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Genetic Diversity Study of Indigenous Rice (*Oryza sativa* L.) Genotypes from Bangladesh

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Key words: Genetic distance, Indigenous rice genotypes, Agronomic traits, PIC, SSR markers

Abstract

Six SSR markers, namely, RM17, RM19, RM25, RM30, RM206 and RM225 were used to study the genetic diversity among the seven rice genotypes which are highly polymorphic, reliable, and have been shown to be linked to important traits in rice. The most alleles were found in locus RM17, whilst the fewest alleles were found in loci RM25 and RM225. The rice varieties Hori and B-11 were shown to have the lowest genetic distance, but various combinations of rice varieties between Lombur, Pokkali, BRRIdhan-29, Kalampazam, and BRRIdhan-47 showed distinct genetic distances. UPGMA dendrogram prefaced partitioning of 7 rice varieties, indigenous Lombur and salt tolerant Pokkali varieties were closely related in one cluster and another cluster containing rest of varieties in one group. It can be concluded that Lombur rice varieties having rich genetic information resources linked with improved agronomic traits and stress tolerance is a significant rice genotypes for sustainable mutation breeding program.

Introduction

Rice is one of the most important cereal crops and approximately half of the world population consumed it as a staple food (Violita 2019). Now-a-day's 7.5 billion people are living in the world, and it will reach 9.7 billion in 2050 (Skaf et al. 2019). To feed the increasing population, the hybrid varieties should be considered as a maximum rice production instead of wide use of chemical fertilizers in agriculture (Tait and Barker 2011).

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Rice has also acquired position next to wheat for its own qualities in the global food grain production. Rice production is an important activity in Bangladesh because more than 80% people involve on agricultural activity directly or indirectly. The agricultural land of this country consists of salinity area, low land area and some hill-tract area. In Bangladesh the farmers grown rice throughout the year with maintaining three distinct cropping seasons i.e., Aus (March-April to June-July), Aman (June-July to November-December) and Boro (December-January to May-June) which is specifically denoted irrigated, rain fed and deep-water condition (Rashid, 1994). According to Bangladesh Rice Knowledge Bank (BRKB) rice is grown in 10.5 million hectares from 14.3 million hectares total cultivated land area. On the other hand, the population of Bangladesh is still growing by two million every year. Maintaining or increasing food production in the face of limitation rice cultivated area is an important future challenge to feed continuous increasing population in Bangladesh. In these circumstances, improvement of new variety which able to adapt adverse condition with higher yield from existing cultivars is the best option for a researcher. Existing morphological traits, biochemical and DNA are a reservoir of enormous variation among rice varieties which could play a wide scope for future improvement (Chakravarthi and Naravaneni 2006).

However, rice germplasm in respect of a rich reservoir of useful genes can be used to estimate significant genetic gains and improve existing variety. In many cases both farmers and researchers may find the variation in their existing varieties or experimental fields that lead to scope selection technique for maintaining the purity or even to screen in order to develop new variety with desired traits. Mostly, it becomes more difficult for giving effort to separate distinctly among them based on agronomical traits due to genotype-environment interaction. In these cases, for the identification of cultivars or new varieties, molecular markers have been used (Mailer et al. 1994). It also to be powerful tool in the clarification of genetic relationship within and among the species. There are many markers available such as RELP, RAPD, SSRs, AFLP and SNPs. DNA based SSR marker is one of them that considers most effective tools to identify variation among germplasm due to their own properties (Gupta et al. 2000, Woodhead M et al. 2005). In plants, it has also been indicated that SSRs are well informative, locus specific markers in many species (Hoshino AA et al. 2012, Akkaya et al. 1992, Lagercrantz and Andersson 1993, Wu and Tanksley 1993). In this investigation a study was conducted to assay the genetic diversity in four indigenous rice genotypes with three other rice cultivars.

