gram per Litre	g/L
International	*Intl.*
Journal	J.
Marker assisted breeding	MAS
Micro liter	µl

ABBREVIATIONS (Cont'd)

FULL WORD	ABBREVIATION
Mili Litre	ml
Mili metre	mm
Mili mole	mM
Molecular	Mol
Namely	*viz.*
Negative logarithm of hydrogen ion concentration (-log[H+])	pH
Percent	%
Polymerase chain reaction	PCR
Polymorphic information content	PIC
Random Amplified Polymorphic DNA	RAPD
Restriction Fragment Length Polymorphism	RFLP
Research	Res.
Rotation per minute	rpm
Science	*Sci*.
Single Nucleotide Polymorphism	SNP
Simple Sequence Repeat	SSR
Sodium chloride	NaCl
Sodium Dodecyl Sulphate	SDS
Species	Sp.
That is	i.e
Tris Boric Acid EDTA	TBE
Tris-EDTA	TE
Tons	t
Unweighted Pair Group of Arithmetic Mean	UPGMA
Ultra Violet	UV
Volt	V

ACKNOWLEDGEMENT

*All praises are due to **"Almighty Allah"** the Creator and the Sustainer of the world for bestowing mercy upon the author and for imbibing confidence on the author to complete this thesis leading to Master of Science (MS) degree in Biotecnlogy.*

This thesis would not have been possible without the assistance from many people who gave their support in different ways. To them I would like to convey my heartfelt gratitude and sincere appreciation.

*The author wishes to express her deep sense of gratitude, ever indebtedness and sincere appreciation to her Research Supervisor **Dr. Md. Ekramul Hoque,** Professor, Department of Biotecnology, Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh, for his constant supervision, scholastic guidance, constructive comments, invaluable advices and suggestions in conducting the research work and constructive criticism in preparing the manuscript.*

*The author wishes to record her deep sense of gratitude, sincere appreciation, enormous indebtedness and thanks to co-supervisor, **Humayra Huq**, Associate Professor, Department of Biotecnology, Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh, for her co-operation, kind comments, invaluable suggestions, encouragement and affectionate behavior that made it possible to complete this study and improve the manuscript.*

*The author wishes to express heartiest respect and profound appreciation to Assistant Professor **Fahima Khatun**, Department of Biotecnology, Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh, for her kind help and supports.*

The author would like to extend her special thanks to Professor Abdullahil Baque, Department of Agronomy, Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh, for providing experimental materials.

The author would like to express her deepest sense of gratitude, sincere appreciation and profound regards to Zahidul Islam, Senior Scientific Officer, GRS Division Bangladesh Rice Research Institute (BRRI), Joydebpur, Gazipur-1701 for giving valuable suggestions during data analysis and thesis paper preparation. Moreover, without his help in statistical computation it would be almost impossible to complete this thesis.

The author thankfully acknowledges the gratitude and respect to all the teachers and staffs of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh, for their valuable suggestions and sympathetic co-operation and inspirations throughout the period of the study.

The author is also grateful to the Ministry of Science and Technology, Govt. of the People's Republic of Bangladesh for granting a NST Fellowship to conduct the research work.

The author wishes to record her deep sense of gratitude and thanks to her friends, labmates and well wishers who always inspired her during her research for their kind help and all-out support in the entire period of the research work and writing thesis.

Finally, the author owes a debt of gratitude and esteemed respect to her beloved Mother Gulesa Islam, Father Md. Zahirul Islam, brother Asfaqul Islam for their blessings, unconditional love, ever willing help, patience, constant source of inspiration, all out sacrifice and moral support throughout the entire period of her academic life and their faith in her which always kept her focused on her objectives and helped to achieve her goals.

The author expresses her immense gratefulness to all of them who assisted and inspired her to achieve higher education and regret for her inability for not to mention every one by name.

Again the author thanks the Almighty for giving her patience and strength to overcome the difficulties which crossed her way in the accomplishment of the endeavor.

The Author

LIST OF CONTENTS

LIST OF CONTENTS (Cont'd)

LIST OF CONTENTS (Cont'd)

LIST OF TABLES

LIST OF PLATES

LIST OF PLATES (Cont'd)

LIST OF FIGURE

POLYMORPHISM STUDY AND MOLECULAR DIVERSITY ANALYSIS OF DIFFERENT HYBRID VARIETIES OF RICE (*Oryza sativa* L.) THROUGH RAPD AND SSR MARKERS

ABSTRACT

Rice (*Oryza sativa* L.) is one of the most important cereal crop around the world. It is the staple food in Bangladesh. This study was conducted in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of November 2018 to April 2019 to determine the molecular diversity among four rice hybrid varieties using Random Amplified Polymorphic DNA (RAPD) and Single Sequence Repeat (SSR) markers. A total of 21 RAPD primers and 4 SSR primers were used to determine polymorphism among the rice hybrids to produce scorable DNA bands. Total 82 bands were amplified through RAPD primers. Among them 26 were polymorphic bands. The range of DNA amplification varied from 1300-150 bp. The rate of polymorphism was obtained about 30.33%. Lower level of polymorphisms in hybrids indicated that there is a basic similarity among the varieties which is due to the common ancestor and the similarity of the selective traits. Genetic diversity ranged from 0 to 0.5000 and the frequency of major allele ranged from 0.5000 to 1.0000. Nei's genetic distance ranged from 0.1190 to 0.2262. The PIC value ranged from 0 to 0.3750 with the average value 0.1052. The PIC value indicates that the studied rice hybrids had low molecular diversity. A dendogram indicating the relative genetic similarity of the rice hybrids was constructed which followed three major clusters (A, B and C) among the studied material. Two SSR primers showed polymorphic band which can be used as an evidence of variety protection data. This experimental findings can be used for the protection of hybrids in commercial purpose and in managing rice genetic resource in Bangladesh.

CHAPTER I

INTRODUCTION

Rice (*Oryza sativa* L.) is a self-pollinated cereal crop. It belongs to the family Gramineae (synonym-Poaceae) having chromosome number 2n=24 under the Order Cyperales and Class Monocotyledon (Hooker, 1979). It is an annual, bisexual cereal crop. The genus *Oryza* includes a total of 22 to 27 species out of which only few are cultivated. *Oryza sativa* have been domesticated nearly 10000 years ago in northestern India, Bangladesh, Burma, Thailand, Vietnam and southern China. The only other cultivated species of rice *Oryza glaberrima* is indigenous to the upper valley of the Niger river in west Africa (Singh, 2000*). Oryza sativa* itself has three sub-species i.e., Indica, Japonica and Javanica.

Rice is an important food for about half of the world's population and 90% of it is being produced and consumed in Asia (Rao *et al*., 2016) and share maximum in grain production. In Bangladesh, rice engages more than 70 % of the rural population and is central to agriculture and the national economy. At present, rice alone constitutes about 93% of the total food grains produced annually in the country (BER, 2013). It provides about 62% of the calorie and 46% of the protein in the average daily diet of the people (HIES, 2010).

The population of Bangladesh is increasing at an alarming rate and the cultivable land is reducing due to urbanization and industrialization resulting in shortage of food. The nation is still adding about 2.3 million mouth every year to its total of 160 million people (Momin and Husain, 2009). Thus, the present population will swell progressively to 223 million by the year 2030 which will require additional 48 million tons of food grains (Julfiquar *et al*., 2008). Population growth demands a continuous increase in rice production in Bangladesh. So, the highest priority has been given to produce more rice (Bhuiyan, 2004). Production of rice has to be increased by at least 60% to meet up food requirement of the increasing population by the year 2020 (Masum, 2009).

Hybrid rice is the first generation (F1) crop grown from the cross of two distantly related rice varieties. Due to hybrid vigor, hybrid rice has 15-30% or more yield advantage over the conventional rice varieties that farmers grown (PANAP, 2007). Hybrid rice is one option for increasing the yield ceiling in rice over the best modern varieties. It is one of the viable and

proven technologies that have been considered as a new frontier to increase rice production for meeting growing demand for staple food in Bangladesh and it has greatly contributed to the growth of rice production in China (Lin, 1991 and 1994 and Virmani *et al*, 1998). Hybrid rice in China and other countries has yielded 20-30% higher than the best inbred varieties. Chinese experience showed that, with efforts to improve seed production techniques, the nationwide average yield of hybrid seed in China increased from 0.27 t ha-1 (1976) to 2.72 t ha-1 in 1997, with a record of 7.391 t ha-1 (Mao, 2006).

The development of hybrid rice technology in Bangladesh began in 1993. Combined efforts of farmers, rice scientists, extension personnel and Government of Bangladesh have enabled the country with a surplus of about 2 MT of rice in 2014-15. In the last few years (2009-10 to 2013-14), rice production has increased by o.34 MT per year (BBS, 2014). The current level of food grain production in Bangladesh is about 20.3 million tones, of which rice alone accounts for 18.04 million tones. Recent breakthroughs in tropical hybrid rice technology provide an economically viable option for raising the yield and sustaining future production growth (FAO, 2017). Now Bangladesh is the fourth largest producer and consumer of rice after China, India, and Indonesia. One hundred and eighteen Hybrid rice varieties have been released in Bangladesh by National Seed Board (NSB) during 1997 to October 2014. Out of these 118 varieties, 110 varieties are imported varieties. Only the source of 8 Hybrid rice varieties is Bangladesh of which BRRI has 4 and BRAC has 4 varieties. Out of the imported 110 varieties, the source of 93, 16 and 1 rice varieties are China, India and Philippines, respectively. Different companies, NGO, Farms, Seed industries, and public sector like BADC are importing the Hybrid rice seeds from abroad commercially. Upto 2010, there were 85 released Hybrid rice varieties. Within 4 years (2011-October 2014) another 33 varieties have been released (NSB, 2015).

Hybrid seed is the product of hybridization between two genetically dissimilar parents. Plants germinated from the hybrid seeds often become vigorous in growth. Hybrid vigor has been primarily exploited in cross-pollinated crops due to obvious advantage of pollinating system and ease of producing hybrid seeds. Hybrids do not bred true and they lose their yield advantage in subsequent generations. Therefore, farmers have to buy the hybrid seeds every year for continuing their production.

Moreover both breeders and farmers in many cases find the variations in their varieties and fields tend to take the advantages of appropriate selection technique in order to maintain the purity or even to screen for a new type. As the number of rice cultivars increases, the ability to distinguish them on the basis of morphological, molecular and biochemical traits becomes more difficult mostly due to genotype and environment interaction.

Molecular markers have been successfully applied in variety and cultivar identification (Mailer *et al*., 1994), or controls of seed purity of hybrid varieties (Marshall *et al*., 1994). The use of DNA markers has been suggested for precise and reliable characterization and discrimination of rice genotypes (Karkousis *et al*., 2003). Molecular markers based on DNA sequence are found to be more reliable (Raghunathachari *et al*., 2000; Karunagoda and Bandara, 2005; Mani *et at*., 2010 and Rasheed, 2005). They serve as a valuable guide for effective collection and use of genetic resources too. Molecular markers provide information that helps in deciding the distinctiveness of species and their ranking according to the number of close relatives and phylogenetic position. Moreover, varietal distinctiveness and relativeness can unambiguously be estimated by DNA fingerprinting in commercially important crops (Thomas *et al*., 2006).

Existance of genetic diversity is an essential requirement for successful hybridization program. Genetic diversity is necessary for crop improvement program as it helps in analyzing and establishing genetic relationship in accessions collection, its monitoring, identification of diverse parental combinations to create segregating progenies with high genetic variability and to obtain potential recombination for further selection and introgression of desirable genes from these diverse accessions (Ramadan *et al*., 2015; Thompson *et al*., 1998 and Islam *et al*., 2012). Determination of genetic diversity can be done by assessing morphological or molecular data. The use of advanced molecular technologies is one possible approach to understand their diversity. Evaluation of genetic diversity using DNA marker technology is non-destructive, not affected by environmental factors, requires small number of samples, and does not require large experimental setup and equipments for measuring physiological parameters (Kanawapee *et al*. 2011).

Recent advances in molecular biology enabled DNA based markers to study genetic basis of crops (Agarwal, 2003; Liu *et al*., 2007; Prabakaran *et al*., 2010 and Yasmin *et al*., 2012). Several types of molecular markers for example, restriction fragment length polymorphisms

(RFLP), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), simple sequence repeats (SSR) and single-nucleotide polymorphisms (SNPs) are now available to assess the variability and diversity at molecular level (Botstein *et al*., 1980; Williams *et al*., 1990; Vos *et al*., 1995; Singh, 2003; Abbas, 2000; Das, 2009; Saker *et al*., 2005; Tehrim *et al*., 2012 and Alessandra *et al*., 2009). Among these DNA fingerprinting methods, RAPD markers are increasingly being employed in genetic research owing to its speedy process and simplicity (Williams *et al*., 1990). This technique always allows the examination of genomic variation without prior knowledge of DNA sequences (Hadrys *et al*., 1992). The polymorphisms detected among the accessions are helpful in selecting genetically diverse cultivars in future breeding programme (Deepu *et al*., 2013).

RAPD (Randomly amplified polymorphic DNA) which is a PCR based marker has many advantages including readily being used, requiring minute amount of genomic DNA, does not need blotting and radioactive detection etc. RAPD markers are based on the amplification of unknown DNA sequences using single, short and random oligonucleotide sequences of arbitrary nature as primers (Chen *et. al*., 2007). RAPD analysis also showed promise as an effective tool in estimating genetic polymorphism in different rice hybrids.

On the other hand, microsatellites (SSRs) are the marker of choice because of their advantages over other markers. These markers are polymorphic, abundant in eukaryotic organisms and well distributed throughout the genome (Tautz, 1989; Morgante and Olivieri 1993). 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, including genetic mapping, germplasm surveys, and determination of the genetic structure and diversity patterns and for marker-assisted breeding (Panaud *et al*., 1996; Temnykh *et al*., 2000; Garris *et al*., 2005; Islam *et al*., 2008, 2012 and Yasmin *et al*., 2012). SSR markers are efficient in detecting genetic polymorphisms and discriminating among genotypes from germplasms of various sources, even they can detect finer level of variation among closely related breeding lines within a same variety (Lapitan *et al*., 2007).

Keeping in view the role of RAPD and SSR markers in the determination of genetic diversity, the present study has been undertaken to provide molecular diversity of different hybrid rice varieties by PCR amplification technique. Thus the present study was conducted with the following objectives:

1. Molecular diversity analysis of different hybrid rice varieties.
2. Polymorphism study among different hybrid rice varieties in Bangladesh.
3. Establishment of dendogram and phylogenetic relationship among the studied varieties.

CHAPTER II
REVIEW OF LITERATURE

Rice (*Oryza sativa* L.) is one of the most important cereal crops around the world. Rice belonging to the family Graminae and subfamily Oryzoidea is the staple food for one third of the world's population and occupies almost one-fifth of the total land area covered under cereals and main staple food of Bangladesh. Hybrid rice, one of the viable and proven technologies has been considered as a new frontier to increase rice production for meeting growing demand for staple food in Bangladesh.

Several researchers throughout the world have performed research activities on rice genetic diversity and relationship, phylogenetic study and characterization through molecular markers like Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeat (SSR) etc. Some of the research works also done in Bangladesh. As literature on genetic analysis of Bangladeshi rice is very scare, present study could help the researchers in this regard in future. Keeping in view the role of RAPD and SSR markers in the determination of genetic diversity, the present study was carried out to determine genetic diversity among different varieties of hybrid rice using RAPD and SSR markers and selection of genetically diverse genotypes for breeding programs. The most relevant literature about the present study has been reviewed and some of the relevant literatures are cited below.

2.1 The concept of molecular marker

Molecular markers are reliable tools to characterize the DNA profile of plant genotypes to study the genetic diversity. According to Datta *et al*. (2011) molecular markers are specific fragments of DNA that can be identified within the whole genome. Molecular markers are found at specific locations of the genome.

Molecular markers have been found to be more dependable than the phenotypic observations for evaluating the variations and in the assessment of the genetic stability (Leroy *et al.*, 2000)

and provide an efficient means to link phenotypic and genotypic variation (Varshney *et al*., 2005).

These methods are being very rapidly adopted by the researchers all over the world for the crop improvement. The molecular marker techniques are diverse and vary in principle, application and amount of polymorphism observed and in time requirements. Molecular markers present an efficient tool for fingerprinting of cultivars, and assessment of genetic resemblance and relationships (Vilanova *et al.*, 2012).

With the advent of molecular biology techniques, molecular markers are effective because they identify an abundance of genetic linkage between identifiable locations within a chromosome and are able to be repeated for verification. A molecular marker is a DNA sequence that can be readily detected and whose inheritance can easily be monitored. Amin *et al*. (2010) stated that, the use of molecular markers is based on naturally occurring DNA polymorphism, which forms the basis for designing strategies to exploit for applied purposes.

Molecular markers can be used for molecular characterization and detecting genetic variation and relationship of plants. These markers can detect the variation that arises from deletion, duplication, inversion, and/or insertion in the chromosomes. Such markers themselves do not affect the phenotype of the traits of interest because they are located only near or linked to genes controlling the traits (Mondini *et al.*, 2009).

According to Karp *et al.* (1997), DNA based marker is classified into three categories depending on technique used. Hybridization based DNA markers, arbitrarily primed polymerase chain reaction (PCR)-base markers, and sequence targeted and single locus DNA marker. Restriction Fragment Length Polymorphism (RFLP) is a hybridization based marker in which DNA polymorphism is detected by digesting DNA with restriction enzyme followed by DNA blotting and hybridizations with probes. Sequence Tagged Sites (STS), Sequence Repeat (SSRs), Single Nucleotide Polymorphism (SNPs) markers belongs to sequence targeted and single locus PCR based DNA markers. Of these, RFLP and micro satellites are co-dominant markers, while RAPD and AFLP markers are largely dominant markers.

The most interesting application of molecular markers is marker-assisted selection (MAS). They have proved to be excellent tools for assessment of genetic diversity in a wide range of plant species (Madhumati, 2014).

These markers are selectively neutral because they are usually located in non-coding regions of DNA. Usually located in non-coding regions of DNA. Unlike morphological and biochemical markers, DNA markers are practically unlimited in number and are not affected by environmental factors. Apart from the use of DNA markers in construction of linkage maps, they have numerous applications in plant breeding such as assessing the level of genetic diversity within cultivars and fingerprinting the germplasms. DNA markers are accepted widely as potentially valuable tools for crop breeding such as rice (Mackill *et al*., 1997 and McCouch *et al*., 1988), wheat and forage species (Jahufer *et al*., 2003).

Among the available molecular markers, microsatellites or simple sequence repeats (SSRs) which are tandem repeats of one to six nucleotide long DNA motifs, have gained considerable importance in plant genetics and breeding owing to many desirable genetic attributes including hypervariability, multiallelic nature, codominant inheritance, reproducibility, relative abundance, extensive genome coverage including organellar genomes, chromosome specific location and amenability to automation and high throughput genotyping (Kalia *et al*., 2011).

Molecular markers have successfully been applied in registration activities like cultivar identification (Mailer *et al*., 1994), or controls of seed purity of hybrid varieties (Marshall *et al*., 1994) and also for the variety identification as a part of seed and grain trade (Bligh *et al*. 1999). A powerful technique for DNA fingerprinting is successful Polymerase Chain Reaction (PCR) amplification of tandem repeat sequences, which have long been known to be polymorphic and widespread in plant genomes referred to as Simple Sequence Repeats (SSR) or Microsatellite polymorphism (Cregan, 1992; Morgante and Olivieri, 1993).

