Molecular Diversity Analysis of Different Hybrid Rice (*Oryza sativa* L.) Varieties through RAPD and SSR Markers

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Abstract

Rice (Oryza sativa L.) is one of the principal cereal crops around the globe. A study was conducted to assess molecular diversity of four rice hybrid varieties released from a private seed company using PCR-based Random Amplified Polymorphic DNA (RAPD) and Single Sequence Repeat (SSR) markers. A total of 20 RAPD primers and 4 SSR primers were used to determine polymorphism among the rice hybrids. Some total 76 DNA bands were amplified through RAPD primers. Among them 24 were polymorphic bands. The rate of polymorphism was obtained about 30.20%. Genetic diversity ranged from 0.083 to 0.438 and the frequency of major allele ranged from 0.625 to 0.917. The PIC value ranged from 0.063 to 0.340 with the average value 0.150. The PIC value indicated that the studied rice hybrids had low molecular diversity. The dendrogram 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 bands which can be used as an evidence of variety protection data. These experimental findings can be used for the protection of hybrids in commercial purpose and in managing rice genetic resource in Bangladesh.

Keywords: *Molecular Diversity, RAPD and SSR Markers, Polymorphism, Rice hybrid.*

I. INTRODUCTION

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. At present, rice alone constitutes about 93% of the total food grains produced annually in the country [1]. It provides about 62% of the calorie and 46% of the protein in the average daily diet of the people [2]. In Bangladesh, rice engages more than 70 % of the rural population and majority of the farmers focused for adoption of hybrid rice which is beneficial to the farmers in both production and profit. A great number of rice varieties are released and notified every year.

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 those farmers grown [3]. 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 [4], [5], [6]. Hybrid rice in China and other countries has yielded 20-30% higher than the best conventional varieties. Bangladesh is the fourth-largest producer and consumer of hybrid 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 110 varieties are imported varieties.

Molecular markers have been successfully applied in variety and cultivar identification [7], or controls of seed purity of hybrid varieties [8]. The use of DNA markers has been suggested for precise and reliable characterization and discrimination of rice genotypes [9]. Recent advances in molecular biology enabled DNA based markers to study genetic basis of crops [10], [11], [12], [13]. Several kinds of molecular markers are available for marker assisted selection and diversity study. Among these RAPD markers are increasingly being employed in genetic research owing to its speedy process and simplicity [14]. It is a PCR based marker which has many advantages including readily being used, requiring minute amount of genomic DNA, does not need blotting and radioactive detection etc. 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 [15], [16]. The SSRs are most suitable for rice because of their reproducibility, multiallelic nature, hypervariablility, co-dominant inheritance, relative abundance and genome-wide coverage [17]. SSR markers are efficient in detecting genetic polymorphisms and discriminating among genotypes from germplasm of various sources, even they can detect finer level of variation among closely related breeding lines within a same variety [18]. Keeping in view the role of RAPD and SSR markers in the determination of genetic diversity, polymorphism was studied done in some rice hybrids through PCR amplification technique.

II. MATERIALS & METHODS

A. Experimental Site and Source Materials

The experimental study was carried out at the Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka-1207, Bangladesh. Four rice hybrids were used as experimental materials. All the genotypes were collected from a Private Seed Company at Dhaka, Bangladesh.

B. DNA Isolation

Good quality, disease free, healthy rice seeds were collected and seed were germinated and sample were collected from two week old seedling. Genomic DNA was extracted from approximately 200 mg of sterilized young leaves by using SDS detergent. Extracted DNA was visualized in 1% agarose gel. Approximate 20- 25 ng of DNA was used as template in PCR reaction.

C. RAPD and SSR Primer Selection

Twenty 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 and OPZ-06 showed minimum smearing, high resolution and maximum reproducible and distinct polymorphic amplified bands. As well, four SSR primers *viz*. RM1, RM17, RM72, OSR16 were used for DNA amplification.

PCR reactions were functioned on each DNA sample. DNA amplification was per formed in oil-free thermal cycler (Esco Technologies swift^{TM} Mini Thermal Cycler and Q-cycler). The PCR reactions were performed in 10 µl reaction mixture containing 5.0 µl 2X Taq Master Mix, 1.50 µl primers, 2.0 µl sample DNA and 1.50 µl de-ionized water. RAPD primers were amplified under the following PCR conditions: Pre-denaturation with 95^0 C for min; de-naturation with 95^0 C for 45sec, annealing at 35^0 C (on the basis of Tm value of primer) for 40 sec, extension at 72^0 C for 1 min, final extension at 72^0 C for 5 min continuing with 31 cycles and finally stored at 4^0C.