Materials and Methods

Four indigenous rice genotypes i.e., Lombur, Hori, Kalampazam, B-11 along with cultivated varieties BRRI dhan-29 and BRRI-47 of Bangladesh and salt tolerant Pokkali were cultivated for evaluating of their genetic diversity. The seeds of Lombur, Hori, Kalampazam, B-11 rice genotypes were collected from Teknaf, Cox`s bazar in coastal area of Bangladesh. Lombur rice genotype has been used for cultivation in the coastal

ecosystem due to its longer plant height which is suitable for flush flood prone areas (Mia et al. 2022). Pokkali rice seeds was obtained from Dr. Yoshihiro Hase, QST, Takasaki, Tokyo, Japan. BRRI-29 and BRRI-47 were obtained from Bangladesh Rice Research Institute (BRRI), Gazipur, Bangladesh. The genotypes of coastal areas are cultivated with shrimp cultivation. Seeds of cultivar were sown in seed bed on June 2019, transplanted on July 2019 and harvested on November 2019 at the Plant Biotechnology and Genetic Engineering Division (PBGED) experimental field, AERE, Savar, Dhaka in a randomized block design (RBD) with three replications for each genotype. Each plot size was 5.0 m × 2.0 m and distance between rows and plants were maintained 20 cm and 15 cm respectively. Recommended dose of fertilizer, cultural and intercultural practices were done as and when required.

Whole genomic DNA was extracted from juvenile leaf of 15 days seedlings by the method of Rezadoost et al. (2016). For this purpose, 50 mg of the leaf with small size was put in a 2 ml tube. Then 400 µl extraction buffer including 200 mM Tris-HCl, 1.4 M NaCl, 0.5% (v/v) Triton X-100, 3% (w/v) CTAB, and 0.1% (w/v) PVP was added and was vortexed for the 20 s and the tube was then transferred to the heat sink at 60°C for 30 min. Then 400 µl chloroform-isoamyl alcohol (24:1, v/v) was added and kept on ice for 2 min. 300 µl of supernatant was transferred to a 2-ml tube after performing centrifuged at 10,000 rpm at 4°C for 20 min. then, 1/2 volume buffer 2 containing 50 mM Tris-HCl, 2 M guanidine thiocyanate, 0.2%(v/v) mercaptoethanol (add to buffer only before use) and 0.2 mg/ml Proteinase K were added to the solution. The mixed solution was centrifuged at 10,000 rpm at 4°C for 20 min. The pellet was then washed with 75% ethanol gently. The extracted DNA was stored at - 20°C. After that 1% agarose gel electrophoresis was used by using the 1X TBE buffer to determine the quality of extracted DNA. After analyzing the bands, nanodrop lite spectrophotometer was used to determine DNA concentration. The light absorption index (A260/ 280 nm) was also read to get desire ratios ranging from 1.8 to 2. DNA samples were diluted up to 10 ng/µl. Highly polymorphic primer i.e., RM 17, RM 19, RM 25, RM 30, RM 206 and RM 225 were used in this experiment (Table 1). Polymerase chain reaction (PCR) was carried out as described by Panaud et al. (1996) in 20 µl reaction mixture containing 20 ng genomic DNA, 2 µl 10x PCR buffer, 1 µM dNTPs, 0.05 µM Taq DNA Polymerase, 1 µM of each primer and a suitable amount of sterile deionized water. This thermal cycle was performed for 32 cycles initial denaturation at 94°C for 20 sec, annealing at 55°C respective primers for 30 sec, primer extension at 72°C for 40 sec, followed by a final extension at 72°C for 2 min.

PCR product was performed by 4% agarose gel electrophoresis. Before electrophoresis each sample was prepared with one-fourth or fifth loading dye of sample volume (0.25% xylene cyanol, 0.25% bromophenol blue, 30% glycerol and 1 mM EDTA). After gel preparation, it was placed in the gel electrophoresis chamber to make sure the level of 1×TBE buffer (running buffer) was ~5 mm above the gel. The amplified PCR products along with a 100 bp DNA marker ladder was performed at 100 V for 30 min. then, agarose gel was placed in ethidium bromide solution for screening 10 min and

carefully washed with distilled water for minimum 5 min. For the documentation, gel was taken out from distillated water and was placed on gel documentation chamber to observe the band of DNA.

Table 1. List of primers used in the PCR with their 5'-3' sequence, chromosomal location, no. of alleles, PIC and annealing temperature.