Conventional breeding is time consuming and depends on environmental conditions. Breeding a new variety takes 8 to 12 years. Molecular marker technology offers a possibility by adopting a wide range of novel approaches to improve the selection strategies in plant breeding (Gosal *et al*., 2010; Choudhary *et al*., 2008).

2.2. Concept of RAPD marker

Random Amplified Polymorphic DNA is a PCR-based technique discovered by Williams *et al*. (1990) and generated by the use of short (10-mer) synthetic oligonucleotides in single strand primer. Kumar and Gurusubramanian (2011) pointed that there is no need to know DNA sequence information for targeted gene. In this technique, a decamer primer of arbitrary sequence is allowed to anneal at a relatively low temperature priming the amplification of DNA fragments distributed at random in the genome (Williams *et al*., 1990).

The amplified products are visualized by separation on agarose gel and stained with ethidium bromide. They usually result in DNA fragment patterns that are polymorphic between genotypes, there by detecting diversity within them (Tommercup *et al*., 1998).

Vierling and Nguyen (1992) pointed out that, the polymorphism detected between amplification products of different individuals using the short, random, single primers made RAPD marker studies good for genetic diversity, genetic relationships, genetic mapping, plant breeding, DNA fingerprinting and population genetics.

There are several advantages of RAPDs compared to other DNA based techniques. It includes non-radioactive detection, multiple loci detection in single reaction, requirement of small quantity of DNA, no requirement of prior sequence information, quick and technically simple (Karp *et al*., 1997).

Main advantages of the RAPD technology include suitability for work on anonymous genomes, involves no blotting or hybridization steps, hence, it is quick, simple and efficient, applicability to problems where only limited quantities of DNA are available and Unit costs per assay are low compared to other marker technologies (Kumari and Thakur, 2014; Madhumati, 2014; Kumar and Gurusubramanian, 2011).

Hadrys *et al.,* 1992 stated that RAPD-PCR technique used for examining variation in the total genome. RAPD analysis is advantageous over isozyme electrophoresis because it generates much greater numbers of loci required for genetic analysis (Kimbeling *et al.,* 1996). RAPD markers can be used as supposedly unbiased; and neutral markers for genetic mapping applications (Michelmore *et al.,* 1991), in population genetics (Haig *et al.,* 1994), taxonomy (Chapco *et al.,* 1992) as well as for genetic diagnostics.

In spite of having many usefulness of RAPD marker it have some limitation. Because of random nature of genome sampling, the RAPD assay is not an appropriate technique when the difference between the two genomes is being compared is limited to an extremely small genomic fraction. The most unavoidable problem is dominancė of RAPD marker because the presence of given RAPD band does not distinguish whether its respect locus is homozygous or heterozygous or co-dominance which is possible when SSR marker is used (Rahman *et al.*, 2006).

Though having such weakness, the relative ease and speed the high degree of polymorphisms and virtually inexhaustible pool of possible genetic marker makes the RAPD technique advantageous over other molecular technique (Clark and Lanigan, 1993; Fristsch and Rieseberg, 1996). RAPD markers, in particular, have been successfully employed for determination of intra-species diversity in several plants, whereas fewer reports are available on determination of inter-species diversity (Goswami and Ranade, 1999).

2.3 Concept of SSR markers

Microsatellites (SSRs) are short tandem repeats of simple (1–6 nt) motifs. Their value for genetic analysis lies in their multi-allelism, co-dominant inheritance, relative abundance, genome coverage and suitability for high-throughput PCR-based platforms. They can serve as highly informative genetic markers, and in conjunction with the use of polymerase chain reaction (PCR) technology enable the detection of length variation (Powell, 1996).

In plants, it has been demonstrated that SSRs are highly informative, locus specific markers in many species (Akkaya *et al*., 1992; Lagarcrantz *et al*., 1993; Wu and Tanksley, 1993, Rahman *et al*., 2006 and 2007).

Microsatellite primers developed for one species can be used to detect polymorphism at homologous sites in related species. They have become one of the most useful molecular marker systems in plant breeding. The development of SSR markers from genomic libraries is expensive and inefficient (Squirrell *et al.*, 2003).

SSR markers are non-isotype based precise PCR technology. Stretches of DNA, consisting of randomly repeating small nucleotide units and conserved regions flanking the repeats are

suitable for designing PCR primer pairs. Used for amplifying the intervening repeat loci were first referred to as Microsatellites by Litt and Luty (1989).

In rice, microsatellites are abundant and well distributed throughout the genome (Akagi *et al*., 1996; McCouch *et al*., 1997; Wu and Tanksley, 1993). They are valuable as genetic markers because they are co-dominant in nature, detect high levels of allelic diversity, and are assayed efficiently by the PCR (McCouch *et al*., 2002).

SSR is a marker of choice for molecular characterization as it is co-dominant, distributed throughout the genome, highly reproducible, variable, reliable, easily scorable, abundant and multiallelic in nature (Salgotra *et al*., 2015).

SSR markers have been used by many researchers (Das *et al*., 2013; Jin *et al*., 2010; Sow *et al*., 2014) for characterization of rice varieties. SSR markers even in less number can give a better genetic diversity spectrum due to their multi allelic and highly polymorphic nature (McCouch *et al*., 1997).

Simple sequence repeat (SSR) is an important tool for genetic variation identification of accessions (Sajib *et al*., 2012 and Ma *et al*., 2011). SSR marker are highly informative, mostly monolocus, co-dominant, easily analyzed and cost effective (Gracia *et al*., 2004) and able to detect high level of allelic diversity (Ni *et al*., 2002), thus being widely applied in genetic diversity analysis, molecular map construction and gene mapping (Zhang *et al*., 2007 and Ma *et al*., 2011) and analysis of germplasm diversity (Zhou *et al*., 2003; Jin *et al*., 2010 and Ma *et al*., 2011).

SSR markers even in less number can give a better genetic diversity spectrum due to their multi-allelic and highly polymorphic nature (Singh *et al*., 2016).

The current level of average genome-wide coverage provided by micro- satellites in rice, one marker every 6 centimorgans (Temnykh *et al*., 2000), is sufficient to be useful for assessment of hybrid seed purity and for genotype identification. Akagi *et al*. (1997) suggested that hyper-variable microsatellites could be used to classify individual rice cultivars and to maintain the purity of rice seeds by eliminating contamination. For characterization and

documentation, this technique has been recently used in 20 crop species including rice, wheat, maize, barley, rapeseed, soybean, potato and other crops by Rahman *et al*. (2007).

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).

SSR markers are efficient in detecting genetic polymorphisms and discriminating among genotypes from germplasms of various sources, even they can detect finer level of variation among closely related breeding lines within a same variety (Lapitan *et al*., 2007). For rice, 18,828 SSR markers throughout the whole rice genome are now available (IRGSP, 2005).

Over the last few centuries, rice has faced diversity loss (Choudhary *et al*., 2013) especially, after the green revolution due to replacement of native varieties with high yielding varieties (Heal *et al*., 2004).

Among various PCR-based markers, microsatellites (SSRs) are more appropriate and successfully used for assessing genetic diversity among closely related rice cultivars compared to other molecular markers; because it can be simply amplified by PCR reaction, abundant, highly informative, mostly mono locus, co-dominant, easily analyzed, cost effective and it can identify higher degree polymorphism in rice (Siddique *et al*., 2016).

2.4 Genetic diversity studies in rice genotypes by RAPD and SSR markers

Genetic diversity study is of prime important in conservation of endangered species and utilization of appropriate plant resources from diverse germplasm. Successful breeding for crop development programmes depends on genetic variability that arises from genetic diversity (Rana and Bhat, 2004). Lack of genetic variability may limit breeding progress and gain from selection. So, knowledge of the genetic diversity of any germplasm collection provides a basis for improvement of crops and development of superior cultivars.

Morphological features are indicative of the genotype but are represented by only a few loci because they are not large enough. Moreover, they can also be affected by environmental factors and cultural practices. To have an accurate and reliable estimate of genetic relationships and genetic diversity assessment, there is a need of polymorphic molecular markers. Therefore, RAPD and SSR techniques (Williams *et al.*, 1990) provides unlimited number of marker loci that can be used for genetic diversity and genetic recombination analysis. It was successfully applied to characterize germplasm lines developed through interspecific hybridization. RAPD and SSR markers have been used for the estimation of genetic similarities and cultivar analysis for introgressed genes through amplified genomic regions.

Mitra *et al*., 2017 studied the genetic diversity and the relationship among 14 Aman rice genotypes by using 15 RAPD primers. A considerable level of variability was observed among different cultivars. A total of 191 reproducible and scorable amplification products were generated across 14 varieties. Out of 191 bands, 153 (80%) were found to be polymorphic. Primers OPB-03 resulted in the highest percentage of polymorphic bands, while the minimum polymorphism was observed using OPA-03 primer. RAPD analyses in *Oryza sativa* accessions were also reported by Muhammad *et al*. (2005), Rahman *et al*. (2007), Malik *et al*. (2008). It was observed that the genetic distance were highly variable among 14 Aman rice varieties, ranged from 0.0373 to 0.5983 as revealed by the genetic distances matrix. Deepu *et al*. (2013) reported 0.19-0.54 genetic distances within Indian cultivars and Yeasmin *et al*. (2013) showed 0.088-0.504 within BRRI varieties. Garris *et al*. (2005) also found overall higher gene diversity for the indica group (0.55) and lower for japonica varieties (0.47). On the contrary, Thomson *et al*. (2007) reported as slightly higher index of gene diversity for the japonica group (0.56), while the indica group was 0.54.

Hasan and Raihan (2015) were studied thirty indigenous rice varieties from different regions of Bangladesh with four RAPD primers. Thirty three bands were generated from which 26 fragments were found to be polymorphic (78.79%).

Rabbani *et al*., 2008 performed an investigation which was carried out analysis of genetic diversity among a total of 40 traditional and improved cultivars of rice from Pakistan

through 25 RAPD markers. A total of 186 (89.4%) polymorphic bands were observed. The primer OPA-17 and OPB-17 gave highest number of polymorphic bands (15), while the minimum number of polymorphic bands (2) using OPA-05 primer. Cluster analysis based on Nei and Li's similarity coefficients using UPGMA grouped 40 cultivars into 3 main clusters I, II and III.

Rajani *et al*., 2013 was studied a considerable level of genetic diversity in ten different cultivars of rice. Among 30 RAPD primers, a total of 428 DNA fragments were generated by 25 primers out of which 363 were polymorphic. The percentage of polymorphism was calculated as 85.02%. The primer OPB-17 produced the maximum number of polymorphic bands, The dendogram classified the cultivars into two distinct clusters.

Ashraf *et al*. (2007) analyzed 34 accessions (aromatic and non-aromatic) to estimate the genetic diversity in the rice germplasm belonging to the South and Southeast Asian regions of diverse agro-climates. Fourteen RAPD primers were used for varietal identification. The 14 primers amplified a total of 108 DNA fragments. Of these, 66.7% bands were polymorphic and 33.3% were monomorphic in nature. The maximum polymorphism was showen by the primer OPJ-01.

Rahman *et al*., 2007 reported that a total of 24 samples (four for each cultivar) of six rice cultivars viz. Basmati 370, DM 25, IRATOM 24, Binadhan 6, TNDB 100 and Y 1281 from Bangladesh Institute of Nuclear Agriculture, Mymensingh were amplified through three RAPD markers. Total 26 bands were observed of which 14 (53.85%) were polymorphic. Primer OPD-05 produced maximum bands whereas OPD-06 produced minimum bands. UPGMA dendogram based on genetic distance grouped six cultivars of rice into three clusters following geographical proximately.

Islam *et al*. (2012) used SSR marker to generate polymorphic band among 14 rice genotypes. 14 rice genotypes were fingerprinted with 40 SSR markers and all markers were polymorphic. The total 168 alleles were detected. Polymorphism information content (PIC) value ranged from 0.21 to 0.76, with an average of 0.57. The highest PIC value was 0.76 and the lowest was 0.21. The gene diversity value ranged from 0.2449 to

0.7901 with an average value of 0.6231.

SSR markers have been widely applied in the genetic diversity analysis, molecular characterization, genotypic identification and population structure estimation in several rice genetic studies including basmati rice (Das *et al*., 2013; Choudhury *et al*., 2013; Allhgholipour *et al*., 2014; Singh *et al*., 2013; Shah *et al*., 2013 and Yadav *et al*., 2013).

Khalequzzaman *et al*. (2017) was investigated the allelic diversity existing among a collection of 31 Aus rice landraces from different district of Bangladesh using 36 SSR markers and 141 alleles were detected from 36 SSR. Similar number of microsatellite markers was previously used as subset for genetic diversity analysis of *Oryza sativa* (Garris *et al*., 2005; Chakrabarthia and Naravaneni, 2006 and Thomson *et al*., 2007). Islam (2014) also detected 140 alleles with an average of 3.11 among 113 aromatic rice accessions by using SSRs.

Sixty five rice genotypes were subjected to SSR marker assay to assess the molecular diversity. Out of twenty primers used, 19 produced reproducible and polymorphic pattern while one primer was monomorphic (Rashmi *et al*., 2017).

Siddique *et al*., 2014 conducted a study with twenty four rice genotypes of rainfed (T. aman) landraces which were successfully amplified with the five SSR primers. The polymorphism information content (PIC) values ranged from 0.65 to 0.91. The PIC values observed, were similar to previous estimates of microsattelite analysis in rice such as 0.34-0.88 (Thomson *et al*., 2007) and 0.20-0.90 with an average of 0.56 (Jain and McCouch, 2003).

Alam *et al*., 2016 performed a study to analyze the molecular diversity of 28 local rice (*Oryza sativa* L.) genotypes of Bangladesh using five SSR markers. All the amplified bands were found to be polymorphic.

CHAPTER III
MATERIALS AND METHODS

The chapter focused on the materials and methods of the experiment. The details of different materials and methodologies followed for the study have been described in this chapter.

3.1 Experimental site and time duration

The experiment was carried out at the Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka-1207, Bangladesh. The period of the experiment was November 2018 to April 2019.

3.2 Name and source of study materials

Four rice hybrids were used as experimental materials (Table 1). All the genotypes were collected from Nilsagor Seed Company Ltd, Dhaka, Bangladesh.

Table 1: Name of hybrids of different Rice genotypes

Sl. No.	Hybrid Name	Source
1.	Nilsagor Rice Hybrid1 (H1)	Nilsagor Seed Company Ltd, Dhaka, Bangladesh
2.	Nilsagor Rice Hybrid2 (H2)	Nilsagor Seed Company Ltd, Dhaka, Bangladesh
3.	Nilsagor Rice Hybrid3 (H3)	Nilsagor Seed Company Ltd, Dhaka, Bangladesh
4.	Nilsagor Rice Hybrid4 (H4)	Nilsagor Seed Company Ltd, Dhaka, Bangladesh

3.3 Collection of leaf sample

Good quality, disease free, healthy rice seeds were collected from Nilsagor Seed Company, Dhaka, Bangladesh. Seeds were soaked in water for one day. Seeds were then germinated in petridish and, after three days of germination, seedlings were sown in pots .Soil was collected

from nearby nursery and different pots were kept in the research farm of Sher-e-Bangla Agricultural University. Leaf samples were collected from 17-day-old seedlings. Those leaves were used as source of genomic DNA extraction.

3.4 Genomic DNA extraction

Genomic DNA was extracted from the leaf sample using appropriate protocol of Phenol-Chloroform-Isoamyl alcohol method described by Islam *et al.* (2013) with some modifications. The following reagents and methods were used for the isolation of total genomic DNA.

3.4.1 Equipments required

1. Morter and pestle
2. Water bath
3. Centrifuge
4. Vortex mixture
5. Ice maker
6. Micropipet
7. PCR machine
8. Electrophoresis system
9. Gel documentation system etc.

3.4.2 Reagents required

1. Extraction buffer, pH =8.0

Composition of extraction buffer is as follows:

- 1 M Tris HCl
- 0.5 M EDT A (Ethylene diamine tetra-acetic Acid) (pH =8.0)
- 5 M NaCI
- Distill H_20
- 1 % SDS (Sodium Dodecyl Sulphate)
- Marcapto-ethanol
- PVP (Polyvinylpyrrolidone)

2. Phenol : Chloroform: Isoamyl Alcohol = 25: 24 : 1
3. TE (Tris-EDTA) buffer, pH =8.0

Composition of extraction TE buffer are as follows:

- 1 M Tris HCI
- 0.5 M EDTA
- Distill H_20

4. Isopropanol

5. 0.3 M Sodium Acetate

6. Absolute (100%) ethanol

7. Ethanol (70%)

8. RNase

9. Ethidium Bromide Solution

3.4.3 Reagent preparation for DNA extraction

Stock solution for 1000 ml Extraction buffer

- 100 ml 1M Tris HCl (pH 8.0) was taken in a measuring cylinder.
- Then 40 ml of 0.5 M EDTA was added.
- 100 ml 5 M NaCl was mixed with the mixture.
- Finally sterilized dd.H_2O was added to make the volume upto 1000 ml.
- Then the mixture was mixed well and autoclaved.

Stock solution for 250 ml

1M Tris-HCI pH 8.0

- At first 30.28 g Tris was taken in a volumetric flask (500 ml)
- 100 ml dd.H_2O was added.
- pH was adjusted to 8.0 by adding HCl.
- Then sterilized dd.H_2O was added to make the volume up to 250 ml.
- The solution was autoclaved.

Stock solution for 250 ml

0.5 M EDTA pH 8.0

- At first 46.53 g EDTA.2H2O was added in a volumetric flask (500 ml)
- 100 ml dd.H_2O was added.
- Then 4 g NaOH was added.

- pH was adjusted to 8.0 with NaOH
- Then sterilized dd.H_2O was added to make the volume up to 250 ml.
- The solution was autoclaved.

Stock solution for 250 ml

5 M NaCl

- Firstly 73.05 g of NaCl was added in 250 ml dd.H_2O.
- It was then mixed well and autoclaved.
- The solution was autoclaved.

2 % SDS Stock solution for 100 ml

- 10 g of SDS was added in 100 ml of extraction buffer solution in a 250 ml beaker.
- As SDS is hazardous, so the mixture was mixed by a hot top magnetic stirrer well but not autoclaved.
- 20 ml of 10% that solution added in 80 ml dd.H_2O in a 250 ml beaker.

Stock solution for 100 ml TE buffer

- 1 ml Tris HCl (pH 8.0) was taken in a volumetric flask (250 ml).
- Then 0.2 ml EDTA (pH 8.0) was added.
- Sterilized dd.H_2O was added to make the volume up to 100 ml.

Composition of 5x TBE buffer (1 litre)

- 54 g Tris-HCl
- 27.5 g of Boric acid
- 4.65 g of EDTA
- pH= 8.3
- Added 1000 ml of dd.H_2O and pH was adjusted at 8.3.

Phenol: Chloroform: Isoamyl Alcohol = 25: 24: 1 (100 ml)

- At first 50 ml Phenol was taken in a volumetric flask (250 ml).
- Then 48 ml Chloroform was added.
- 2 ml Isoamyl Alcohol was also added and mixed well.
- The solution was stored at 4°C.