SSR primers were amplified under the following PCR conditions: Pre-denaturation with 95^0 C for 5 min; de-naturation with 95^0 C for 50 sec, annealing at 60^0 C (on the basis of Tm value of primer) for 45 sec, extension at 72^0 C for 1 min, final extension at 72^0 C for 5 min continuing with 31 cycles and finally stored at 4^0 C.

D. Electrophoretic Separation of the Amplified Products

PCR products for each sample were confirmed by running in 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. Under Ultra Violet (UV) light on a trans-illuminator RAPD and SSR bands were observed and documented by taking photograph using a Gel Cam Polaroid camera.

E. RAPD Data Analysis

One molecular weight marker, 100 bp DNA ladder (Bio Basic, Cat. No. M-1070, Canada) was used to evaluate the size of the amplified 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 position 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 [19]. 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 dendrogram construction by NTSYS-pc software [20]. The unweighted pair grouping method, using arithmetic average (UPGMA), was used to determine similarity matrix following Dice coefficient with SAHN sub program.

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

$PIC = 1-\sum pi^2$

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) [21]. The dendrogram was constructed using a computer program, POPGENE; (Version 1.31) [22].

III. RESULTS

Highly polymorphic and repeatable PCR based markers Random Amplified Polymorphic DNA (RAPD) were used here to assess the polymorphism, diversity and similarity identification within those rice hybrids. Results obtained from the experiment have been presented below under the following headings.

A. DNA amplification by RAPD primer and its polymorphism

Twenty RAPD primers were screened on four rice hybrids to evaluate their suitability for amplification of DNA. Among them thirteen primers OPB-17, OPBA-06, OPBB-03, OPBB-05, OPBB-06, OPBB-09, OPBC-05, OPBD-18, OPD-20, OPG-03, OPX-10, OPY-11 and OPZ-06 showed reproducible and distinct polymorphic amplification. A total of 24 polymorphic bands were detected with an average number of alleles of 1.20 and a range between 1 to 4 alleles. (Table 1). The number of polymorphic band (2) was produced by the primer OPB-17, OPBA-06, OPBB-05 and OPZ-06 of each respectively and lowest (1) number of polymorphic band was produced by the primer OPBC-05, OPBD-18, OPD-20, OPG-03, OPX-10 and OPY-11. The primer OPBB-03 was produced 4 DNA fragments in total which ranged from 300 to 650 bp (base pair). All bands were polymorphic (Figure 3). The primer OPBB-06 was showed total no. of 7 bands. Among them 3 DNA fragments were polymorphic which ranged from 250 to 1100 bp (Figure 6). Four DNA fragments amplification were noticed by the primer OPBB-09 in four rice hybrids which were ranged from 300 to 650 bp and 3 were polymorphic in nature and another band was monomorphic (Figure 7). Maximum 100% of polymorphism was recorded in the primer OPBB-03 and it was followed by primer OPY-11 (17%) which was lowest polymorphism.

Table 1: RAPD primers with corresponding banding pattern and polymorphism observed in four rice hybrids.

Sl. No.	Name of RAPD Primer	Sequence of primer (5′-3′)	GC content (%)	No. of bands scored	Number of Polymorphic Bands	Percentage of Polymorphic loci %	DNA fragment size (bp)
1.	OPB-17	AGGGAACGAG	60	5	2	40	375-1300
2.	OPBA-03	GTGCGAGAAC	60	2	0	0	300-600
3.	OPBA-06	GGACGACCGT	70	7	2	29	175-1100
4.	OPBB-03	TCACGTGGCT	60	4	4	**100**	300-650
5.	OPBB-05	GGGCCGAACA	70	3	2	67	300-500
6.	OPBB-06	CTGAAGCTGG	60	7	3	43	250-1100
7.	OPBB-09	AGGCCGGTCA	70	4	3	75	300-650
8.	OPBB-12	TTCGGCCGAC	70	4	0	0	300-1500
9.	OPBC-05	GAGGCGATTG	60	3	1	33	350-600
10.	OPBC-14	GGTCCGACGA	70	4	0	0	250-750
11.	OPBC-16	CTGGTGCTCA	60	2	0	0	400-900
12.	OPBD-16	GAACTCCCAG	60	3	0	0	250-900
13.	OPBD-18	ACGCACACTC	60	4	1	25	275-600
14.	OPD-20	ACCCGGTCAC	70	3	1	33	250-800
15.	OPG-03	GAGCCCTCCA	70	4	1	25	375-600
16.	OPG-05	CTGAGACGGA	60	3	0	0	150-400
17.	OPG-17	ACGACCGACA	60	3	0	0	275-450
18.	OPX-10	CCCTAGACTG	60	2	1	50	250-600
19.	OPY-11	AGACGATGGG	60	6	1	**17**	300-800
20.	OPZ-06	GTGCCGTTCA	60	3	2	67	300-650
Total		-	-	76	24	604	-
Mean		**-**	**-**	**3.80**	**1.20**	**30.20**	**-**