Primer name	Forward sequence	Reverse sequence	Chr. No.	PIC value	AT (°C)
RM17	TGCCCTGTTATTTTCTTCTCTC	GGTGATCCTTTCCCATTTCA	12	0.53	55
RM19	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA	12	0.14	
RM25	GGAAAGAATGATCCTTTTCATGG	CTACCATCAAAACCAATGTTC	8	0.60	
RM30	GGTTAGGCATCGTCACGG	TCACCTCACCACACGACACG	6	0.41	
RM206	TAGTTTAACCAAGACTCTC	GGTTGAACCCAAATCTGCA	2	0.57	
RM225	TGCCCATATGGTCTGGATG	GAAAGTGGATCAGGAAGGC	6	0.72	

PIC = polymorphism information content, AT = annealing temperature

The aplegen (omega lum W) imager was used to photograph the DNA band profiles of our tested rice varieties against six SSR primers. The most intensely amplified band size (in nucleotides) for each microsatellite marker was calculated since its migration in relation to the molecular weight (mw) of size markers (100 bp DNA ladder) using the software Alpha Ease FC (Alpha Innotech). Sample size represent the target population whose traits are included at study (Table 2). The equation for calculating sample size is shown below.

$$n = \frac{z^2 \times \hat{p}(1-\hat{p})}{\varepsilon^2}$$

Where, z is the z score estimated from confidence level, ε is the margin of error, p̂ is the population proportion.

The bands of the each SSR loci represent the alleles and designated the bands as A, B, C etc. from the gel. The genotypes of different individuals were hypothetically scored as AA, BB, CC etc. for homozygous or as AB, AC, BC etc. for heterozygous. Genetic diversity studies (allele number, allele frequency, gene flow, Shannon index, expected homozygosity and heterozygosity etc.) data of our targeted primer were congregated through diploid data analysis for co-dominant marker by population genetic analysis software POPGENE 1.31 (Yeh et al. 1999). It is better to mention that the genetic variability depends on the value of heterozygosity; those are positively correlated with each other. As they are indigenous rice varieties, it is usual to have less heterozygosity (Table 2). Estimation of Nei's genetic distance values (Nei 1983) and construction of UPGMA dendrogram was constructed to infer genetic relationships using power marker software 3.25 (Liu and Muse 2005).

Table 2. Summary of genetic variation for all loci.

Locus	Sample size	na	I	Nm	Exp_Hom	Exp_Het	Gene diversity	Het
RM17	14	7	0.41	0.00	0.08	0.92	0.86	0.00
RM19	14	5	0.48	0.00	0.17	0.84	0.77	0.00
RM25	14	4	0.55	0.00	0.21	0.79	0.73	0.00
RM30	14	6	0.52	0.00	0.21	0.79	0.82	0.00
RM206	14	5	0.44	0.00	0.12	0.88	0.74	0.00
RM225	14	6	0.47	0.00	0.27	0.73	0.82	0.00
Mean		5.17	0.48	0.00	0.17	0.83	0.79	0.00
Standard deviation		1.17	0.13		0.07	0.07	0.21	

na = observed number of alleles/genotypes, I = Shannon's Information Index (Lewontin 1972), Nm = gene flow estimated from Fst = 0.25(1 - Fst)/Fst., Expected homozygosity and heterozygosity were computed using Levene (1949).

The following formula was used to calculate the polymorphism information content (PIC) value of SSR markers (Anderson et al. 1993):

$$PIC = 1 - \sum_{i=1}^{K} p_i^2$$

Where k is the total number of alleles (bands) detected for one SSR locus, and p is the fraction of variety or genotypes in all samples studied that have the allele (band). PIC calculates a marker's discriminatory power by considering the expressed allele number and relative frequencies of those alleles. PIC values range from 0.00 (monomorphic) to 1.00 (very high discriminative).

Results and Discussion

In this study, four indigenous rice genotypes collected from different regions of salt prone areas in Bangladesh was evaluated for genetic correlation with the cultivated rice varieties using six SSR primers in terms of varied agronomic traits. Upon comparison, the best rice genotypes in terms of better yield and yield attributing traits on the basis of genetic correlation would be significant material for mutation breeding program for further improvement. The obtained results were presented and expressed as table and figure for getting easy to understand that consider as a source of diversity information for a researcher.