RNase

- 10 mg of RNase was added to 1 ml of dd.H_2O.
- Then it was dissolved completely with the help of necessary heat (at 65℃ in water bath for 30 minutes).

70% Ethanol (1000 ml)

- 700 ml absolute ethanol was mixed with 300 ml dd.H_2O

0.3 M Sodium Acetate

- 2.05 gm of Na acetate dissolved in 50 ml sterilized d.H_20 then we get 0.3M Na acetate.

3.5 Sequential steps for DNA extraction

1. For isolation of genomic DNA, vigorous, young, actively growing fresh leaf tissues were collected from 4 different rice hybrids.
2. Initially, healthy youngest leaves were washed thoroughly by tap water followed by washing with deionized water. Then sterilized by ethanol to remove wastes and any source of foreign DNA and leaves are then dried on tissue paper.
3. Approximately 150 mg of young leaves were cut into small pieces and then taken in morter. 600 µl of extraction buffer was added to it. The ground samples were taken into the 1.5 ml eppendorf tube and then it was vortexed for 20 seconds in a vortex mixture and then incubated at 65°C for 20 minutes in hot water bath.
4. Equal volume (600µl) of Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1) was added to the tube. Then it was vortexed for 20 seconds.
5. The solution was then centrifuged for 10 minutes at 13000 rpm. The supernatant was recovered using a micro pipette tip without disturbing the lower portion and transferred into a new eppendorf tube. Approximately 400-450 µl was taken and then equal volume of Chloroform: Isoamyl Alcohol (24: 1) was added to it. The solution was vortexed for 10 seconds.
6. Again the solution was centrifuged at 13000 rpm for 10 minutes.
7. The supernatant was taken in a separate eppendorf tube and the lower layer was discarded.

8. The amount of the solution was multiplied with 0.6 and then same volume of Isopropanol (0.6 volume of the liquid) was added.

9. It was then tapped by finger for 20-30 seconds (The genomic DNA was visible as cotton like structure).

10. After tapping the sample was again centrifuged at 13000 rpm for 15 minutes. The liquid was discarded completely and re-precipitation of the DNA solution was done by adding 500µl of absolute (100%) cold ethanol plus 20 µl 0.3 M Sodium acetate.

11. It was shaken gently. Tapping was done to separate pellet. The sample was centrifuged at 13000 rpm for 15 minutes. The liquid was removed completely by pouring and blotting the open tube end on fresh tissue paper.

12. The DNA pellet was then air dried for 2-3 hours. It was then dissolved in an appropriate volume (30 to 40 µl) of TE buffer and treated with 3µl of RNase at 37^0C in hot water bath for 15-20 minutes for removing RNA. Then it was spinned for 4-5 seconds.

13. Finally, the DNA samples were stored in freezer at -20°C.

3.6 Confirmation of extracted DNA

To confirm the extracted DNA sample 1% Agarose gel, working sample of each genomic DNA, 2x loading dye and de-ionized H_20 was needed.

3.6.1 Agarose gel preparation (1%)

Reagents

- Agarose powder
- 5x TBE buffer (pH 8.3)
- Ethidium Bromide

Gel preparation procedure

800g of agarose powder was taken in a 500 ml Erlenmeyer flask containing 100 ml electrophoresis buffer (1x TBE buffer) prepared by adding 20 ml of 5x TBE buffer in 80 ml of de-ionized water. The flask was enclosed with aluminum foil paper to prevent excessive evaporation. The flask was heated in a microwave oven for about 3 minutes with occasional swirling to generate uniform suspension until no arose particle was seen to generate homogenous and crystal clear suspension. The agarose solution was cooled to about 45-50^{0}C (flask was cool enough to hold comfortably with bare hand) and 1µl ethidium bromide (DNA stain) was added and mixed well by gentle shaking to make the DNA visible under ultraviolet light box (Trans-illuminator). The molten gel was poured immediately on to a clean gel bed (l5×15×2 cm^3 n size). That was placed on a level bench and appropriate comb was inserted parallel to the plate's edge with the bottom of the teeth about 2 mm above the plate. After 25 minutes, gel was completely cooled at room temperature and solidified and the comb was removed gently. The gel was then ready for loading the DNA samples.

3.6.2 Preparation of DNA samples for electrophoresis

The samples were all in the same concentration in buffer. For each sample, 3 µl de-ionized H_20 and 2 µl 2x loading dye (0.25% xylene ethanol, 0.25% bromophenol blue, 30% glycerol and 1mM EDTA) and 3.0 µl of sample DNA was taken in an eppendorf tube using 0.5-10 µl adjustable micropipette. Loading dye was used for monitoring loading and the progress of the electrophoresis and to increase the density of the sample so that it stayed in the well. Finally, 8.0 µl expected DNA was added to it and mixed well. The sample was, then loaded into the well of the gel and allowed them to sink to the bottom of the well. The gel was placed in the electrophoresis chamber keeping the gel horizontal and submerged in 1x TBE buffer (running buffer). The gel tank was covered and the electrophoresis power supply was connected and turned on to move DNA from negative to positive electrode (black to red) through the gel. Electrophoresis was carried out at 75 volt for about 60 minutes.

Table 2: DNA confirmation reagents with amount

Components	Amount (μl)
Working DNA sample	3.0
De-ionized water	3.0
2x loading dye	2.0
Total	**8.0**

3.6.3 Documentation of the DNA samples

The gel was carried out from the gel chamber and was placed on an ultraviolet light box (UV trans-illuminator) to examine and photographed by a GelCam camera, DNA samples showing better quality bands were taken for quantification and working solution preparation for next process.

3.7 Working solution of DNA samples preparation

DNA concentration were adjusted to 25 ng/μl for doing PCR using the following formula:

$V_1 \times S_1 = V_2 \times S_2$

Where,

V_1 = Initial volume of DNA solution (μl)

S_1 = Initial DNA concentration (ng/μl)

V_2 = Final volume of DNA solution (μl)

S_2 = Final DNA concentration (ng/μl)

3.8. Amplification of RAPD markers by PCR

3.8.1 Principle of RAPD primer amplification

For amplification of RAPD, a single oligonucleotide of arbitrary DNA sequence is mixed with genomic DNA in the presence of a thermo-stable DNA polymerase and a suitable buffer and then it is subjected to temperature cycling conditions typical to the Polymerase Chain Reaction (PCR). The products of the reaction depend on the sequence and length of the oligonucleotide, as well as the reaction conditions. At an appropriate annealing temperature the single primer binds to sites on opposite strands of the genomic DNA that are within an amplifiable distance of each other (e.g., within a few thousand nucleotides) and a discrete

DNA segment is produced. The presence or absence of this specific product, although amplified with an arbitrary primer, will be diagnostic for the oligonucleotide binding sites on the genomic DNA. In practice, the DNA amplification reaction is repeated on a set of DNA samples with several different primers, under conditions that result in several amplified bands from each primer. Often a single primer can be used to identify several polymorphisms, each of which matches to a different locus.

3.8.2 Selection of RAPD primers

Twenty seven RAPD primers were tested, they resulting in in faint or irreproducible DNA fragments. From them, twenty one primers were selected for this study. Twenty decamer RAPD primers were OPB 17, OPBA 03, OPBA 06, OPBB 03, OPBB 05, OPBB 06, OPBB 09, OPBB 12, OPBC 05, OPBC 14, OPBC 16, OPBD 16, OPBD 18, OPG 03, OPG 05, OPG 17, OPD 20, OPX 10, OPY 11, OPZ 01, OPZ 06 (Operon Technologies, INC., Alameda, California, USA) and it was screened for PCR reaction in 4 hybrid varieties of rice. The detail of RAPD primers are given in Table 3.

Table 3: Name of RAPD primers with GC content and sequence information

Sl. No.	Primer name	Sequence (5′ to 3′)	(G+C) %
1.	OPB-17	AGGGAACGAG	60
2.	OPBA-03	GTGCGAGAAC	60
3.	OPBA-06	GGACGACCGT	70
4.	OPBB-03	TCACGTGGCT	60
5.	OPBB-05	GGGCCGAACA	70
6.	OPBB-06	CTGAAGCTGG	60
7.	OPBB-09	AGGCCGGTCA	70
8.	OPBB-12	TTCGGCCGAC	70
9.	OPBC-05	GAGGCGATTG	60
10.	OPBC-14	GGTCCGACGA	70
11.	OPBC-16	CTGGTGCTCA	60
12.	OPBD-16	GAACTCCCAG	60

Cont'd

Sl. No.	Primer name	Sequence (5′ to 3′)	(G+C) %
13.	OPBD-18	ACGCACACTC	60
14.	OPD-20	ACCCGGTCAC	70
15.	OPG-03	GAGCCCTCCA	70
16.	OPG-05	CTGAGACGGA	60
17.	OPG 17	ACGACCGACA	60
18.	OPX-10	CCCTAGACTG	60
19.	OPY-11	AGACGATGGG	60
20.	OPZ-01	TCTGTGCCAC	60
21.	OPZ-06	GTGCCGTTCA	60

3.8.3 PCR amplification

PCR reactions were performed on each DNA sample. 2x Taq ready Master Mix was used. DNA amplification was per formed in oil-free thermal cycler (Esco Technologies swift™ Mini Thermal Cycler and Q-cycler). To prepare a 10.0 µl reaction mixture containing ready mix Taq DNA polymerase and other compositions were given in Table 4.

Table 4: PCR mixture composition for for each hybrid rice

Reagents	Amount(µl)
2x Taq Master Mix	5.00
RAPD primer	1.50
De-ionized water	1.50
Sample DNA	2.00
Total Reaction volume	**10.00**

From frozen stocks of the PCR reagents i.e., 2x Taq Master Mix, primer and DNA working samples were melt, mixed by vortexing and kept on ice for maintain good quality. DNA samples were pipetted first into PCR tubes compatible with the thermo-cycler used (0.2 ml). A pre-mixture was then prepared in the course of the following order: reaction mixture, DNA

sample and de-ionized water. Then the mixture was mixed up well and aliquoted into the tubes containing primer. The tubes were then sealed and placed in a thermal cycler and the cycling was started immediately.

3.8.4 Thermal profile for PCR

DNA amplification was performed in an oil-free thermal cycler (Esco Technologies Q-cycler). The PCR tubes were kept in the thermal cycler and the following programs were run:

Step- 1: 95 ˚C for 4 minutes – Initial denaturation

Step- 2: 95˚ C for 45 seconds – Denaturation

Step- 3: 35˚ C for 40 seconds – Primer annealing

Step- 4: 72˚ C for 1 minutes – Extension

Step- 5: 72˚ C for 5 minutes – Final extension

Step- 6: 4˚ C for 10 minutes – Hold

} 31 cycles

The amplified products were loaded on two per cent agarose gel using 50x TAE buffer stained with ethidium bromide along with marker (100bp Invitrogen). Electrophoresis was performed at 70V for two hours. The profile was visualized under UV transilluminator and documented using gel documentation system (BIORAD, USA). The documented RAPD profiles were carefully examined for amplification of DNA as bands. The size of polymorphic bands in kb/bp of bases were recorded in comparison with marker.

3.8.5 Electrophoresis of the amplified products

After completion of thermal cycler reaction, each sample of PCR products were confirmed by running 2% agarose gel containing 1 µl ethidium bromide in 1x TBE buffer at 75 V for 80 minutes. Loading dye (3.0 µl) was added to the PCR products and loaded in the wells. One molecular weight marker 100 bp DNA ladder (Bio Basic, Cat. No. M-1070-1, Canada) was also loaded on left side of the gel respectively. Under Ultra Violet (UV) light on a trans-illuminator RAPD bands were observed and documented by taking photograph using a Gel Cam Polaroid camera.

3.8.6 Documentation of the DNA samples

After staining, the gel was taken out carefully from the gel chamber and was placed on high performance ultraviolet light box (UV trans-illuminator) of gel documentation for checking the band of DNA and photographed by a Gel Cam Polaroid camera.

3.9 Data analysis

Since RAPD markers are dominant, we assumed that each band represented the phenotype at a single allelic locus (Williams *el at.*, 1990). One molecular weight marker, 100 bp DNA ladder (Bio Basic, Cat. No. M-1070, Canada) was used to estimate the size of the amplification products by comparing the distance traveled by each fragment with known sized fragments of molecular weight markers. All distinct bands or fragments (RAPD markers) were thereby given identification numbers according to their on gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. The band-size for each of the markers was scored using the Alpha Ease FC 4.0 software. The scores obtained using all primers in the RAPD analysis were then pooled to create a single data matrix. The individual fragments were assigned as alleles of the appropriate loci. This was used to estimate polymorphic loci using Power Marker version 3.25 software (Liu K. J., 2005). The summary statistics that were determined included the following: the number of alleles, the major allele size and its frequency, gene diversity, and the polymorphism information content (PIC) value**.** The allele frequency data from POWERMARKER was used to export the data in binary format (presence of allele as "1" and absence of allele as "0"). Binary data form of allele frequency used for dendogram construction by NTSYS-pc software (Rholf F., 2002). The unweighted pair grouping method, using arithmetic average (UPGMA), was used to determine similarity matrix following Dice coefficient with SAHN subprogram.

Polymorphic Information Content (PIC) value of a primer is calculated as:

$PIC = 1-\sum pi^2$

Where pi is the frequency of the i^{th} allele. Polymorphic Information Content was used to confirm the suitability of the primers selected for DNA fingerprinting.

Nei's genetic distance and genetic identity values were computed from frequencies of polymorphic markers to estimate genetic relationship among the studied four rice hybrids using the Unweighted Pair Group Method of Arithmetic Means (UPGMA) (Sneath and Sokal,

1973). The dendrogram was constructed using a computer program, POPGENE; (Version 1.31) (Yeh *et al*., 1999).

3.10 Amplification of SSR markers by PCR

3.10.1 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 abundant 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. The polymorphism can be detected by agarose gel electrophoresis if differences are large enough (agarose gels can detect differences greater than 10 base pair). SSRs were first used as markers for use in genetic mapping in humans (Litt *and Lutty*., 1989).

3.10.2 List of SSR primers

Five SSR primers were tested. From them four primers *viz*. RM 1, RM 17, RM 72 and OSR 16 were selected for PCR reaction on 4 hybrid rice varieties for their ability to produce polymorphic band.

Table 5: The list of SSR primers with their sequences and GC content

Sl. No.	Name of SSR primers	Sequences of the primer (5'-3')	(G+C) %
1.	RM 1	For. GCGAAAACACAATGCAAAAA Rev. GCGTTGGTTGGACCTGAC	47
2.	RM 17	For. TGCCCTGTTATTTTCTTCTCTC Rev. GGTGATCCTTTCCCATTTCA	43
3.	RM 72	For. CCGGCGATAAAACAATGAG Rev. GCATCGGTCCTAACTAAGGG	51
4.	OSR 16	For. AAAACTAGCTTGCAAAGGGGA Rev. TGCCGGCTGATCTTGTTCTC	49

3.10.3 PCR amplification

PCR reactions were performed on each DNA sample. 2x Taq ready Master Mix was used. DNA amplification was per formed in oil-free thermal cycler (Esco Technologies swift^{TM} Mini Thermal Cycler and Q-cycler). To prepare a 10.50 µl reaction mixture containing ready mix Taq DNA polymerase and other compositions were given in Table 6.

Table 6: Reaction mixture composition for PCR for each hybrid rice.

Reagents	Amount (µl)
2x Taq Master Mix	5.00
SSR Forward primer	1.25
SSR Reverse primer	1.25
De- ionized water	1.00
Sample DNA	2.00
Total Reaction volume	**10.50**

From frozen stocks of the PCR reagents i.e., 2x Taq Master Mix, primer and DNA working samples were melt, mixed by vortexing and kept on ice for maintain good quality. DNA samples were pipetted first into PCR tubes compatible with the thermo-cycler used (0.2 ml). A pre-mixture was then prepared in the course of the following order: reaction mixture, DNA sample and de-ionized water. Then the mixture was mixed up well and aliquoted into the tubes containing primer. The tubes were then sealed and placed in a thermal cycler and the cycling was started immediately.

3.10.4 Thermal profile for PCR

DNA amplification was performed in an oil-free thermal cycler (Esco Technologies Q-cycler). The PCR tubes were kept in the thermal cycler and the following programs were run:

Step- 1: 95 ˚C for 5 minutes – Initial denaturation

Step- 2: 95˚ C for 50 seconds – Denaturation

Step- 3: 60˚ C for 45 seconds – Primer annealing

Step- 4: 72˚ C for 1 minutes – Extension

Step- 5: 72˚ C for 5 minutes – Final extension

Step- 6: 4˚ C for 10 minutes – Hold

} 31 cycles

The amplified products were loaded on two per cent agarose gel using 50x TAE buffer stained with ethidium bromide along with marker (100bp Invitrogen). Electrophoresis was performed at 70V for two hours. The profile was visualized under UV transilluminator and documented using gel documentation system (BIORAD, USA). The documented SSR profiles were carefully examined for amplification of DNA as bands. The size of polymorphic bands in kb/bp of bases were recorded in comparison with marker.

3.10.5 Electrophoretic separation of the amplified products

PCR products for each sample were confirmed by running it in 2% agarose gel containing 1 µl ethidium bromide in 1x TBE buffer at 75 V for 1 hour. 3 µl loading dye was added to the PCR product and spinned them well. Then loaded them in the wells and one molecular weight maeker 100 bp DNA ladder (Bio Basic, Cat. No. M-1070-1, Canada) was also placed in left side of the gel. Under ultra-violet light on a trans-illuminator SSR bands were observed.

3.10.6 Documentation of PCR amplified DNA products:

The gel was taken out carefully from the gel chamber and was placed on high performance ultra-violet light box (UV trans-illuminator) of gel documentation for checking the DNA band and photographed by a Gel Cam Polaroid camera.

Since SSR markers are co-dominant hence, each band represented the phenotype at a single allelic locus (Williams *et al.*, 1990). One molecular weight marker, 100 bp (Bio Basic, Cat. No. M-1070-1, Canada) DNA ladder was used to estimate the size of the amplification products by comparing the distance traveled by each fragment with known sized fragments of

molecular weight markers. The band size for each markers was scored. The scores obtained for the SSR primers were then used to assess the polymorphism of different hybrids.

3.11 Precautions

- To maintain a strategic distance from all types of contaminations and keep DNA pure, all dishes, micropipette tips, eppendorf tubes, glass pipettes, deionized water and buffer solutions were legitimately autoclaved. Metal supplies i.e., scissors, forceps were cleaned with absolute ethanol.

- Since Ethidium Bromide (Et-Br) is an intense mutagen and carcinogenic in nature, hand gloves were utilized when taking care of anything that has been presented to Et-Br.

- Always power pack was kept turn off and the leads were unplugged before opening the electrophoresis unit to avoid electrical hazard.

- Eye protector was used while working with trans-illuminator as it produces UV radiation of 254 nm range which can cause eye damage.

- The common safety measures were kept up when performing PCR responses. All the disposables such as PCR tubes, tips, eppendorf tubes and reagents used during preparation of PCR reactions were autoclaved. Freezing condition was maintained when necessary. Hand gloves were worn amid treatment of PCR segments. Contamination of PCR segments was maintained a strategic distance from.