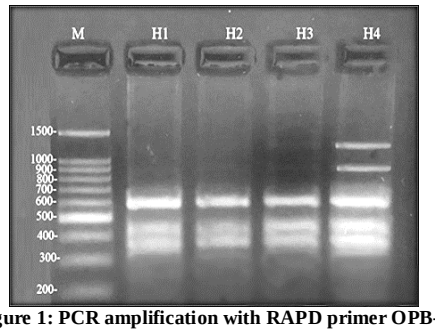


Figure 1: PCR amplification with RAPD primer OPB-17

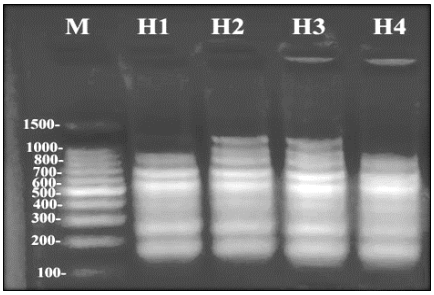


Figure 2: PCR amplification with RAPD primer OPBA-06

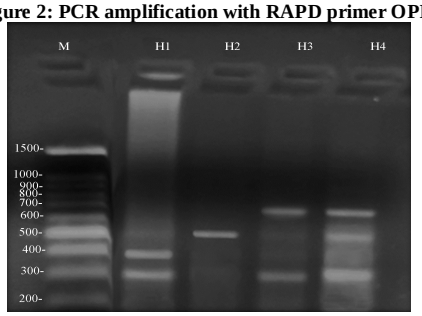


Figure 3: PCR amplification with RAPD primer OPBB-03

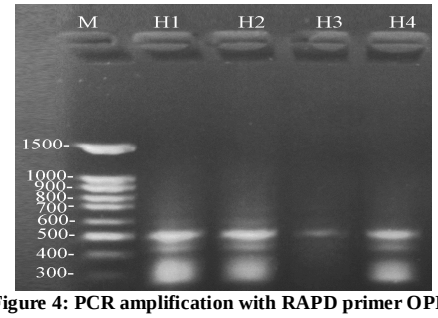


Figure 4: PCR amplification with RAPD primer OPBB-05

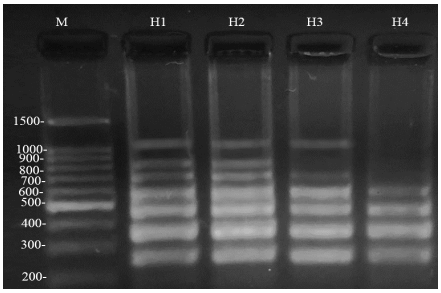


Figure 5: PCR amplification with RAPD primer OPBB-06

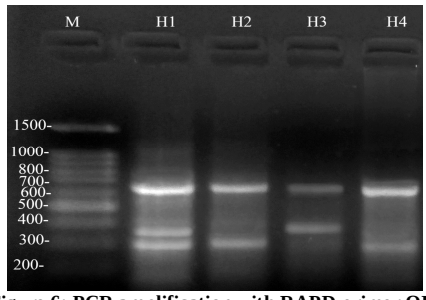


Figure 6: PCR amplification with RAPD primer OPBB-09

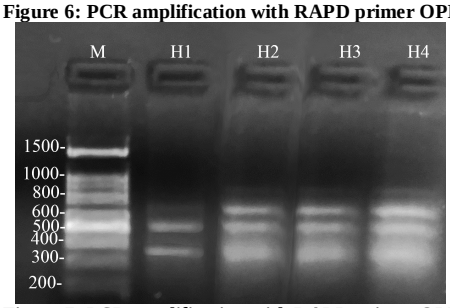


Figure 7: PCR amplification with RAPD primer OPBC-05

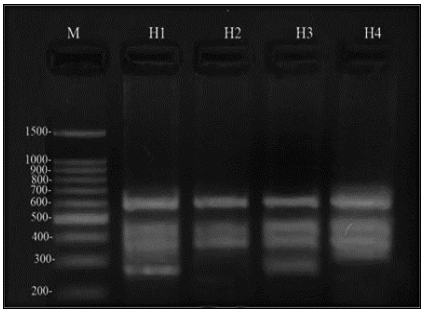


Figure 8: PCR amplification with RAPD primer OPBD-18

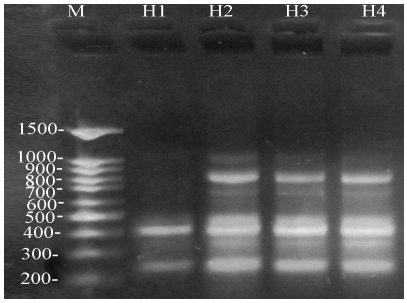


Figure 9: PCR amplification with RAPD primer OPD-20

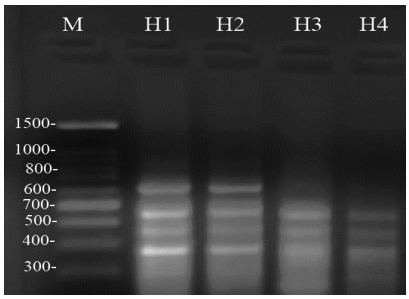


Figure 10: PCR amplification with RAPD primer OPG-03