To evaluate the yield and yield attributing characters, all rice genotypes that assigned earlier were grown in the experimental field at Institute of Food and Radiation Biology (IFRB), AERE, Savar, Dhaka, Bangladesh. According to our previous study (Das et al. 2019), we found variation in different agronomic traits such as yield production, height, and grain quantities etc. we also found in the same paper that these rice genotypes

showed varied resistance to salinity under *in vitro* condition. These findings indicated that diversification of agronomic traits among the indigenous rice genotypes that leads us to investigate genetic variation among these cultivars.

SSR is one of the most widely utilized molecular markers in genetic research. Because of their abundance and relatively low technique requirements, SSRs have been successfully used for molecular variation of rice variety genomes. (Temnyk et al. 2000). All the SSR loci amplified (RM17, RM19, RM25, RM30, RM206 and RM225) were polymorphic. Fig. 1 shows the SSR profiles of loci RM17 and RM206. Using 6 SSR primers across 7 varieties, 31 alleles were identified. The number of alleles ranged from 4 to 7 per locus. The locus RM17 was shown the highest number of alleles (7.00) while the locus RM25 and RM225 was found the lowest number of alleles (4.00). Alam et al. 2016 identified 62 alleles using five SSR primers across 28 local rice varieties in Bangladesh.

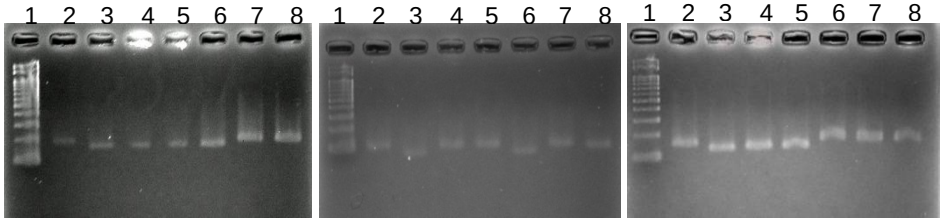

Fig. 1. SSR profiles of 7 local rice varieties at locus RM17, RM30 and RM206 (lane1: 100 bp ladder; lane 2: Pokkhali; lane3: Kalampazam; lane4: Hori; lane5: B11; lane6: Lombur; lane7: BRRI dhan29; lane8: BRRIdhan47).

Genetic diversity of seven rice genotypes for six SSR universal primers are given in Table 2. The highest Nei's genetic diversity (0.86) was shown in RM17 locus whilst the lowest (0.73) was found in RM25 locus. The average value of genetic diversity was 0.7892 and the average value of gene flow (Nm) was 0.000. From the investigation it is suggesting that seven indigenous rice genotypes have a higher level of genetic variation and a lower level of gene flow values, revealing variability among the varieties investigated. Rice genotypes differed in expected homozygosity, heterozygosity, and Shannon's information index. Hardy-Weinberg expectation of average heterozygosity in sub-population (Ht) was obtained 0.83 where homozygosity was found 0.17. This result indicates that significant levels of genetic variations was existed among this rice genome. The lowest Shannon's information index score for RM17 indicates higher genetic diversity for this locus.

The seven rice genotypes were all homozygous, with no heterozygosity. Variation also appeared in allele frequency and PIC values (Table 3). The range of allele frequency was from 0.1429 to 0.5000. In the present report, the polymorphism information content (PIC) value ranged from 0.68 to 0.83, with an average of 0.76. The higher PIC index value was 0.83965 for RM17. This result similar with Ahmed et al. (2016) reported a range of

0.44 to 0.89 and average of 0.74 and partially closed to previous values: 0.34 to 0.88 and an average of 0.66 (Thomson et al. 2007), as well as 0.04 to 0.59 (Shah et al. 2013) and 0.05 to 0.67 (Islam 2014). This result indicated that it can be attributed to the use of more informative markers (Akkaya and Buyukunal-Bal 2004). Closely related genotypes result in lower PIC value and diverse genotypes result in higher PIC values (Jeung et al. 2005).

Table 3. The Frequency of allele of SSR markers.