CHAPTER IV

RESULT AND DISCUSSION

This chapter comprises the presentation and discussion of the results of the experiment. The results were obtained from 4 hybrid varieties of rice using twenty one RAPD primers and four SSR primers. In the RAPD analysis significant genetic variation and polymorphisms was noticed in different hybrid rice varieties. The results of the experiment were presented and expressed in Table 7 to 9 and Plate 2 to 26 for ease of understanding.

4.1 Extraction of genomic DNA

The genomic DNA extraction of four hybrids rice was done by using the phenol-chloroform method with minor modification. RNA sharing was removed by applying RNase treatment. Finally, the purified DNA was stored at - 20^{o}C freezer for further use.

4.2 DNA confirmation

The extracted genomic DNA of four samples was loaded on 1% agarose gel for confirmation and quantification of DNA sample. It revealed that, all the samples showed clear DNA band in each well (Plate 1.). Hence, the genomic DNA of each sample was diluted on the basis of concentration. The working DNA sample was prepared for PCR works.

DNA confirmation

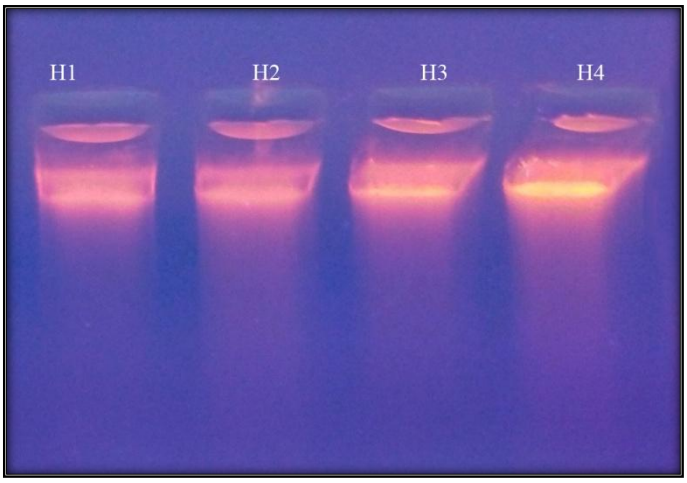

Plate 1: Isolated genomic DNA of 4 rice hybrids

(Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3 = Hybrid3, Lane 4 = Hybrid4)

4.3 Banding pattern and Polymorphism survey of four rice hybrids by RAPD primers

Initially twenty seven DNA decamer RAPD primers were screened on randomly 4 rice hybrid varieties to evaluate their suitability for amplification of the rice DNA fragments. The primers, which gave minimum smearing, high resolution and maximum reproducible and distinct polymorphic amplified bands were selected. It revealed that, out of twenty seven RAPD primers, twenty one decamer RAPD primers *viz*. OPB 17, OPBA 03, OPBA 06, OPBB 03, OPBB 05, OPBB 06, OPBB 09, OPBB 12, OPBC 05, OPBC 14, OPBC 16, OPBD 16, OPBD 18, OPG 03, OPG 05, OPG 17, OPD 20, OPX 10, OPY 11, OPZ 01 and OPZ 06 showed reproducible amplified bands.

The RAPD primer OPB-17 produced different DNA fragments in rice varieties. It produced total 6 DNA fragments which ranged from 1300 bp to 275 bp and 2 of them were polymorphic and rest of all were monomorphic bands (Plate 2). The primer OPBA-03 produced 2 DNA fragments which ranged from 600 to 300 bp and all were monomorphic (Plate 3). The primer OPBA-06 was able to produce 7 DNA fragments in total and ranged from 1100 to 175 bp. Two bands out of 7 bands were polymorphic (Plate 4). The primer OPBB-03 was produced 4 DNA fragments in total which ranged from 625 to 300 bp. Three bands out of 4 bands were polymorphic (Plate 5). Three DNA fragments were amplified by the primer OPBB-05 which ranged from 500 to 300 bp and out of 3 bands 2 were polymorphic and 1 was monomorphic (Plate 6). The primer OPBB-06 was showed total no. of 7 bands. Three bands were polymorphic and others were monomorphic band which ranged from 1100 to 250 bp (Plate 7). Four DNA fragments amplification were noticed by the primer OPBB-09 in four rice hybrids which were range from 650 to 275 bp and 3 were polymorphic in nature and another band was monomorphic (Plate 8). The RAPD primer OPBB-12 was able to amplify 4 DNA fragments among all the individuals. The DNA fragments ranged from 1500 to 300 bp and all were monomorphic in nature (Plate 9). The primer OPBC-05 was produced 3 bands which ranged from 650 to 350 and one was polymorphic and 2 bands were monomorphic (Plate 10). Four DNA fragments were amplified by the primer OPBC-14 in rice varieties which ranged from 750 to 250 bp and all were monomorphic (Plate 11). The primer OPBC-16 was produced 3 DNA fragments in total which ranged from 900 to 400 bp and all were monomorphic in nature (Plate 12). The primer OPBD-16 was produced 2 DNA fragments which ranged from 600 to 275 bp and all of were monomorphic in nature (Plate 13). Five DNA fragments amplification were noticed by the primer OPBD-18 in four rice

hybrids which were range from 975 to 275 bp and out of 5 bands, 2 were polymorphic and rest of all were monomorphic in nature (Plate 14). The primer OPD 20 amplified among the different rice varieties scored 3 DNA fragments which ranged from 800 to 250 bp in which 1 band was polymorphic and 2 were monomorphic (Plate 15). The primer OPG-03 showed total no. of 4 bands. One of band was polymorphic and others were monomorphic band which ranged from 625 to 375 bp (Plate-16). The primer OPG-05 was produced 3 DNA fragments which ranged from 400 to 150 bp and all were monomorphic in nature (Plate 17). Three bands were amplified by primer OPG-17 which ranged from 450 to 275. It was noticed that all were monomorphic band (Plate 18). The primer OPX-10 was able to produce 4 bands and which ranged from 1100 to 200 bp. Two bands out of 4 bands were polymorphic (Plate 19). The primer OPY-11 was able to produce 6 DNA fragments in total and which ranged from 800 to 300 bp. Out of 6 bands. 1 was polymorphic (Plate 20). Two bands were showed by the primer OPZ-01 which ranging from 190 to 150 and 1 band was showed polymorphic band (Plate 21). Three DNA fragments amplification were noticed by the primer OPZ-06 in four rice hybrids which were range from 650 to 295 bp and out of 3 bands, 2 were polymorphic and another one was monomorphic in nature (Plate 22). Seven primers *viz.* OPBA-03, OPBB-12, OPBC-14, OPBC-16, OPBD-16, OPG-05 and OPG-17 showed all monomorphic bands in their amplifications and no polymorphism was detected. For this reason, this 7 primers were discarded for next statistical analysis.

The 21 primers regenerated total 82 DNA fragments with an average 3.90 per primer among the four rice hybrids. Out of 82 DNA bands, 26 DNA fragments were polymorphic and the average percent of polymorphism was 30.33. The highest (3) was produced number of polymorphic band by the primer OPBB-03, OPBB-06 and OPBB-09, the (2) was produced number of polymorphic band by the primer OPB-17, OPBA-06, OPBB-05, OPBD-18, OPX-10 and OPZ-06 of each respectively and lowest (1) number of polymorphic band by the primer OPBC-05, OPD-20, OPG-03, OPY-11 and OPZ-01. The primer OPBA-03, OPBB-12, OPBC-14, OPBC-16, OPBD-16, OPG-05 and OPG-17 were not able to regenerate any polymorphic band. Maximum 75% of polymorphism was recorded in the primer OPBB-03 and OPBB-09 and it was followed by primer OPY-11 (17%) which was lowest polymorphism. Twenty one of RAPD markers scored for each individual of four rice hybrids for each primer is presented in Table 7.

Table 7: RAPD primers with corresponding banding pattern and polymorphism observed in four rice hybrids.

Sl. No.	Primer	Primer Sequence (5′-3′)	(G+C) %	Band Scored	Number of Polymorphic Bands	Polymorphism %	Size ranges
1.	OPB-17	AGGGAACGAG	60	6	2	33	1300-275
2.	OPBA-03	GTGCGAGAAC	60	2	0	0	600-300
3.	OPBA-06	GGACGACCGT	70	7	2	29	1100-175
4.	OPBB-03	TCACGTGGCT	60	4	3	**75**	625-300
5.	OPBB-05	GGGCCGAACA	70	3	2	67	500-300
6.	OPBB-06	CTGAAGCTGG	60	7	3	43	1100-250
7.	OPBB-09	AGGCCGGTCA	70	4	3	**75**	650-300
8.	OPBB-12	TTCGGCCGAC	70	4	0	0	1500-300
9.	OPBC-05	GAGGCGATTG	60	3	1	33	650-350
10.	OPBC-14	GGTCCGACGA	70	4	0	0	750-250
11.	OPBC-16	CTGGTGCTCA	60	2	0	0	900-400
12.	OPBD-16	GAACTCCCAG	60	3	0	0	900-250
13.	OPBD-18	ACGCACACTC	60	5	2	40	975-250
14.	OPD-20	ACCCGGTCAC	70	3	1	33	800-250
15.	OPG-03	GAGCCCTCCA	70	4	1	25	625-375
16.	OPG-05	CTGAGACGGA	60	3	0	0	400-150
17.	OPG-17	ACGACCGACA	60	3	0	0	450-275
18.	OPX-10	CCCTAGACTG	60	4	2	50	1100-200
19.	OPY-11	AGACGATGGG	60	6	1	**17**	800-300
20.	OPZ 01	TCTGTGCCAC	60	2	1	50	190-150
21.	OPZ-06	GTGCCGTTCA	60	3	2	67	650-295
Total		-	-	82	26	622	-
Mean		**-**	**-**	**3.90**	**1.24**	**30.33**	**-**

In this study, the level of polymorphism (30.33%) indicated the effectiveness of RAPD technique to study less amount of polymorphisms or diversity among the different varieties of hybrid rice. Rahman *et al*. (2007) found 53.85% polymorphism using three RAPD primers.

However, higher level of polymorphism is also found in various experiments. Rahman *et al*. (2009) reported four primers which gave reproducible and distinct polymorphic amplified products. Selected four primers generated 41 bands where 37 bands (90.24%) were polymorphic and 4 bands (9.76%) were monomorphic. A diverse level of polymorphism in rice genotypes were reported by Qian *et al*. (2006) and Shivapriya and Shailaja (2006) 83.5% and 74.1% respectively. Fourteen Aman rice genotypes were evaluated by using 15 RAPD primers. A total of 191 reproducible and scorable amplifications were generated and out of 191 bands, 153 (80.1%) were found to be polymorphic (Mitra *et al*., 2017).

Polymorphism in rice in different studies could be attributed to the nature of the genetic material under investigation. The low degree of polymorphism in our study could be due to the less diverse material among the rice hybrids.

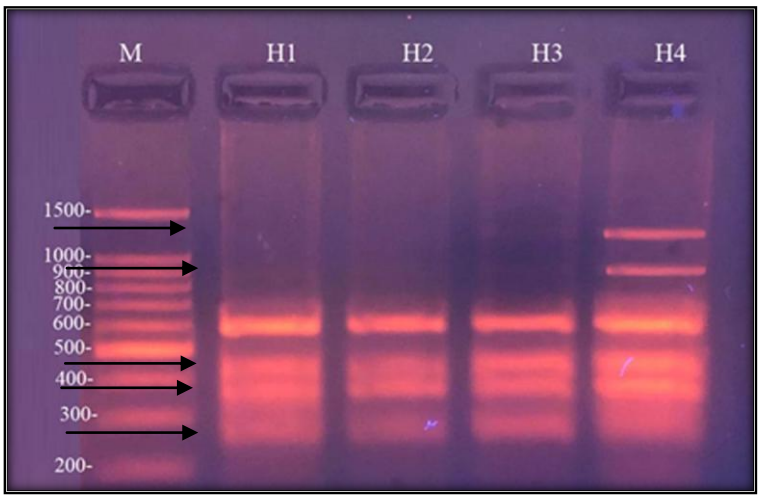

Plate 2: PCR amplification with RAPD primer-OPB 17

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

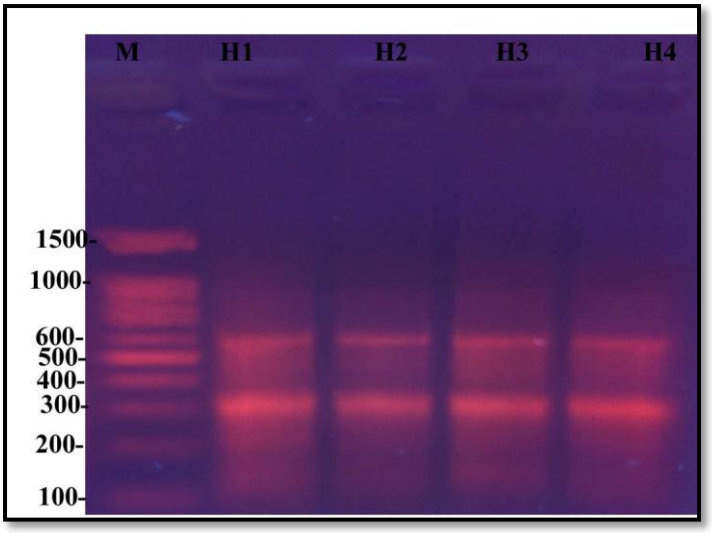

Plate 3: PCR amplification with RAPD primer OPBA-03

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

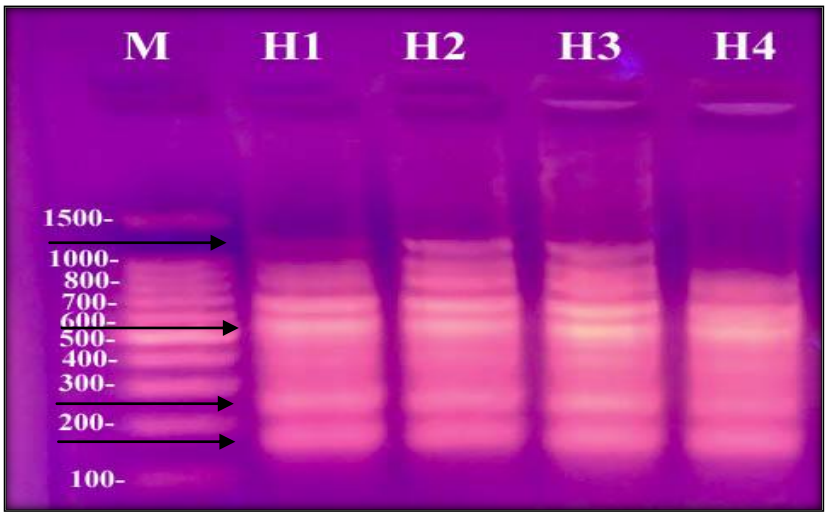

Plate 4: PCR amplification with RAPD primer OPBA-06

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

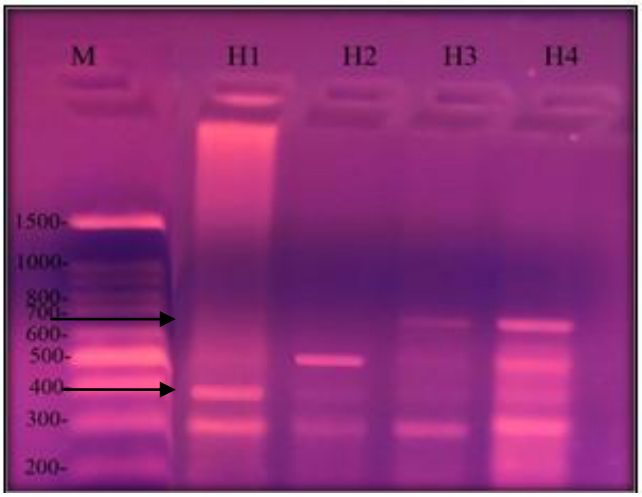

Plate 5: PCR amplification with RAPD primer OPBB-03

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

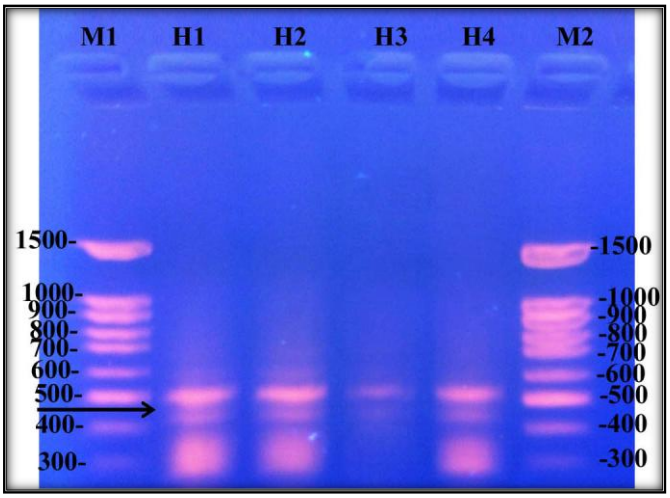

Plate 6: PCR amplification with RAPD primer OPBB-05

M1=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4, M2=1 kb DNA ladder (Bio Basic, Canada)