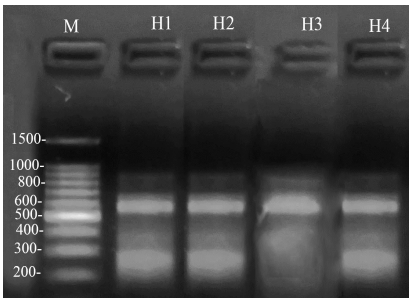


Figure 11: PCR amplification with RAPD primer OPX-10

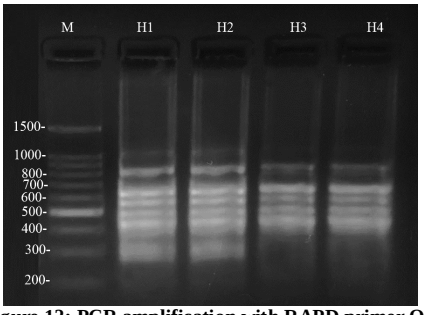


Figure 12: PCR amplification with RAPD primer OPY-11

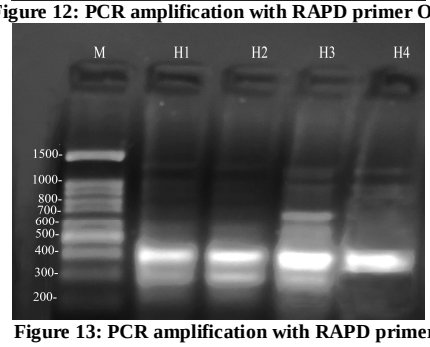


Figure 13: PCR amplification with RAPD primer OPZ-06 [M= 1kb DNA ladder (Bio Basic, Canada), Lane 1= Hybrid1, Lane 2= Hybrid2, Lane 3= Hybrid3, Lane 4= Hybrid4]

B. Gene diversity, gene frequency and PIC values

DNA polymorphisms were detected according to band presence and absence. Seven primers *viz.* OPBA-03, OPBB-12, OPBC-14, OPBC-16, OPBD-16, OPG-0

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.

Out of twenty primers, thirteen primers used in the present study showed different levels of major allele frequency, gene diversity and polymorphism information content (PIC) values which are presented in Table 2.

Table 2: Gene diversity, gene frequency and PIC value of different hybrid varieties of rice by RAPD primers.