Allele	RM17	RM19	RM25	RM30	RM206	RM225
Allele A	0.1429	0.1429	0.2857	0.1429	0.1429	0.1667
Allele B	0.1429	0.2857	0.1429	0.1429	0.1429	0.1667
Allele C	0.1429	0.2857	0.2857	0.1429	0.1429	0.5000
Allele D	0.1429	0.1429	0.2857	0.1429	0.1429	0.1667
Allele E	0.1429	0.1429	-	0.4286	0.1429	-
Allele F	0.1429	-	-	-	0.2857	-
Allele G	0.1429	-	-	-	-	-
PIC	0.8396	0.7396	0.6847	0.7913	0.6997	0.7913
ONA	7	5	4	5	6	4
ONA (Mean)	-	-	5.17	-	-	-

ONA = Observed number of alleles

The Nei's genetic distance value ranged from 0.6938 to 1.7918 (Table 4). The highest Nei's genetic distance (1.00) was calculated in 12 combinations, the lowest genetic distance (0.500) was estimated in Hori and B 11 varieties. Genetic distance is an important factor that influences the genetic relatedness of populations (Nei and Roychoudhury 1974).

Table 4. Nei's genetic distance among 7 rice varieties.

Variety	Pokkhali	Kalampazam	Hori	B 11	Lombur	BRRIdhan 29	BRRIdhan 47
Pokkhali							
Kalampazam	1.00						
Hori	1.00	1.00					
B 11	1.00	0.83	0.50				
Lombur	0.67	1.00	0.83	0.83			
BRRIdhan 29	1.00	1.00	1.80	1.80	1.00		
BRRIdhan 47	1.00	1.00	1.00	1.00	0.83	0.83	

UPGMA dendrogram prefaced partitioning of 7 rice varieties into two main clusters (Fig. 2) based on Nei's (1983) genetic distance. The closely related Lombur and Pokkali varieties are in one cluster and another cluster containing other 5 related rice varieties in

one group. The one cluster again separated into two sub-clusters. BRRIdhan-29 and BRRIdhan-47 which are closely related are in one group and other sub cluster containing Hori, B 11, and Kalampazam rice varieties. Again, the one sub cluster subsequently separated into two groups. One group contains closely related Hori and B 11; and other group contains distance variety Kalampazam. Although the genotypes Lombur, Kalampazam, B-11, and Hori were collected with the same place in shrimp cultivation area, but Lombur is far different from other genotypes resulting Lombur has conserved additional characteristics than others. Interestingly, the Lombur cultivar was similar with Pokkali which was collected from Japan leading this genotype may be ability to adapt in different environments. The highest yield production was found in Pokkhali which is similar to Lombur by clustering leading Lombur could be improved in term of production as well as adaptation to different stress conditions by good management practices. On the other hand, BRRIDhan-29 and BRRIDhan-47, collected from BRRI, Gazipur, Dhaka, Bangladesh, showed similar patterns by clustering. This finding suggested that the features of these genotypes are similar and Lombur rice genotype might have different genetic makeup which require further study for the improvement of this genotype.

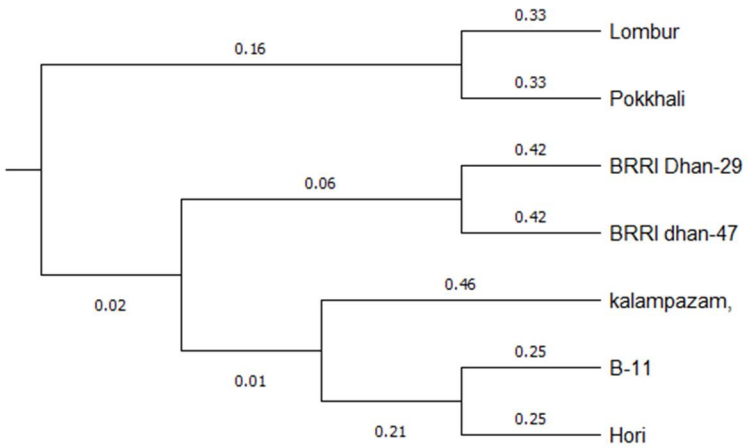

Fig. 2. UPGMA dendrogram based on Nei's (1983) genetic distance of 7 rice varieties.

From the investigation, it could be concluded that the parental source or genotype would be similar in the same sub-cluster that refers to less genetic diversity comparatively. In the meantime, the genotypes that showed genetic distance in close level could be used for further study to get genomic information of corresponding genotypes. Moreover, this study signifies the importance for conserving these indigenous rice genotypes having diversified gene pool which would be useful for sustainable crop improvement using modern breeding techniques.

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