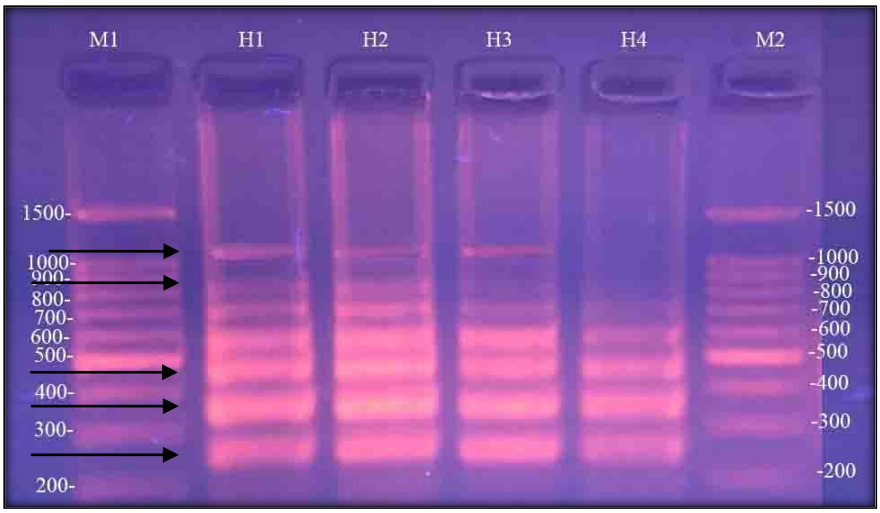

Plate 7: PCR amplification with RAPD primer OPBB-06

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

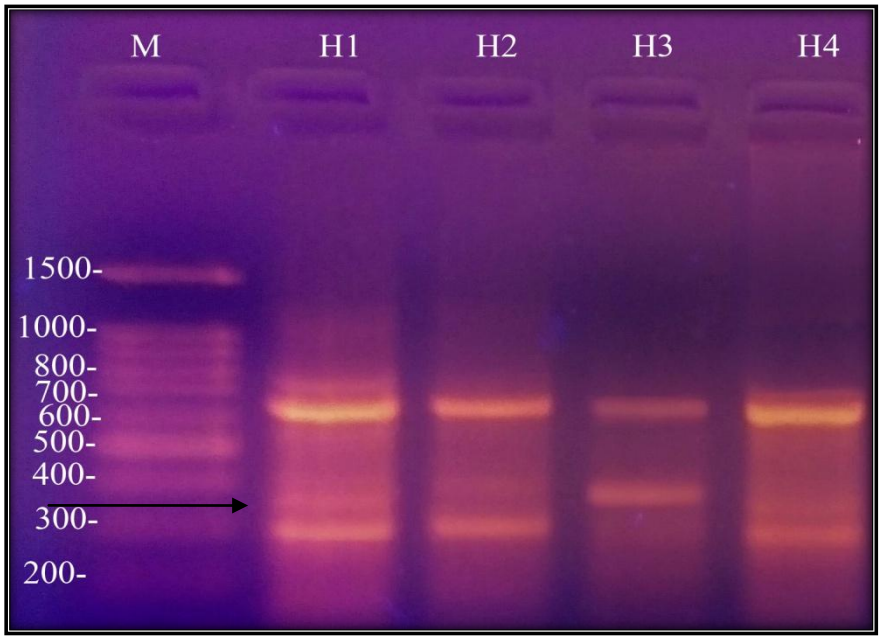

Plate 8: PCR amplification with RAPD primer OPBB-09

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid

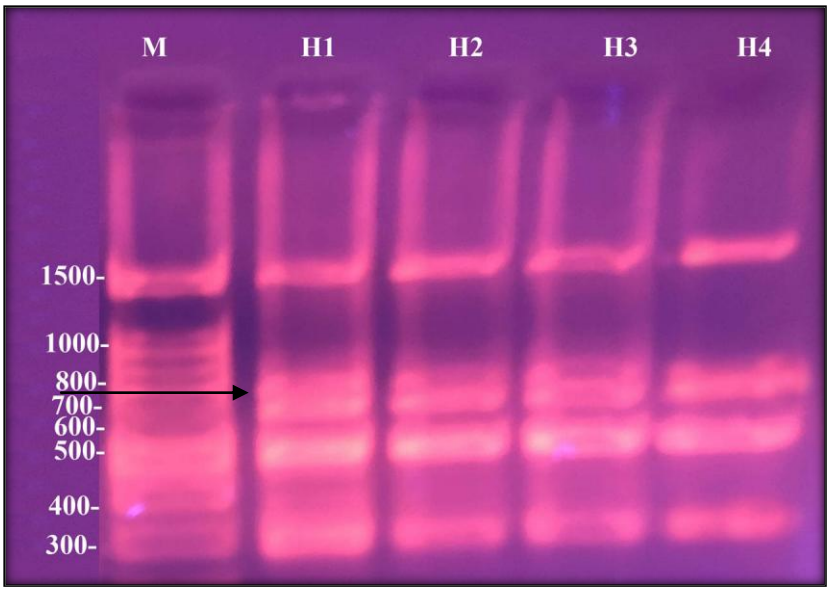

Plate 9: PCR amplification with RAPD primer OPBB-12

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

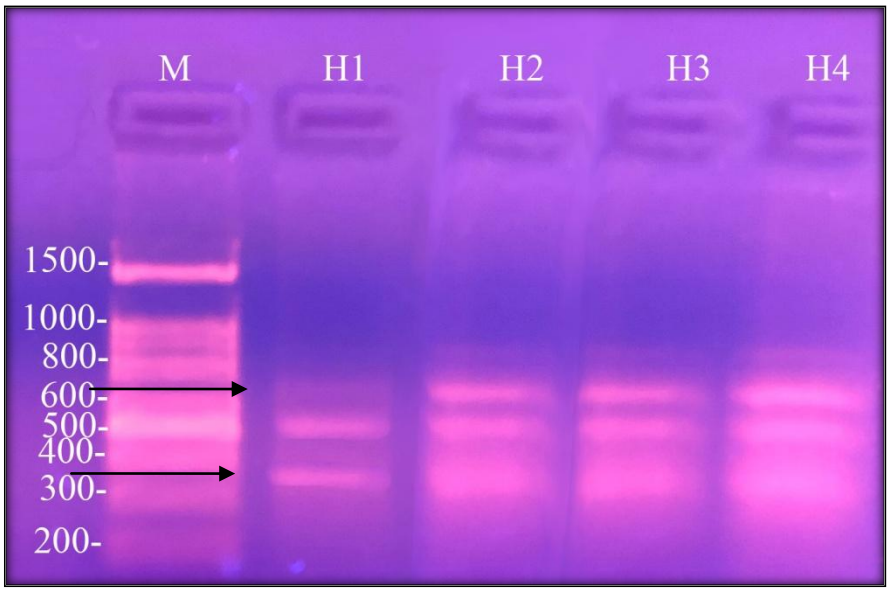

Plate 10: PCR amplification with RAPD primer OPBC-05

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

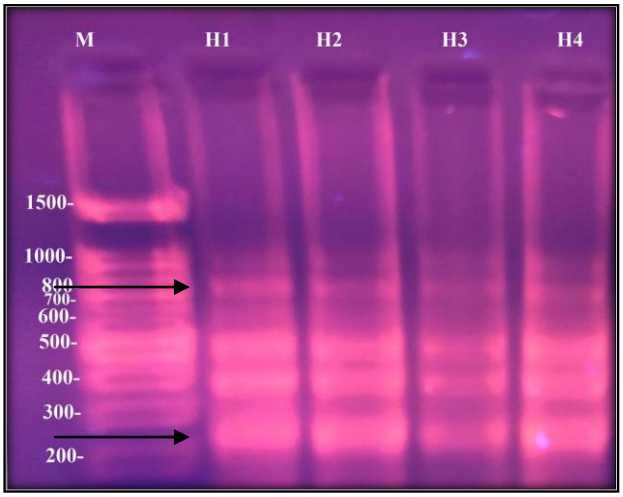

Plate 11: PCR amplification with RAPD primer OPBC-14

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

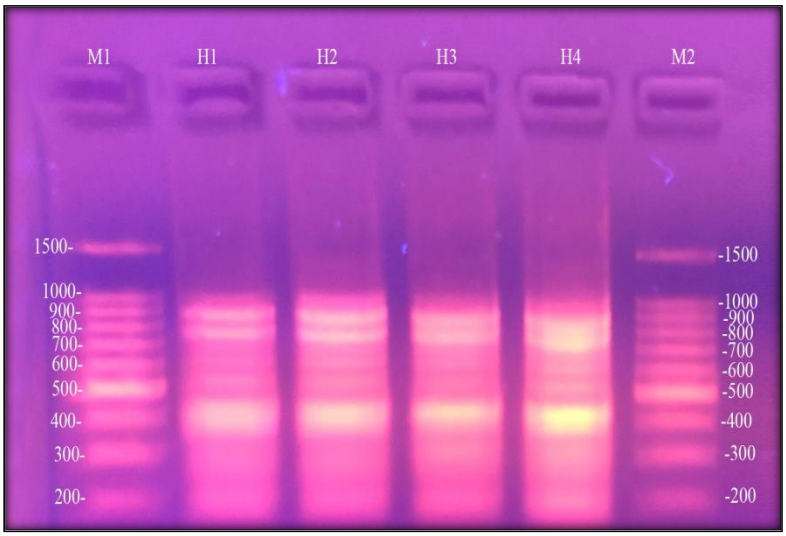

Plate 12: PCR amplification with RAPD primer OPBC-16

M1=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4, M2=1 kb DNA ladder, (Bio Basic, Canada)

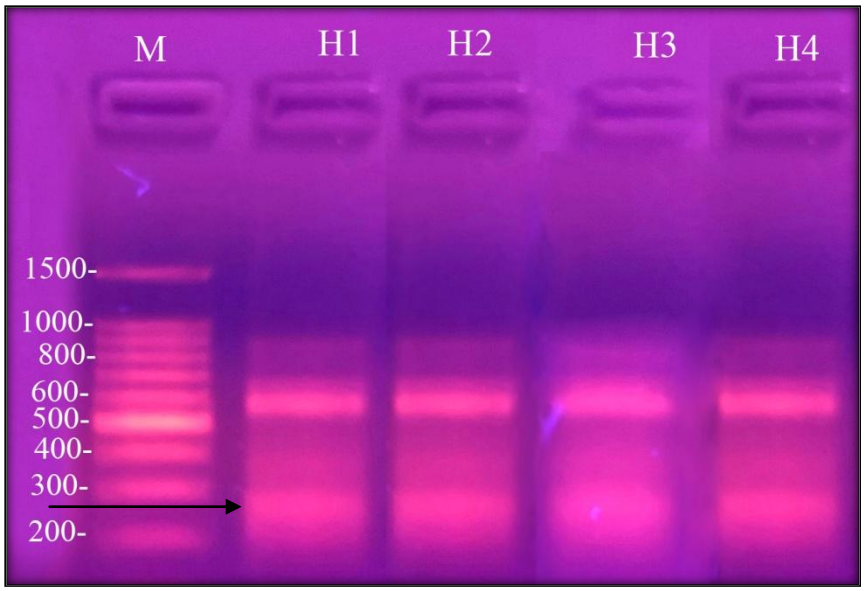

Plate 13: PCR amplification with RAPD primer OPBD-16

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

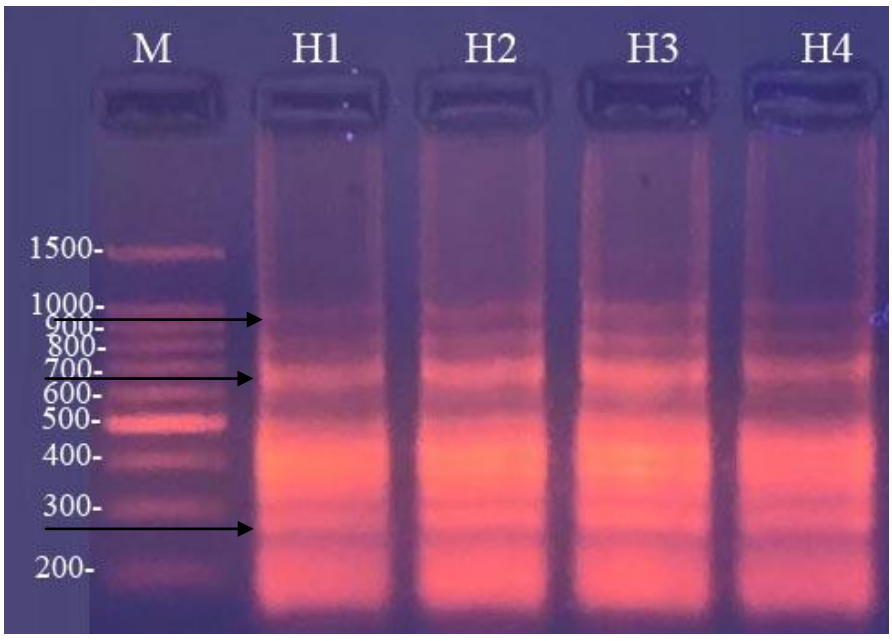

Plate 14: PCR amplification with RAPD primer OPBD-18

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

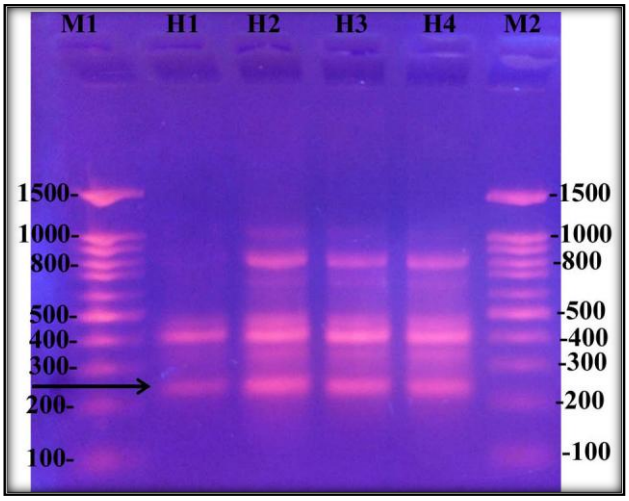

Plate 15: PCR amplification with RAPD primer OPD-20

M1=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4, M2=1 kb DNA ladder, (Bio Basic, Canada)

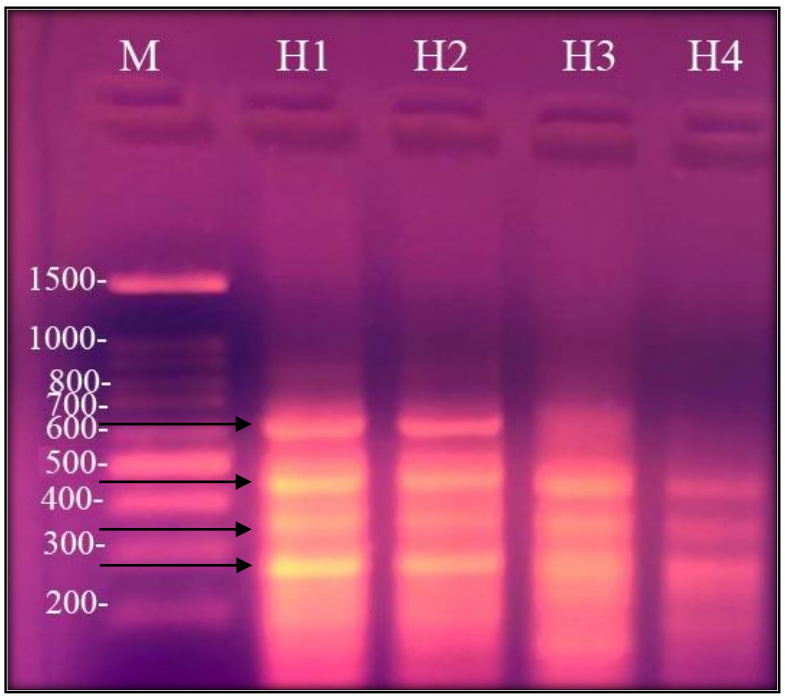

Plate 16: PCR amplification with RAPD primer OPG-03

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

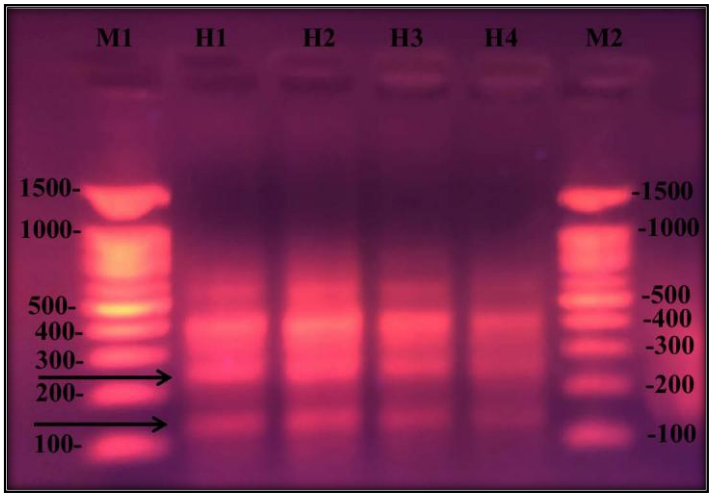

Plate 17: PCR amplification with RAPD primer OPG-05

M1=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4, M2=1 kb DNA ladder, (Bio Basic, Canada)