Sl. No.	Primer	Major Allele frequency	Gene Diversity	PIC
1.	OPB-17	0.900	0.150	0.122
2.	OPBA-06	0.857	0.143	0.107
3.	OPBB-03	0.625	0.438	0.340
4.	OPBB-05	0.833	0.250	0.203
5.	OPBB-06	0.857	0.179	0.141
6.	OPBB-09	0.813	0.281	0.229
7.	OPBC-05	0.917	0.125	0.102
8.	OPBD-18	0.875	0.125	0.094
9.	OPD-20	0.917	0.125	0.102
10.	OPG-03	0.875	0.125	0.094
11.	OPX-10	0.875	0.188	0.152
12.	OPY-11	0.917	0.083	0.063
13.	OPZ-06	0.833	0.250	0.203
Mean		**0.853**	**0.189**	**0.150**

PCR products of thirteen RAPD markers were characterized. The frequency of the major allele ranged between 0.625 to 0.917 with an average value of 0.853. The primer OPBB-03(0.438) was considered as the best marker for diversity analysis in rice hybrids followed by OPBB-09(0.281). Gene diversity ranged between 0.083(OPY-11) to 0.438(OPBB-03) with an average of 0.189. The highest Polymorphism Information Content (PIC) value was observed in the primer OPBB-03(0.340) and it was lowest (0.063) in the primer OPY-11 and the average PIC value was 0.150.

The PIC was a good index for genetic diversity evaluation. Reference [23] 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. The primer OPBB-03 showed highest (0.340) PIC value and can be considered as the best marker for rice hybrids evaluation. The second highest PIC value (0.229) was recorded in the primer OPBB-09. All of the primers noticed low PIC value (maximum 0.340) revealed that, the studied materials had a low degree of genetic diversity.

C. Nei's Genetic Distance and Genetic Identity

Genetic distance refers to the genetic deviation between species or between populations within a species. Similarity indices measure the amount of closeness between two individuals, the larger the

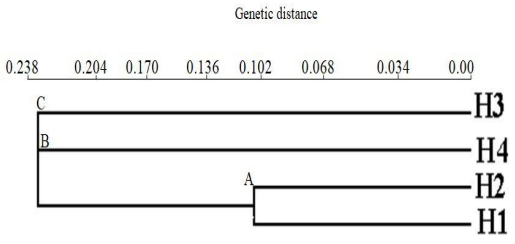
value the more similarity between two individuals. Genetic distance can be used to compare the genetic similarity between different species. The value of pair-wise comparisons Nei's [24] genetic distance between four rice hybrids were computed from combined data through twenty one primers, ranging from 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 3). The result also reveals that the genetic base among these rice hybrids is rather narrow.

Table 3: Summary of Nei's genetic identity (above diagonal) and genetic distance (below diagonal) values among four rice hybrids.

	Hybrid1	**Hybrid 2**	**Hybrid3**	**Hybrid4**
Hybrid 1	0.0000	0.1190	0.2024	0.1667
Hybrid 2	0.1190	0.0000	0.1548	0.1667
Hybrid 3	0.2024	0.1548	0.0000	0.2262
Hybrid 4	0.1667	0.1667	0.2262	0.0000

D. Genetic Relationship (UPGMA Dendrogram)

Dendrogram based on Nei's [25] genetic distance using Unweighted Pair Group Method of arithmetic Mean (UPMGA) segregated the 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.



E. DNA amplification by SSR

Four SSR primers were screened on randomly 4 rice hybrid variety genotypes to evaluate their suitability for amplification of the rice DNA fragments. A 125 bp DNA fragment was common in hybrid-01, 02 and 03 (Figure 14). The hybrid-04 produced 100 bp DNA fragment by the rice specific SSR primer RM1. 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 rice hybrids.

Rice specific SSR primer RM17 used for the DNA fingerprint of rice hybrids. A 150 bp DNA band is common in H1, H2 and H4 varieties (Figure 15). The hybrid H3 showed 175 bp DNA band by the same primer. Only 25 bp polymorphism was observed in hybrid-04. This molecular diversity can be used as an evidence of variety protection data.