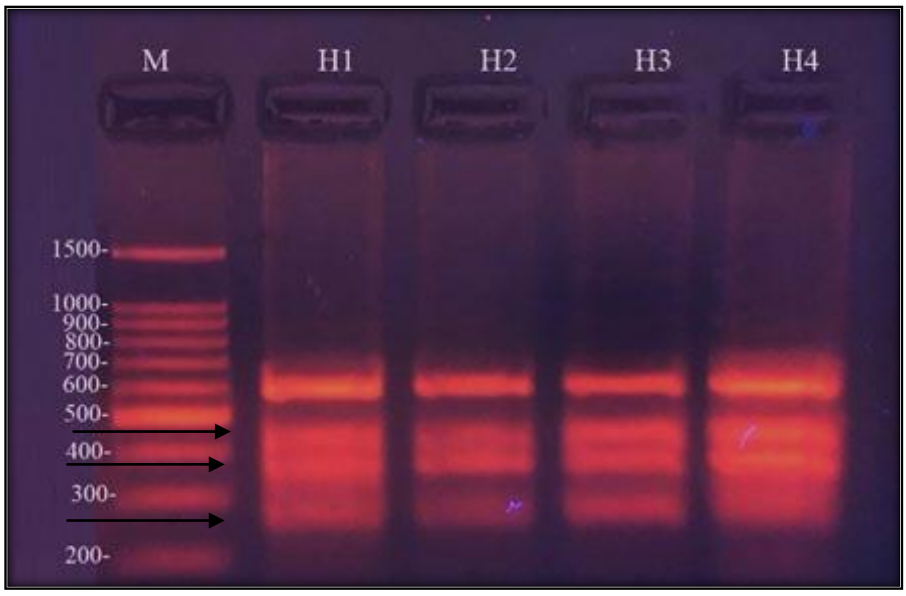

Plate 18: PCR amplification with RAPD primer OPG-17

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

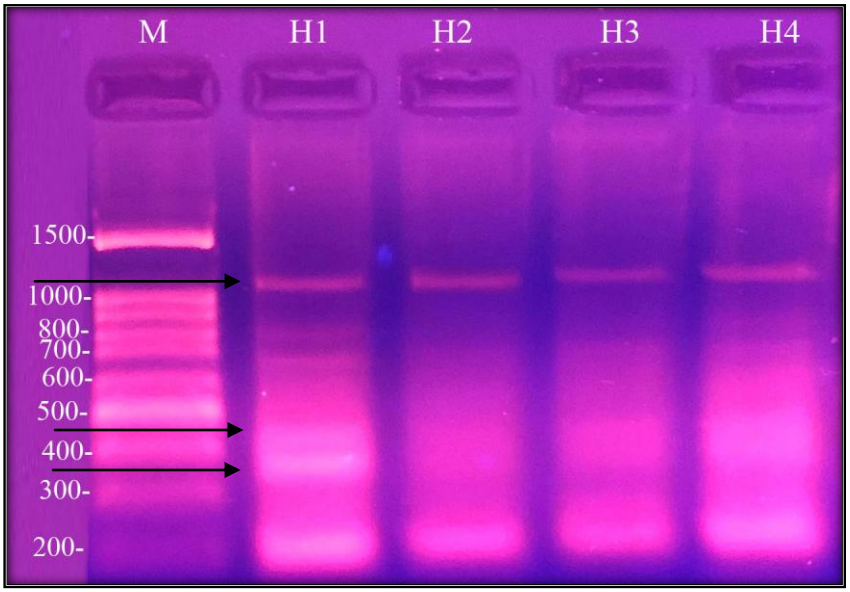

Plate 19: PCR amplification with RAPD primer OPX-10

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

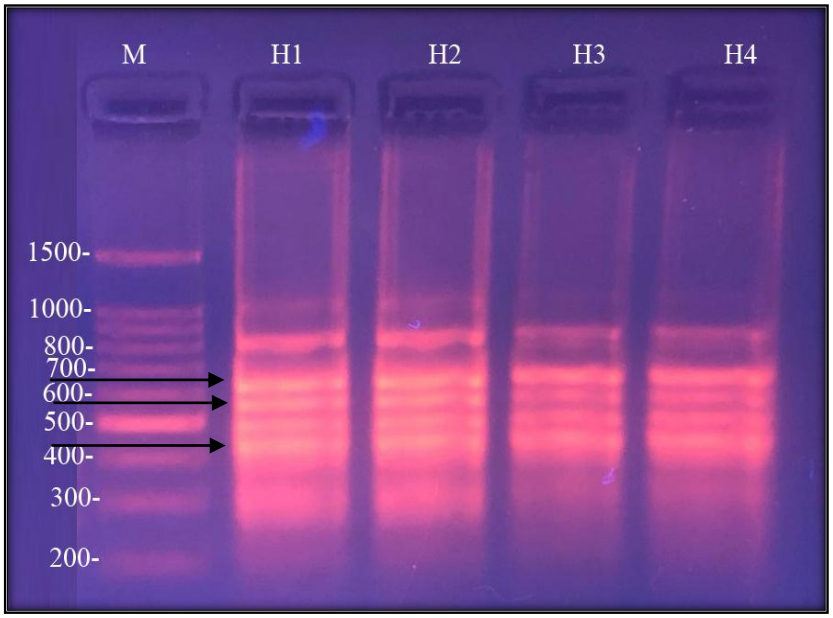

Plate 20: PCR amplification with RAPD primer OPY-11

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

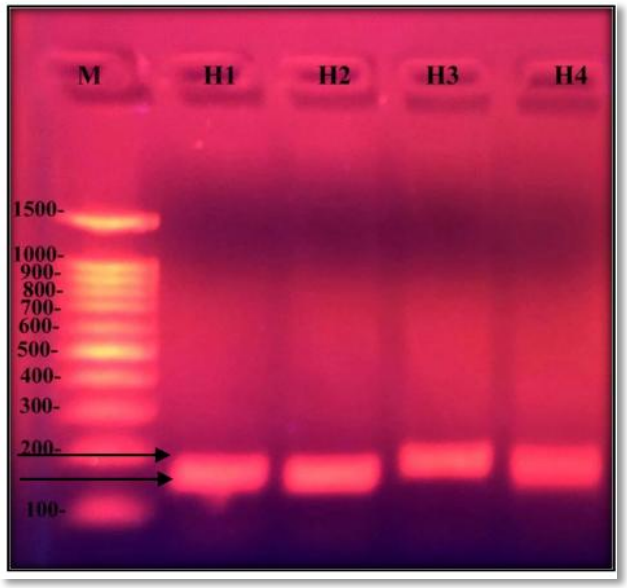

Plate 21: PCR amplification with RAPD primer OPZ-01

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

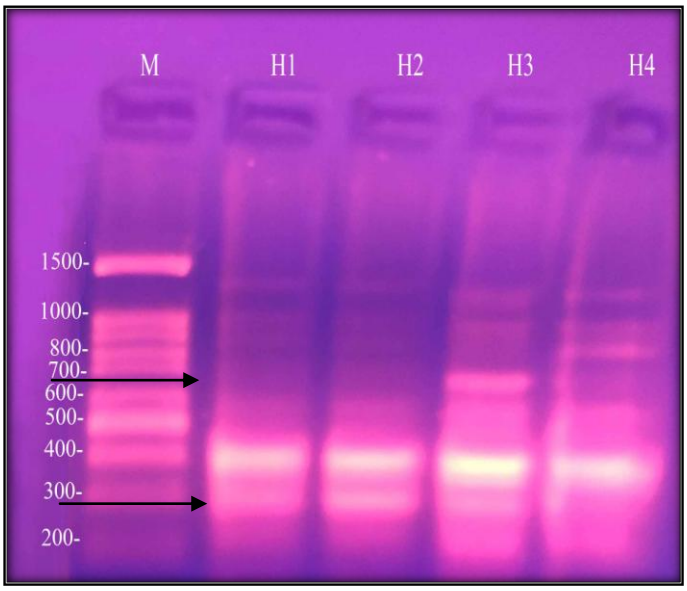

Plate 22: PCR amplification with RAPD primer OPZ-06

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

4.4 Gene diversity, gene frequency and PIC values

DNA polymorphisms were detected according to band presence and absence. Absence of bands may be caused by failure of primers to anneal a site in some individuals due to nucleotide sequence differences or by insertions or deletions between primer sites (Clark and Lanigan, 1993).

The twenty one primers used in the present study showed different levels of major allele frequency, gene diversity and polymorphism information content (PIC) values which are given in Table 8.

Table 8: Gene diversity, gene frequency and PIC value of different hybrid varieties of rice by RAPD primers.

Sl. No.	RAPD Primers	Locus No.	Locus Size (bp)	Major Allele frequency	Gene Diversity	PIC
1.	OPB-17	1	1300	0.7500	0.3750	0.3047
		2	900	0.7500	0.3750	0.3047
		3	600	**1.0000**	0.0000	0.0000
		4	490	**1.0000**	0.0000	0.0000
		5	375	**1.0000**	0.0000	0.0000
		6	275	**1.0000**	0.0000	0.0000
2.	OPBA-03	1	600	**1.0000**	0.0000	0.0000
		2	300	**1.0000**	0.0000	0.0000
3.	OPBA-06	1	1100	**0.5000**	**0.5000**	**0.3750**
		2	900	0.7500	0.3750	0.3047
		3	800	**1.0000**	0.0000	0.0000
		4	700	**1.0000**	0.0000	0.0000
		5	550	**1.0000**	0.0000	0.0000
		6	250	**1.0000**	0.0000	0.0000
		7	175	**1.0000**	0.0000	0.0000

Cont'd

Sl. No.	RAPD Primers	Locus No.	Locus Size (bp)	Major Allele frequency	Gene Diversity	PIC
4.	OPBB-03	1	625	**0.5000**	**0.5000**	**0.3750**
		2	500	**0.5000**	**0.5000**	**0.3750**
		3	390	0.7500	0.3750	0.3047
		4	300	**1.0000**	0.0000	0.0000
5.	OPBB-05	1	500	**1.0000**	0.0000	0.0000
		2	450	0.7500	0.3750	0.3047
		3	300	0.7500	0.3750	0.3047
6.	OPBB-06	1	1100	0.7500	0.3750	0.3047
		2	850	**0.5000**	**0.5000**	**0.3750**
		3	700	0.7500	0.3750	0.3047
		4	600	**1.0000**	0.0000	0.0000
		5	475	**1.0000**	0.0000	0.0000
		6	350	**1.0000**	0.0000	0.0000
		7	250	**1.0000**	0.0000	0.0000
7.	OPBB-09	1	650	**1.0000**	0.0000	0.0000
		2	400	0.7500	0.3750	0.3047
		3	350	0.7500	0.3750	0.3047
		4	300	0.7500	0.3750	0.3047
8.	OPBB-12	1	1500	**1.0000**	0.0000	0.0000
		2	75O	**1.0000**	0.0000	0.0000
		3	500	**1.0000**	0.0000	0.0000
		4	300	**1.0000**	0.0000	0.0000
9.	OPBC-05	1	600	0.7500	0.3750	0.3047
		2	500	**1.0000**	0.0000	0.0000
		3	350	**1.0000**	0.0000	0.0000

Cont'd

Sl. No.	RAPD Primers	Locus No.	Locus Size (bp)	Major Allele frequency	Gene Diversity	PIC
10.	OPBC-14	1	750	**1.0000**	0.0000	0.0000
		2	500	**1.0000**	0.0000	0.0000
		3	400	**1.0000**	0.0000	0.0000
		4	250	**1.0000**	0.0000	0.0000
11.	OPBC-16	1	900	**1.0000**	0.0000	0.0000
		2	800	**1.0000**	0.0000	0.0000
		3	400	**1.0000**	0.0000	0.0000
12.	OPBD-16	1	600	**1.0000**	0.0000	0.0000
		2	275	**1.0000**	0.0000	0.0000
13.	OPBD-18	1	975	**0.5000**	**0.5000**	**0.3750**
		2	800	**0.5000**	**0.5000**	**0.3750**
		3	675	**1.0000**	0.0000	0.0000
		4	400	**1.0000**	0.0000	0.0000
		5	290	**1.0000**	0.0000	0.0000
14.	OPD-20	1	800	0.7500	0.3750	0.3047
		2	400	**1.0000**	0.0000	0.0000
		3	250	**1.0000**	0.0000	0.0000
15.	OPG-03	1	625	**0.5000**	**0.5000**	**0.3750**
		2	550	**1.0000**	0.0000	0.0000
		3	450	**1.0000**	0.0000	0.0000
		4	375	**1.0000**	0.0000	0.0000
16.	OPG-05	1	400	**1.0000**	0.0000	0.0000
		2	250	**1.0000**	0.0000	0.0000
		3	150	**1.0000**	0.0000	0.0000
17.	OPG-17	1	450	**1.0000**	0.0000	0.0000
		2	390	**1.0000**	0.0000	0.0000
		3	275	**1.0000**	0.0000	0.0000

Cont'd

Sl. No.	RAPD Primers	Locus No.	Locus Size (bp)	Major Allele frequency	Gene Diversity	PIC
18.	OPX-10	1	1100	**1.0000**	0.0000	0.0000
		2	450	**0.5000**	**0.5000**	**0.3750**
		3	350	**0.5000**	**0.5000**	**0.3750**
		4	200	**1.0000**	0.0000	0.0000
19.	OPY-11	1	800	**1.0000**	0.0000	0.0000
		2	650	**1.0000**	0.0000	0.0000
		3	575	**1.0000**	0.0000	0.0000
		4	500	**1.0000**	0.0000	0.0000
		5	425	**1.0000**	0.0000	0.0000
		6	300	**0.5000**	**0.5000**	**0.3750**
20.	OPZ-01	1	190	0.7500	0.3750	0.3047
		2	150	**1.0000**	0.0000	0.0000
21.	OPZ-06	1	650	0.7500	0.3750	0.3047
		2	400	**1.0000**	0.0000	0.0000
		3	300	0.7500	0.3750	0.3047
Mean	**-**	**-**	**-**	**0.8902**	**0.1341**	**0.1052**

The gene frequency of the major allele ranged between 0.5000 to 1.0000 with an average value of 0.8902 and Nei's (1972) Gene diversity ranged from 0 to 0.5000. The highest gene frequency (1.0000) as well as the lowest gene diversity (0) was shown by the primers OPB-17 (at 600, 490, 375, 275 bp), OPBA-03 (at 600 and 300 bp), OPBA-06 (at 800, 700, 550, 250 and 175 bp), OPBB-03 (at 300 bp), OPBB-05 (at 500 bp), OPBB-06 (at 600, 475, 350 and 250 bp), OPBB-09 (at 650 bp), OPBB-12 (at 1500, 750, 500 and 300 bp), OPBC-05 (at 500 and 350 bp), OPBC-14 (at 750, 500, 400 and 250 bp), OPBC-16 (at 900, 800 and 400 bp), OPBD-16 (at 600 and 275 bp), OPBD-18 (at 675, 400 and 290 bp), OPD-20 (at 400 and 250 bp), OPG-03 (at 550, 450 and 375 bp), OPG-05 (at 400, 250 and 150 bp), OPG-17 (at 450, 390 and 275 bp), OPX-10 (at 1100 and 200 bp), OPY-11 (at 800, 650, 575, 500 and 425 bp) OPZ-01 (at 150 bp) and OPZ-06 (at 400 bp).

The highest gene diversity was shown by the primer OPBA-06 (at 1100 bp), OPBB-03 (at 625 and 500 bp), OPBB-06 (at 850 bp), OPBD-18 (at 975 and 800 bp), OPG-03 (at 625 bp), OPX-10 (at 450 and 350 bp) and OPY-11 (at 300 bp). On the other hand, the lowest gene frequency was observed at 1100 bp by OPBA-06, 625 and 500 bp by OPBB-03, at 850 bp by OPBB-06, at 975 and 800 bp by primer OPBD-18, at 625 bp by OPG-03, at 450 and 350 bp by OPX-10 and at 300 bp by OPY-11.

Polymorphic Information Content (PIC) value for the twenty one markers ranged from 0 to 0.3750 and the average PIC value was 0.1121. The highest PIC value (0.3750) was obtained for OPBA-06 (at 1100), OPBB-03 (at 625 and 500 bp), OPBB-06 (at 850 bp), OPBD-18 (at 975 and 800 bp), OPG-03 (at 625 bp), OPX-10 (at 450 and 350 bp) and OPY-11 (at 300 bp). The lowest PIC value (0) was obtained for OPB-17 (at 600, 490, 375, 275 bp), OPBA-03 (at 600 and 300 bp), OPBA-06 (at 800, 700, 550, 250 and 175 bp), OPBB-03 (at 300 bp), OPBB-05 (at 500 bp), OPBB-06 (at 700, 550, 475, 350 and 250 bp), OPBB-09 (at 650 bp), OPBB-12 (at 1500, 620, 470 and 300 bp), OPBC-05 (at 500 and 350 bp), OPBC-14 (at 750, 500, 350 and 250 bp), OPBC-16 (at 900, 750 and 400 bp), OPBD-16 (at 600 and 275 bp), OPBD-18 (at 675, 400 and 250 bp), OPD-20 (at 400 and 250 bp), OPG-03 (at 550, 450 and 375 bp), OPG-05 (at 400, 250 and 150 bp), OPG-17 (at 450, 390 and 275 bp), OPX-10 (at 1100 and 200 bp), OPY-11 (at 800, 650, 575, 500 and 425 bp) and OPZ-06 (at 400 bp).

PIC value revealed that OPBB-03 was considered as the best marker for four rice hybrids followed by OPX-10, OPBD-18, OPBB-06, OPBA-06, OPG-03 and OPY-11 respectively. The results indicated that the four rice hybrids present a low degree of homozygosity and a certain degree of genetic differentiation and polymorphism.

The PIC was a good index for genetic diversity evaluation. Botstein *et al*. (1980) reported that PIC index can be used to evaluate 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 intermediate diversity at PIC between 0.25 and 0.5. Lower PIC value indicates that the varieties under study are closely related types, while the higher value of PIC indicates higher diversity of materials, which is better for development of new varieties.

4.6 Nei's genetic distance and genetic identity

Genetic distance refers to the genetic deviation between species or between populations within a species. It is measured by a variety of parameters like Nei's standard genetic distance. This distance measure is known to give more reliable population trees than other distances particularly for DNA data. Similarity indices measure the amount of closeness between two individuals, the larger the value the more similarity between two individuals. Smaller genetic distances indicate a close genetic relationship whereas large genetic distances indicate a more distant genetic relationship. Genetic distance can be used to compare the genetic similarity between different species. Genetic diversity studies help in selection of material for breeding program.

The value of pair-wise comparisons Nei's (1983) genetic distance between four rice hybrids were computed from combined data through twenty one primers, ranging from 0.1190 to 0.2262. The highest Nei's genetic distance (0.2262) was observed in Hybrid4 vs Hybrid3 varietal pair whereas lowest value (0.1190) was observed in Hybrid2 vs Hybrid1 varietal pair (Table 9).

Table 9: Summary of Nei's genetic identity (above diagonal) and genetic distance (below diagonal) values among four rice hybrids

	Hybrid1	**Hybrid2**	**Hybrid3**	**Hybrid4**
Hybrid1	0.0000	0.1190	0.2024	0.1667
Hybrid2	0.1190	0.0000	0.1548	0.1667
Hybrid3	0.2024	0.1548	0.0000	0.2262
Hybrid4	0.1667	0.1667	0.2262	0.0000

This variation can be occurred by geographical origin. The result also reveals that the genetic base among these rice hybrids is rather narrow. Collection of diverse germplasm from centers of diversity may boarden the genetic base. RAPD markers provide a fast, efficient technique for variability assessment that complements methods currently being used in genetic resource management.

4.7 UPGMA Dendrogram

Dendogram based on Nei's (1983) genetic distance using Unweighed Pair Group Method of Arithmetic Means (UPGMA) indicated the segregation of four hybrids of rice into three main clusters: A, B and C. The first major cluster 'A' had divided only two minor groups, genotypes Hybrid1 and Hybrid2 and the second cluster 'B' had Hybrid4 varieties. The third major cluster 'C' had Hybrid3 varieties (Figure 1).

The main objectives for this study were to study genetic diversity among four rice hybrid varieties using RAPD analyses. Consideration of estimated genetic distance is important for comparative analysis of diversity levels (Rodriguze *et al*., 1999). The result indicates that the low or high level genetic distance exists between varieties with their same or different origins. Hybrid4 vs Hybrid3 showed highest Nei's genetic distance (0.2262) as they are released from different parental origin. On the other hand, Hybrid1 vs Hybrid2 varietal pair showed lowest genetic distance (0.1190) as they are released from same parental origin.

Cluster analysis on rice varieties was also performed by several scientists. The study conducted by Hasan and Raihan (2015) on genetic distance by UPGMA dendrogram segregated 30 aromatic rice into 6 clusters. Cluster I includes 9 varieties. The cluster II included 6 varieties and cluster III had 5 varieties. Cluster IV consisted of 4 varieties, Cluster V included 5 and Cluster VI contained two varieties.

Mitra *et al*. (2017) constructed an Unweighted Pair-Group Method of Arithmetic Means (UPGMA) dendrogram from genetic distance and 14 Aman rice cultivars grouped into 6 major clusters. Rabbani *et al*. (2008) grouped 40 traditional and improved cultivars of rice into 3main clusters I, II and III by using UPGMA.

Rahman *et al*. (2007) conducted Unweighted Pair-Group Method of Arithmetic Means (UPGMA) dendrogram from genetic distance clearly separated the six rice cultivars into three major clusters.

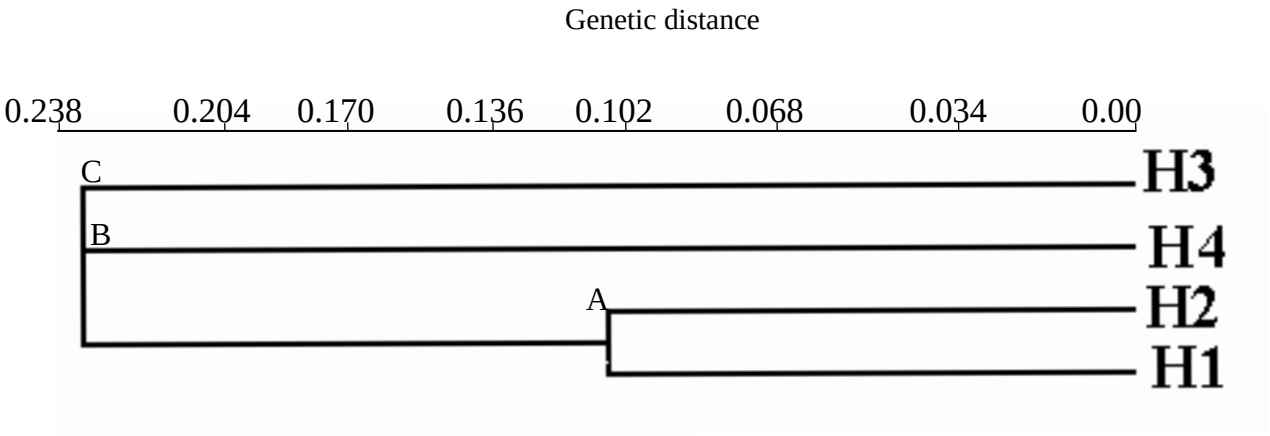

Figure 1. Dendogram of four rice hybrids was based on RAPD marker, according to the un-weighted pair group method of arithmetic means (UPGMA) method based on a similarity matrix by PAST software

4.8 DNA amplification and polymorphism in four rice hybrids through SSR markers

Initially five primers were screened on randomly 4 rice hybrid variety genotypes to evaluate their suitability for amplification of the rice DNA fragments. The primers, which gave minimum smearing, high resolution and maximum reproducible and distinct polymorphic amplified bands were selected. It revealed that, out of five SSR primers, four SSR primers *viz*. RM17, OSR16, RM1 and RM72 showed reproducible amplified bands.

Rice specific SSR primer RM17 used for the DNA fingerprint of Nilsagor Rice Hybrid. A 150 bp DNA band is common in H1, H2 and H4 varieties. The hybrid H3 showed 175 bp DNA band by the same primer (Plate 23). Only 25 bp polymorphism was observed in hybrid-03. This molecular diversity can be used as a evidence of variety protection data.

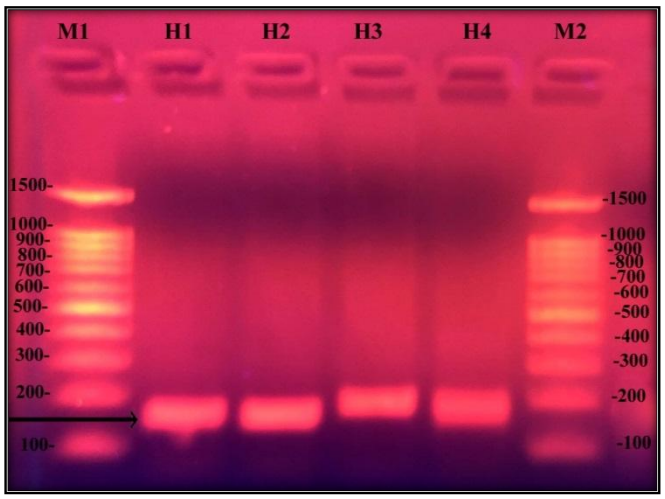

Plate 23: PCR amplification with SSR primer RM 17

M1=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4, M2=1 kb DNA ladder, (Bio Basic, Canada)

In Plate 24, all the hybrids produced 125 bp DNA fragment by the SSR primer OSR16. It is a monomorphic DNA fragment. Hence, this SSR primer was not suitable for hybrid identification and documentation.

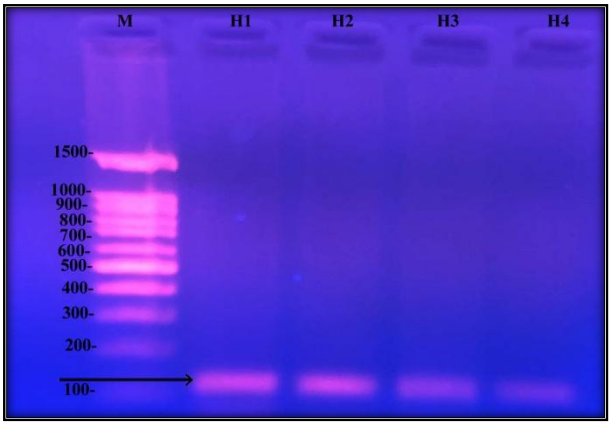

Plate 24: PCR amplification with SSR primer OSR16

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

A 125 bp DNA fragment was common in hybrid-01, 02 and 03. The hybrid-04 produced 100 bp DNA fragment by the rice specific SSR primer RM1 (Plate 25). A 25 bp DNA fragment variation was noticed in H4. Hence, it is a polymorphic SSR marker. DNA fingerprint of this marker can be used as rice hybrid characterization through molecular approach. The amplified DNA banding pattern is the molecular character of Nilsagor Rice Hybrid.

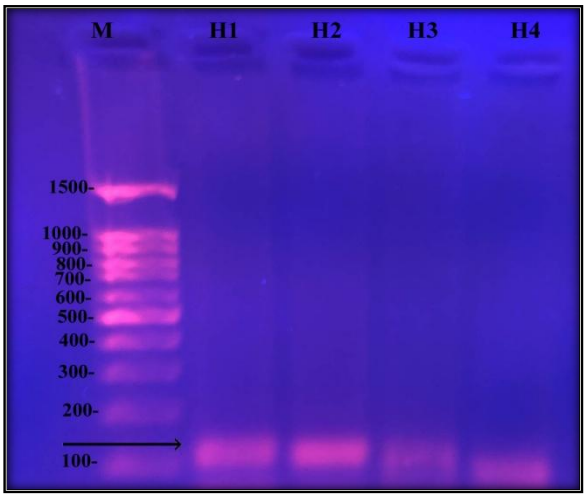

Plate 25: PCR amplification with SSR primer RM1

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

In Plate 26, all the hybrids showed 175bp DNA fragment through the SSR primer RM72. It is a monomorphic primer. Polymorphism study was not possible by using this molecular marker.

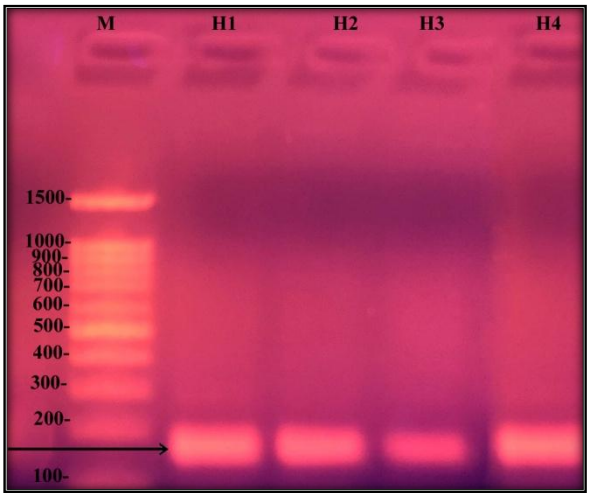

Plate 26: PCR amplification with SSR primer RM72

M=1 kb DNA ladder (Bio Basic, Canada), Lane 1 = Hybrid1, Lane 2 = Hybrid2, Lane 3= Hybrid3, Lane 4 = Hybrid4

4.9 Discussion

Rice hybrid characterization through molecular marker is a new technique of hybrid documentation. Very few works have been done in this area. Literature was not available for interpretation of data. Some commercial seed companies are doing this type of DNA profiling work to protect their own hybrid from any kind of corruption. Now a day the concern authority of Bangladesh Government is aware about the piracy of newly released hybrid variety. Hence, it is essential to submit DNA fingerprint data of a new hybrid for registration and documentation. DNA passport data or DNA blue print information can help to protect the hybrid variety from any kind of chatting.

It is a new avenue of molecular biology. Marker assisted selection and DNA fingerprinting can be a reliable documentation for protection of hybrid in commercial purpose.

CHAPTER V

SUMMARY AND CONCLUSION

Rice (*Oryza sativa* L.) is an important cereal food grain crop of the world and most widely consumed staple food for a large part of the world's human population. Agriculture is the mainstay of Bangladesh economy. Rice is the principal crop and the dominant staple food of the country and is the largest sectorial source of income, employment, savings and investment in the economy. Majority of the farmers focused for adoption of hybrid rice which is beneficial to the farmers in both production and profit. A large number of rice varieties are released and notified every year. Precise identification of crop cultivars is required for variety registration, preventing misappropriation and for protection of plant breeder's as well as farmers' rights. Genetic diversity is one of the most important factors considered in plant breeding and molecular approaches are well accepted and precise to determine the diversity. Despite most commercially important crop in Bangladesh, research findings on genetic analysis of different rice hybrids using molecular markers are scarce. Hence, it is a need to conduct studies to evaluate the genetic diversity of hybrid rice for breeding and documentation purposes.

This research investigation presented mainly the molecular characterization of four rice hybrids. The present study was conducted at Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207. Twenty one RAPD primers generated total 83 distinct and differential amplification bands with an average of 3.95 bands per primer. The highest no. of bands (7) generated by OPBA-06 and OPBB-06 and lowest no. of bands (2) generated by primer OPBA-03, OPBC-16 and OPZ-01. The highest percentage of polymorphic bands (75%) was generated by primer OPBB-03 and OPBB-09 while no polymorphic bands generated by primer OPBA-03, OPBB-12, OPBC-14, OPBC-16, OPBD-16, OPG-05 and OPG-17.

The frequency of the major allele ranged between 0.5000 to 1.0000 with an average value of 0.8902. Polymorphic Information Content (PIC) value for the 21 markers ranged from 0 to 0.3750 and the average PIC value was 0.1052. The overall gene diversity ranged between 0 to 0.5000 with an average of 0.1431. PIC value revealed that OPBB-03 was considered as the best marker for four rice hybrids followed by OPX-10, OPBD-18, OPBB-06, OPBA-06,

OPG-03 and OPY-11. OPBA-03, OPBB-12, OPBC-14, OPBC-16, OPBD-16, OPG-05 and OPG-17 could be considered as the least powerful markers.

The value of pair-wise comparisons Nei's (1972) genetic distance between four rice hybrids were computed from combined data for the 21 primers, range from 0.1190 to 0.2262. The highest genetic distance 0.2262 was observed in Hybrid4 vs Hybrid3 varietal pair whereas lowest value (0.1190) observed in Hybrid2 vs Hybrid1 varietal pair. Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of arithmetic Mean (UPMGA) segregated the segregation of four rice hybrids into three major clusters (Cluster A, B and C). The first major cluster 'A' was subdivided into two clusters containing two hybrids (Hybrid1 and Hybrid2), second major cluster 'B' had only one hybrid (Hybrid4) and third major cluster 'C' had only one hybrid (Hybrid3).

The result indicates that the low level genetic distance exists between varieties with their same or different origins. Rice Hybrid4 vs Rice Hybrid3 showed highest Nei's genetic distance (0.2262) as they are exit from different parental origin. On the other hand, Rice Hybrid2 vs Rice Hybrid1 varietal pair showed lowest genetic distance (0.1190) as they are cultivated from same parental origin. This variation can be created by geographical origin. The result also reveals that the genetic base among these rice hybrids is rather narrow. RAPD markers provide a fast, efficient technique for variability assessment that complements methods currently being used in genetic resource management.

SSR primers showed few polymorphic bands which can be used as a evidence of variety protection data. It is essential to submit DNA fingerprint data of a new hybrid for registration and documentation. DNA passport data or DNA blue print information can help in variety protection of the hybrid varieties. Being a poorly studied genome, little information is available on the molecular characterization of hybrid rice. The result of the present study can be used as a guideline for future diversity assessment and genetic analysis of hybrid rice.

RECOMMENDATION

Though, larger number of samples and higher number of primers would be necessary to generate and construct an appropriate genetic relationship, sample identification and analysis of genetic variation among different varieties and cultivars are widely acceptable by all concern. Using larger number of samples and higher number of primers could be useful in future study.

The present work was the preliminary study to assess genetic variation of rice hybrids and it had some limitations in term of limited number of individuals and varieties as well as number of primers used. The results indicate that the present study might be used as a guideline for further study and the following points might be considered for sustaining the genetic qualities of hybrid rice in Bangladesh:

1. Large number of varieties and higher number of primers should be studied with adequate replications to increase accuracy.
2. Details survey work should be conducted using more molecular markers for obtaining diagnostic loci for hybrid varieties.
3. Other molecular markers such as SNP, AFLP etc. should be developed for rice hybrid varieties of Bangladesh.

REFERENCES

Abbas S. T. (2000). Development of Rachna basmati- a new fine, aromatic rice variety– through biotechnological tools. *Sc.Vis*. **5**: 74-84.

Agarwal M., Sahi C., Katiyar-Agarwal S., Agarwal S., Young T., Gallie, Sharma V. M., Ganesan K. and Grover A. (2003). Molecular characterization of rice hsp101: Complementation of yeast hsp104 mutation by disaggregation of protein granules and differential expression in indica and japonica rice types. *Plant Mol. Biol*. **51**: 543-53.

Akagi H., Yokozeki Y., Inagaki A. and Fujimura T. (1997). Highly polymorphic microsatellites of rice consist of AT repeats, and a classification of closely related cultivars with these microsatellite loci. *Theor. Appl. Genet*. **94**: 61-67.

Akkaya M. S., Bhagwat A. A. and Cregan P. B. (1992). Length polymorphism of simple sequence repeat DNA in soybean. *Genetics*. **132**: 1131-1139.

Alam M. K., Rahman M. S., Mustakima Q. K. J., Hassan M. K., Ahmed N. U., Nath U. K., Sha-E-Alam M. and Rahman L. (2016). SSR based molecular diversity study in local rice genotypes of Bangladesh. *Intl. J. Innovative Res*. **1**(2): 17-24.

Alessandra C., Stefania D. G., Giovanni D. B., Antonino C., Marilena C. (2009). Molecular characterization of Italian rice cultivars. *Eur. Food Res. Technol*. **6:** 875-882.

Allhgholipour M., Farshdfar E. and Rabiei B. (2014). Molecular characterization and genetic diversity analysis of different rice cultivars by microsatellite markers. *Genetika*. **46**: 187-198.

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

Amin A., Razvi1 S. M., Dar Z. A., Zafar G., Bhat M. A., Mir M. R., Wani N. and Bhat K. A. (2010). Molecular markers and their application in improvement of vegetable crops. *Intl. J. Curren. Res*. **4**: 38-48.

Ashraf M., Cheema A. A., Qamar Z. U. and Rashid M. (2007). Detection of Polymorphism in Rice Germplasm using RAPD marker. *Pak. J. Bot*. **39**(7): 2483-2493.

BBS (Bangladesh Bureau of Statistics). (2014). Statistical Yearbook Bangladesh. Ministry of Planning, Government of the People's Republic of Bangladesh, Sher-e-Bangla Nagar, Bangladesh.

BER (Bangladesh Economic Review). (2013). Ministry of Finance, Finance Division. Economic Advisory wing. Government of the People's Republic of Bangladesh.

Bhuiyan N. I. (2004). The Hybrid Rice Program for Bangladesh. In: 'Hybrid Rice in Bangladesh: Progress and Future Strategies'. *Bangladesh Rice Res. Inst. Pub*. **138**: 3-5.

Bligh H. F. J., Blackhall N. W., Edwards K. J. and McClung A. M. (1999). Using amplified fragment length polymorphisms and simple sequence length polymorphisms to identify cultivars of brown and white milled rice. *Crop Sci*. **39**:1543-1635.

Botstein D., White R. L., Skolnick M. and Davis R. W. (1980). Construction of genetic linkage map in man using restriction fragment length polymorphisms. *Amer. J. Hum. Genet.* **32**: 314-333.

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

Chapco W., Ashton N. W., Martel R. K. B. and Antonishyn N. (1992). A feasibility study of the use of random amplified polymorphic DNA in the population genetics and systematics of grasshoppers. **35**: 569-574.

Chen H., He H., Zou Y., Chen W., Yu R., Liu X., Yang Y., Gao Y. M., Xu J. L., Fan L. M., Li Y., Li Z. K. and Deng X. W. (2011). Development and application of a set of

breeder-friendly SNP markers for genetic analyses and molecular breeding of rice (*Oryza sativa* L.). *Theor. Appl. Genet*. **123**: 869-879.

Choudhury B., Khan M. L. and Dayanandan S. (2013). Genetic structure and diversity of indegenous rice (*Oryza sativa*) varieties in the Eastern Himalyan region of Northeast India. *Springer Plus*. **2**(1): 228–38.

Choudhary G., Ranjitkumar N., Surapaneni M., Deborah D. A., Vipparla A. and Anuradha G. (2013). Molecular genetic diversity of major Indian rice cultivars over decadal period. *PLoS One*. **8**(6): 66197.

Choudhary K., Choudhary O. P. and Shekhawat N. S. (2008). Marker assisted selection: A novel approach for crop improvement. *Amer. Eurasian J. Agron*. **1**(2): 26-30.

Clarck A. G. and Lanigan C. M. S. (1993). Prospects for estimating nucleotide divergence with RAPDs. *Mol. Biol. Evol*. **10**: 1069-1111.

Cregan P. B. (1992). Simple sequence repeat DNA length polymorphisms. **2**: 18-22.

Das B. K., Jena R. C, Samal K. C. (2009). Optimization of DNA isolation and PCR protocol for RAPD analysis of banana / plantain (*Musa spp*.). *Intl. J. Agri. Sc*. **1**: 21-25.

Das B., Sengupta S., Parida S. K., Roy B., Ghosh M. and Prasad M. (2013). Genetic diversity and population structure of rice landraces from Eastern and North Eastern States of India. *BMC Genet*. **14**(1): 71.

Datta D., Gupta S., Chaturvedi S. K. and Nadarajan N. (2011). Molecular markers in crop improvement. *Indian Inst. of Pulses Res*. pp. 229-23.

Deepu V., Rajani J., Nair G. M. and Nair A. J. (2013). Molecular characterization of selected cultivars of rice, Oryza sativa L. using Random Amplified Polymorphic DNA (RAPD) markers. *Intl. Food Res. J*. **20**(2): 919-923.

FAO. Food and Agriculture Organization of the United Nations. (2017), Hybrid rice: progress, issues and outlook for Bangladesh. http://www.fao.org/3/x7164t/x7164t08.htm

Fristsch P. and Rieseberg L. H. (1996). The use of random amplified polymorphic DNA (RAPD) in conservation genetics. In: Smithers T. B. and R. K. Wanye, (eds.). Mol. genetic approaches in Conservation, Oxford University Press, Oxford. pp. 54-73.

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

Gosal S. S., Wani S. H. and Kang M. S. (2010). Biotechnology and crop improvement. *J. Crop Improv*. **24**(2): 153-217.

Goswami M. and Ranade S. A. (1999). Analysis of variations in RAPD profiles among accessions of Prosopsis. *J. Genet*. **78**: 141-147.

Gracia A. A. F., Benchimol L. L., Antonica M. M., Geraldi I. O. and Deuza A. P. (2004). Comparison of RAPD, RFLP, AFLP and SSR marker for diversity studies in tropical maize inbred lines. *Euphytica*. **108**: 53-63.

Hadrys H., Balicki M. and Schierwater B. (1992). Application of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol. Ecol*. **1**: 55-63.

Haig S. M., Rhymer J. M. and Heckel D. G. (1994). Population differentiation in Random Amplified Polymorphic DNA of red-cockaded woodpeckers Picoides borealis. *Mol. Ecol*. **3**: 581-595.

Hasan M. and Raihan M. S. (2015). Genetic Variability in Bangladeshi Aromatic Rice through RAPD Analysis. *Turkish J. Agri*. **3**(3): 107-111.

Heal G., Walker B., Levin S., Arrow K., Dasgupta P. and Dailyd G. (2004). Genetic diversity and interdependent crop choices in agriculture. *Resour. Energy Econ. **26***: 175–84.

HIES. Household Income and Expenditure Survey. (2010). Bangladesh Bureau of statistics, Ministry of Planning, Government of the People's Republic of Bangladesh, Dhaka.

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

IRGSP (2005). The map-based sequence of the rice genome. Nature **436**: 793-800.

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

Islam A. S. M. F., Ali M. R., Gregorio G. B. and Islam M. R. (2012). Genetic diversity analysis of stress tolerant rice (*Oryza sativa* L.). *Afr. J. Biotecnol*. **11**(85): pp. 15123-15129.