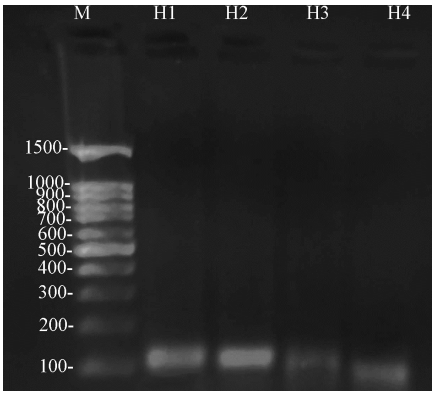


Figure 14: PCR amplification with SSR primer RM1

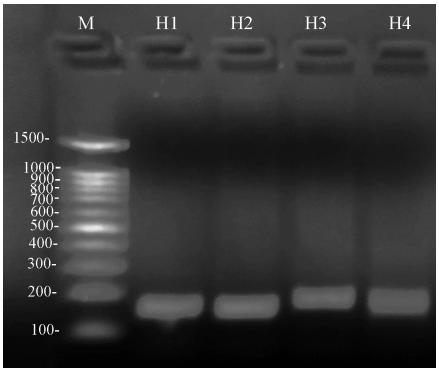


Figure 15: PCR amplification with SSR primer RM17

IV. DISCUSSION

Molecular characterization is a selection criteria for breeding technique as they're more consistent, reliable, fewer tormented by environmental variations, requires small number of samples, and does not require large experimental setup and

equipments for measuring physiological parameters [26]. 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 [27] [28] [29]. This research investigation presented mainly the molecular characterization of four rice hybrids. Twenty RAPD primers generated total 76 distinct and differential amplification bands with an average of 3.80 bands per primer. Among them 24 DNA fragments were polymorphic and the chances of polymorphism ranged from 17% to 100%. The highest percentage of polymorphic bands (100%) was generated by primer OPBB-03. In this study, the level of polymorphism (30.20%) indicated the effectiveness of RAPD technique to investigate less amount of polymorphism or diversity among the different varieties of hybrid rice. Reference [30] found 53.85% polymorphism in six different rice cultivars using three RAPD primers. The lower level of polymorphism in this study indicated that there is a basic similarity among the varieties which is due to the common ancestor and the similarity of selective traits.

The frequency of the major allele ranged between 0.625 to 0.917 with an average value of 0.853. The overall gene diversity ranged between 0.083 to 0.438 with a median of 0.189. The result indicated that the four rice hybrids present a low degree of homozygosity and a certain degree of genetic differentiation and polymorphism. Reference [31] found overall higher gene diversity for the indica group of rice (0.55) and lower for japonica varieties (0.47). On the contrary, Thomson and co-workers [32] reported as slightly higher index of gene diversity for the japonica group (0.56), while the indica group was 0.54. Polymorphic Information Content (PIC) value obtained in present study varied from 0.063 to 0.340 with a median of 0.150. PIC value revealed that OPBB-03 was considered as the best marker for four rice hybrids followed by OPBB-09 and OPY-11 could be considered as the least powerful marker.

Genetic distance refers to the genetic deviation between species or between populations within a species. It is measured by a spread of parameters like Nei's genetic distance. The value of pair-wise comparisons Nei's [25] genetic distance between four rice hybrids were computed from combined data through twenty primers, starting from 0.1190 to 0.2262. Our observation were partially supported by [33], [30], [34]. They analyzed 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 and co-workers [35] reported 0.19-0.54 genetic distances within Indian cultivars and reference [36] showed 0.088-0.504 within BRRI varieties. Dendrogram figure revealed that, four rice hybrids segregated into three major clusters. 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' consisted of only one hybrid (Hybrid3). Our observation was partially supported by Rahman and co-workers [30] conducted the UPGMA dendrogram based on Nei's genetic distance grouped the six cultivars of rice into three clusters. It is concluded that RAPD markers have been proved to be as a fast, efficient technique for variability assessment that complements methods currently being used in genetic resource management. SSR primer RM1 and RM17 showed few polymorphic bands which can be used as an evidence of variety protection data.

V. CONCLUSIONS

Hybrid rice characterization through molecular marker is a new technique of hybrid variety documentation. Some commercial seed companies are doing this type of DNA profiling work to protect their own hybrid from any kind of corruption. DNA passport data or DNA blue print information can help in variety protection of the concern crop documentation. Little information is available on the molecular characterization of hybrid rice. Molecular characterization of rice hybrid was carried out with 20 RAPD and 4 SSR primers. The polymorphism was obtained about 30.20%. Nei's genetic distance starting from 0.1190 to 0.2262. PIC value ranged from 0.063 to 0.340 with an average of 0.150. The fingerprint data, DNA banding pattern, molecular characterization, genetic distance, genetic identity or similarity of the four rice hybrid supplied by a private seed company are the basic documentation for the protection of their own hybrid.

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Author contributions

Absana Islam conducted the research and wrote the article; Shamim Ara Sumi and Abdul Wahab helped author in lab works and analyzing the data and M. E. Hoque supervised the study and edited the manuscript.

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