Islam M.N., Molla M.R. and Rahman L. (2013). Genetic characterization of sesame varieties through RAPD fingerprinting technique in Bangladesh. *J. Sher-e-Bangla Agric. Univ*. **7**(1): 1-5.

Islam M. Z. (2014). Variability assessment of aromatic and fine rice (*Oryza sativa* L.) genotypes through morphological, physicochemical traits and microsatellite DNA markers. Gazipur, Bangladesh: Bangabandhu Sheikh Mujibur Rahman Agricultual University: 115-141.

Jahufer M., Barret B., Griffiths A. and Woodfield D. (2003). DNA fingerprinting and genetic relationships among white clover cultivars. Proceedings of the New Zealand Grassland Association.

Jain S., Rajinder K. J. and McCouch S. R. (2003). Genetic analysis of Indian aromatic and quality rice (*Oryza sativa* L.) germplasm using panels of fluorescently labeled microsatellite markers. *Theor. and Appl Genet*. **109**: 965-977.

Jin L., Lu Y., Xiao P., Sun M., Corke H. and Bao J. (2010). Genetic diversity and population structure of a diverse set of rice germplasm for association mapping. *Theor Appl Genet*. **121**(3): 475–87.

Julfiquar A. W., Haque M. M. E. and Rahsid, M. A. (2008). Current status of hybrid rice research and future programme in Bangladesh. A country report presented in the workshop on Use and Development of hybrid rice in Bangladesh .

Kalia R. K., Rai M. K., Kalia S., Singh R. and Dhawan A. K. (2011). Microsatellite markers: an overview of the recent progress in plants. *Euphytica*. **177**:309–334.

Kanawapee N., Sanitchon J., Srihaban P. and Theerakulpisut P. (2011). Genetic diversity analysis of rice cultivars (*Oryza sativa* L.) differing in salinity tolerance based on RAPD and SSR markers. *Elect. J Biotech*. **6**: 25-31.

Karkousis A., Barr A. R., Chalmers K. J., Ablett G. A., Holton T. A., Henry R. J., Lim P. and Langridge P. (2003). Potential of SSR markers for plant breeding and variety identification in Australian Barley germplasm. *Aust. J. Agric. Res*. **54**: 1197-1210.

Karp A., Kresovich S., Bhat K., Ayad W., and Hodgkin T. (1997). Molecular tools in plant genetic resources conservation: a guide to the technologies. IPGRI. Rome, Italy.

Karunagoda R. P. and Bandara C. S. (2005). Molecular characterization of some consumer-preferred rice varieties using randomly amplified polymorphic dnas (RAPDs). *Sri Lankan J. Agric. Sci*. **42**: 71-80.

Khalequzzaman M., Islam M. Z., Siddique M. A., Prince M. F. R. K., Rashid E. S. M. H. and ahamed M. S. (2017). Genetic diversity in aus rice landraces of Bangladesh using SSR markers. *Bangladesh J. Pl. Breed. Genet*. **30**(1): 11-20.

Kimberling D. N., Ferreira A. R., Shuster S. M. and Keim P. (1996). RAPD marker estimation of genetic structure among isolated northern leopard frog populations in the southwestern USA. *Mol. Ecol*. **5**(4): 521-529.

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

Kumar N. S. and Gurusubramanian G. (2011). Random amplified polymorphic DNA (RAPD) markers and its applications. *Sci. Vis*. **11**: 116-124.

Kumari N. and Thakur S. K. (2014). Randomly Amplified Polymorphic DNA A brief review. *Amer. J. Anim. Vet. Sci*. **9**(1): 6-13.

Lagercrantz U., Ellegren H. and Andersson L. (1993). The abundance of various polymorphic microsatellite motifs differs between plants and vertebrates. *Nucleic Acids Res*. **21**: 1111-1115.

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

Leroy X. J., Leon K., Charles G. and Branchard M. (2000). Cauliflower somatic embryogenesis and analysis of regenerant stability by ISSRs. *Plant Cell Rep.* **19**: 1102-1107.

Lin J. Y. (1991). The household Responsibility System Reform and Adoption of Hybrid Rice in China. *J. Develop. Econom*. **36**: 353-372.

Lin J. Y. (1994). Impact of Hybrid Rice on Input Demand and Productivity. *Agricultural Economics*. **10**: 154-164.

Litt M. and Luty J. A. (1989). A hypervariable microsatellite revealed by *in vitro* amplication of a dinucleotide repeat within the cardiac muscle action gene. *Amer. J. Hum. Genet*. **44**: 388-396.

Liu G., Bernhardt J., Jia M. H., Wamishe Y. and Jia Y. (2007). Molecular characterization of rice recombinant inbred line population derived from a japonica-indica cross. *Euphytica*. **159**: 73-82.

Mackill D., Nguyen H. and Zhang J. (1997). Use of molecular markers in plant improvement programs for rainfed lowland rice. Field crops research. **64**(1): 177-85.

Masum M. (2009). Impact of hybrid rice in Bangladesh. *The Guardian*. **19**: 56-58.

Madhumati B. (2014). Potential and application of molecular markers techniques for plant genome analysis. *Int. J. Pure App. Biosci*. **2**(1): 169-188.

Ma H., Yin Y., Guo Z. F., Cheng L. J., Zhang L., Zhong M. and Shao G. J. (2011). Establishment of DNA fingerprinting of Liaojing series of japonica rice. Middle-East Journal of Scientific Research. **8**: 384-392.

Mailer R. J., Scarth R., and Fristensky B. (1994) Discrimination among cultivars of rapeseed (*Brassica napus* L.) using DNA polymorphism amplified from arbitrary primers. *Theor. Appl. Genet*. **87**: 697-704.

Malik A. R., Zahida H. P. and Muhammad S. M. (2008). Genetic diversity analysis of traditional and improved cultivars of Pakistani rice (*Oryza sativa* L.) using RAPD markers. *Electronic J. Biotechnol*. **11**(3): 1-10.

Mani P., Bastin J., Arun kumar R. and Ahmed A. B. A. (2010). RAPD analysis of genetic variation of four important rice varieties using two OPR primers. *ARPN J. Agric. Biol. Sc*. **5**: 25-31.

Mao C. (2006). Improving seed production to speed up the global commercialization of hybrid rice. Rice research for food security and poverty alleviation- Proceedings of the International Rice Research Conference.

Marshall P., Marchand M. C., Lisieczko Z. and Landry B. S. (1994). A simple method to estimate the percentage of hybridity in canola (*Brassica napus*) F1 hybrids. *Theor. Appl. Genet*. **89**: 853858.

McCouch S., Kochert G. and Yu Z. (1988). Molecular mapping of rice chromosomes. *Theor. Appl. Genet*. **76**(6): 815-29.

McCouch S., Chen X., Panaud O., Temnykh S. and Xu Y. C. Y. (1997). Microsatellite marker development, mapping and applications in rice genetics and breeding. *Plant Mol Biol*. **35**(1–2): 89–99.

McCouch S. R., Teytelman L., Xu Y., Lobos K. B., Clare K., Walton M., Fu B., Maghirang R., Li Z. and Xing Y. (2002). Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). *DNA Res*. **9**: 199-207.

Michelmore R. W., Paran I. and Kesseli R. V. (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci. USA.* **88**(21): 9828-9832.

Mitra S., Islam T., Sarker R. H. and Hoque M. I. (2017). RAPD Profile Analysis of Single and Multigrain Aman Rice (*Oryza sativa* L.) Varieties Available in Bangladesh. *Plant Tissue Cult. and Biotech*. **27**(2): 195-205.

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

Mondini L., Noorani A. and Pagnotta M. A. (2009). Assessing plant genetic diversity by molecular tools. *Diversity*. **1**(1): 19-35.

Morgante M. and Olivieri A. (1993). PCR-amplified microsatellites as markers in plant genetics. *Plant J.* **3**:175-182.

Muhammad A., Samina K., Muhammad A. B., Anjuman A. and Yusuf Z. (2005). Genetic diversity among rice genotypes of Pakistan through Random Amplified Polymorphic DNA (RAPD) analysis. *Pakistan J. Bot*. **37**(3): 585-592.

Nei M. and Chesser R. K. (1983). Estimation of fixation indices and gene diversities. *Annual Hum. Genet*. **47**: 253-259.

Ni J., Colowit P. M., Mackill D. J. (2002). Evaluation of genetic diversity in rice subspecies using microsatellite markers. *Crop Sci*. **42**(2):601-07.

NSB (National Seed Board). (2015). Hybrid rice varieties released in Bangladesh 1997 to October 2014.

PANAP. (2007). Hybrid Rice in Asia: Sowing the Seeds of Dependency, A Fact Sheet on Hybrid Rice. Pesticide Action Network Asia and the Pacific (PANAP), Penang, Malaysia.

Panaud O., Chenayd X., McCouch S. R. (1996). Development of microsatellite markers and characterization of simple sequence length polymorphism (SSLP) in rice (*Oryza sativa* L.). *Mol. Gen. Genet*. **252**(5): 597-607.

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

Prabakaran A., Paramasivam K., Rajesh T. and Rajarajan D. (2010). Molecular characterization of rice land races using SSR markers. *Elec. J. Plant Breed*. **1**: 512-516.

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

Qian W., Song G., Yuan H. D. (2006). Genetic diversity in accessions of wild rice *Oryza* granulate from South and Southeast Asia. *China Genet. Res. Crop Evol*. **53**(1): 197204.

Rabbani M. A., Pervaiz Z. H. and Masood M. S. (2008). Genetic diversity analysis of traditional and improved cultivars of Pakistani rice (*Oryza sativa* L.) using RAPD markers. *Elect. J. Biotech*. **11**: 3.

Raghunathachari P., Khanna V. K., Singh U. S. and Singh N. K. (2000). RAPD analysis of genetic variability in Indian scented rice germplasm (*Oryza sativa* L.). *Curr. Sci*. **79**: 994-998.

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

Rahman M. M., Rahman L., Begum S. N., Nur F. (2009). Molecular characterization and genetic diversity study in F3 population of rice. *Progress. Agric*. **20**: 1-8.

Rahman S. N., Islam M. S., Alam M. S. and Nasiruddin K. M. (2006). Genetic polymorphism in rice (*Oryza sativa* L.) through RAPD analysis. *Indian J. Biotech*. pp. 224-229.

Rajani J., Deepu V., Nair G. M. and Nair A. J. (2013). Molecular characterization of selected cultivars of rice, *Oryza sativa* L. using Random Amplified Polymorphic DNA (RAPD) markers. *Int. Food Res. J*. **20**(2): 919-923.

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

Ramadan E. A., Elmoghazy A. M. and El-Mowafi H. F. (2015). Molecular markers based genetic diversity analysis for drought tolerance in rice (*Oryza Sativa* L.) Using SSR Markers. *Intl. J. Sci. Res. Agric. Sci*. **2**: 137-146.

Rana M. K. and Bhat K. V. (2004). A comparison of AFLP and RAPD markers for genetic diversity and cultivar identification in cotton. *J. Plant Biochem. Biotech*. **13**: 19-24.

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

Rao M., Grithlahre S., Bisen P., Singh N. K., Dar M. H., Singh U. S. and Singh P. K. (2016). Genetics of marker assisted backcross progenies of the cross HUR-105 X SwarnaSUB1. *Intl. J. Agri., Environ. Biotech*. **9**: 499-505.

Rasheed S., Fatima T., Husnain T., Bashir K. and Riazuddin S. (2005). RAPD characterization of somaclonal variation in indica basmati rice. *Pak. J. Bot*. **37**: 249-262.

Rashmi D., Bisen P., Saha S., Loitongbam B., Singh S., Pallavi and Singh P. K. (2017). Genetic Diversity Analysis in Rice (*Oryza sativa* L.) accessions using SSR Markers. *Int. J. Agri. Environ. Biotech*. **10**(4): 457-467.

Rholf F. (2002). Numerical Taxonomy and Multivariate Analysis System Version 2.2. New York, USA: Department of Ecology and Evolution, State University of New York.

Rodriguez J. M., Berke T., Engle L. and Nienhuis J. (1999). Variation among and within Capsicum species revealed by RAPD markers. *Theor. Appl. Genet*. **99**: 147-156.

Sajib A. M., Hossain M. M., Mosnaz A. T. M., Hossain H., Islam M. M., Ali M. S. and Prodhan S. H. (2012). SSR marker-based molecular characterization and genetic diversity analysis of aromatic landreces of rice (*Oryza sativa* L.). *J. BioSci. Biotech*. **1**: 107-116.

Saker M. M., Youssef S. S., Abdallah N. A., Bashandy H. S., Sharkawy A. M. E. (2005). Genetic analysis of some Egyptian rice genotypes using RAPD, SSR and AFLP. *Afr. J. Biotech.* **4**: 882-890.

Salgotra R. K., Gupta B. B., Bhat J. A. and Sharma S. (2015). Genetic diversity and population structure of Basmati rice (*Oryza sativa* L.) germplasm collected from North Western Himalayas using trait linked SSR markers. *PLoS One*. **10**(7): e0131858.

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

Shah S. M, Naveed S. A. and Arif M. (2013). Genetic diversity in Basmati and non-Basmati rice varieties based on microsatellite markers. *Pak J Bot*. **45**: 423–31.

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

Shivapriya M. and Shailaja H. (2006). Detection of genotype specific fingerprints and molecular diversity of selected Indian locals and landraces of rice (*Oryza sativa* L.) using DNA markers. *Indian J. Genet. Plant Breed*. **66**(1): 1-5.

Siddique M. A., Rashid E. S. M. H., Khalequzzaman M., Bashar M. K. and Khan L. R. (2014). Molecular Characterization and Genetic Diversity in T. Aman Landraces of Rice (*Oryza sativa*) Using Microsatellite Markers. *Thai J. Agric. Sci*. **47**(4): 211-220.

Siddique M. A., Khalequzzaman M., Islam M. M., Fatema K. and Latif M. A. (2016). Molecular characterization and genetic diversity in geographical indication (GI) rice (*Oryza sativa* L.) cultivars of Bangladesh. *Braz. J. Bot*. **10**: 1007.

Singh A. (2003). Genetic analysis of rice (*Oryza sp.*) accession using Randomly Amplified Polymorphic DNA (RAPD) and Microsatellites. *Trop. Agric. Res*. **15**: 77-86.

Singh B. D. (2000). Center of origin, Plant breeding. Kalyani Publishers. New delhi-Ludhiana India. Pp. 27-31.

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

Singh N., Choudhury D. R., Singh A. K., Kumar S., Srinivasan K. and Tyagi R. K. (2013). Comparison of SSR and SNP markers in estimation of genetic diversity and population structure of Indian rice varieties. PLOSone. **10:** 1371.

Sneath P. H. A. and Sokal R. R. (1973). Numerical taxonomy: the principles and practice of numerical classification. San Francisco: Freeman. pp. 573.

Sow M., Ndjiondjop M. N., Sido A., Mariac C., Laing M. and Bezançon G. (2014). Genetic diversity, population structure and differentiation of rice species from Niger and their potential for rice genetic resources conservation and enhancement. *Genet Resour Crop Evol*. **61**(1): 199-13.

Squirrel J., Hollingsworth P. M., Woodhead M., Russell J., Lowe A. J., Gibby M. and Powell W. (2003). How much effort is required to isolate nuclear microsatellites from plants. *Molecular Ecolgoy*. **12**: 1339-1348.

Tautz D. (1989). Hypervariabflity of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Res*. **17**: 6463-6471.

Tehrim S., Pervaiz Z. H. and Rabbani M. A. (2012). Molecular characterization of traditional and improved rice cultivars based on random amplified polymorphic DNAs (RAPDs) markers. *African J. Biotech*. **11**: 10297-10304.

Temnykh S., Park W. D., Ayes N., Carthinour S., Hauck N., Lipovich L., Cho Y. G., Ishii T. and McCouc S. R. (2000). Mapping and genome organization of microsatellite sequences in rice (*Oryza sativa* L.). *Theor. Appl. Genet*. **100**(5):697-712.

Thomas G., Mohapatra T., Rao A. R. and Sharma R. P. (2006). Distinguishing Indian commercial wheat varieties using RAPD based DNA fingerprints. *Indian J. Biotechnol*. **5**: 200-206.

Thompson J. A., Nelson R. L. and Vodkin L. O. (1998). Identification of diverse soybean germplasm using RAPD markers. *Crop Sci*. **38**:1348-1355.

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

Tommercup I. C., Barton J. E. and Obrein P. A. (1998). Reliability of RAPD fingerprinting of three basidiomycetes fungi, Laccaria, Hydnangium and Rhizoctonia. *Mycol. Res*. **99**: 179-186.

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

Varshney R. K., Sigmund R., Borner A., Korzun V., Stein N., Sorrells M. E., Langridge P. and Graner A. (2005). Interspecific transferability and comparative mapping of barley EST–SSR markers in wheat rye and rice. *Plant Sci*. **168**: 195-202.

Vierling R. and Nguyen H. T. (1992). Use of RAPD markers to determine the genetic relationships of diploid wheat genotypes. *Theor. Appl. Genet*. **84**: 835-838.

Vilanova S., Manzur J. P. and Prohens J. (2012). Development and characterization of genomic simple sequence repeat markers in eggplant and their application to the study of diversity and relationships in a collection of different cultivar types and origins. *Mol. Breed.* **30:** 647-660.

Virmani S. S., Siddiq E. A. and Muralidaran K. (1998). Advances in Hybrid Rice Technology, IRRI, Los Banos.

Vos P., Hogers R., Bleeker M., Reijans M., Van de Lee T., Hornes M., Frijters J. P., Peleman J., Kuiper M. and Zabeau M. (1995). AFLP: A new fingerprinting technique for DNA fingerprinting. *Nucleic Acids Res*. **23**: 4407-4414.

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

Williams J. G. K., Kubelik A. E., Levak K.J., Rafalski J. A. and Tingey S. V. (1990). Genetic analysis using random amplified polymorphic DNA markers. Methods Enzymology. **1218**: 704-740.

Wu K. S. and Tanksley S. D. (1993). Abundance, polymorphism and genetic mapping of microsatellites in rice. *Mol. Gen. Genet*. **241**: 225-235.

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

Yasmin F., Islam M. R., Rehana S., Mazumder R. R., Anisuzzaman M., Khatun H., Rayhan R. and Gregorio G. B. (2012). Molecular characterization of inbred and hybrid rice genotypes of Bangladesh. *SABRAO J. Breed. Genet*. **44**: 163-175.

Yeasmin S., Islam M. M. and Begum S. N. (2013). Molecular characterization of some rice genotypes using RAPD markers. *Bangladesh J. Nuclear Agric*. **29:** 1-11.

Yeh F. C., Yang R. C. and Boyle T. (1999). POPGENE VERSION 1.31: Microsoft Window-based free Software for Population Genetic Analysis.

Zhang D., Zhang H., Wei X., Qi Y., Wang M. and Sun J. (2007). Genetic structure and diversity of *Oryza sativa* L. in Guizhou, China. *Chinese Sci Bull*. **52**(3): 343–51.

Zhou H. F., Xie Z. W. and Ge S. (2003). Microsatellite analysis of genetic diversity and population genetic structure of a wild rice (*Oryza rufipogon* Griff) in China. *Theor. Appl. Genet*. **107**: 332-339.