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Genetic polymorphism in rice (*Oryza sativa* L.) through RAPD analysis

Shamsun Naima Rahman, Md Shahidul Islam*, Md Samsul Alam[1] and Khondoker M Nasiruddin

Department of Biotechnology and [1]Department of Fisheries Biology & Genetics
Bangladesh Agricultural University, Mymensingh 2202, Bangladesh

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Random Amplified Polymorphic DNA (RAPD) assay was performed to estimate genetic polymorphism in six different rice (*Oryza sativa* L.) cultivars viz. Basmati 370, DM 25, IRATOM 24, Binadhan 6, TNDB 100 and Y 1281. Three out of the 15 decamer random primers showed amplification of genomic DNA in 24 individuals. The primers produced a total of 26 bands of which 14 were polymorphic. Proportion of polymorphic bands and gene diversity estimates were 26.92% and 0.09 for Basmati 370, 11.54% and 0.04 for DM 25, 11.54% and 0.05 for IRATOM 24, 7.69% and 0.02 for Binadhan 6 and 23.08% and 0.11 for TNDB 100 whereas Y 1281 cultivar was monomorphic indicating the existence of high level of intra cultivar genetic variation in Basmati 370 and TNDB, respectively 100. High levels of population differentiation (G_{ST} = 0.75) and low levels of gene flow (N_m = 0.16) estimates across all the loci indicate sufficient existence of genetic variation among these six cultivars. Low intra cultivar variation and significant differentiation in different cultivar pairs was observed at a number of loci. Nei's genetic distances estimated among the different pairs of cultivars were correlated with geographical distances. The UPGMA dendrogram based on Nei's genetic distance clubbed the cultivars into three clusters. RAPD analysis showed promise as an effective tool in estimating genetic polymorphism in different rice cultivars.

Keywords: RAPD, polymorphism, similarity index, genetic distance, rice

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Introduction

Rice (*Oryza sativa* L.), belonging to the family Poaceae, is one of the most commercially important food crops in world. High temperature during the monsoon season with high rainfall favours cultivation of rice in Bangladesh. About 75% of the total cropped area and 83% of the total irrigated area is presently used for rice cultivation[1]. It accounts for 92% of production of total food grains of the country. More than 90% of the population consumes rice in Bangladesh. A large number of rice cultivars, compatible to varied agro-ecological conditions have been evolved and are being cultivated in the country. About 5000 rice accessions are available in the Germplasm Bank of Bangladesh Rice Research Institute. However, all these varieties are not grown in Bangladesh. Developmental activities and exploitive land-use pattern are destroying natural habitats, and furthermore, modern varieties (e.g. HYVs in rice) are replacing indigenous cultivars and land races, resulting in a substantial reduction of genetic diversity too.

* Author for correspondence:
Tel: 880-91-55695-7, Ext. 2560; Fax: 880-91-55810
E-mail: shahidj1@yahoo.com

Discernible improvements have been observed in tools and techniques for classifying rice into modern and traditional (local) varieties. Selection of plant varieties based on morphological characters is not very reliable because major characters of interest possess low heritability and are genetically complex. Molecular markers based on DNA sequence are found to be more reliable[2]. They represent an opportunity to provide information on the variation that exists in a particular species within a local region as well as among different countries. 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 RAPD fingerprinting in commercially important crops[3]. RAPD markers are considered to be unbiased and neutral markers for genetic mapping applications[4], in population genetics[5], taxonomy[6] as well as for genetic diagnostics. This technique always allows the examination of genomic variation without prior knowledge of DNA sequences[7] and is especially useful for unzipping the variations in species with low

genetic variability when other techniques such as isozyme analysis fail to reveal differences among the individuals[8,9]. Keeping all these in purview the present work was undertaken to estimate genetic variation in the germplasm of six rice cultivars using RAPD technique for future use in selection, hybridization, biodiversity assessment, evaluation and conservation of diverse gene pools, etc.

Materials and Methods

Sample Collection

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 were collected from Bangladesh Institute of Nuclear Agriculture, Mymensingh in the month of April 2004. To undertake RAPD analysis, young growing tillers were collected randomly from each cultivar and fresh leaf from each tiller was used for isolation of genomic DNA. Cultivars were distinctly different from each other in respect of their origin. Basmati 370 and DM 25 cultivars were aromatic rice whereas the other four were high yielding non-aromatic lines. Basmati 370 is scented *indica* rice whereas DM 25 is an advanced mutant of Basmati 370. IRATOM 24 is a released variety from Bangladesh (a mutant of IR 8) while Binadhan 6 is a mutant of IRATOM 24 × Dular. TNDB 100 is an advanced mutant line of Vietnam and Y 1281 is a mutant in advance generation originated from Malaysia having high yield and long grain characteristics.

Extraction of Genomic DNA

Genomic DNA from each individual was extracted from young leaf tissues following SDS extraction, phenol:chloroform:isoamyl alcohol purification and ethanol precipitation methods. About 2 cm of leaf tissue was cut into small pieces, taken into centrifuge tube, homogenized and digested with extraction buffer (50 m*M* Tris HCl, 25 m*M* EDTA, 300 m*M* NaCl and 1% SDS) at 65º C for 15 min. Tissue lysate was purified with phenol:chloroform:isoamyl alcohol (25:4:1). DNA was precipitated using absolute ehanol, pelleted by centrifugation and suspended in TE buffer (10 m*M* Tris. HCl, 1 m*M* EDTA, *p*H = 8.0). DNA was reprecipitated by adding 70% ehanol in the presence of 0.3 *M* sodium acetate, and pelleted by centrifugation. Pelletes were air-dried and resuspended in TE buffer. Extracted DNA samples were checked by using 1% agarose gel electrophoresis and quantified by using a spectrophotometer.

Primer Selection

Initially 15 decamer custom primers (Banglore Genei, India) were evaluated for two randomly chosen individuals in each cultivar to test their suitability in amplifying rice. Primers were selected on the basis of intensity or resolution of bands, repeatability of markers and consistency within individual and potential to differentiate populations (polymorphism). Finally three primers displaying appreciable banding patterns were selected for the analysis of all six cultivars (Table 1).

PCR Amplification

Amplification conditions were set based on a standard method[10] with some modifications. PCR reactions were performed on each DNA sample in a 10 µL reaction mix containing 1 µL of 10x Ampli Taq polymerase buffer, 2 µL of 10 µ*M* primer, 1 µL of 250 µ*M* dNTPs, 1 unit of Ampli Taq DNA polymerase (Bangalore Genei, India) and 75 ng of genomic DNA and a suitable amount of sterile deionized water. DNA amplification was performed in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf). The reaction mix was preheated at 94ºC for 3 min followed by 40 cycles of 1 min denaturation at 94ºC, 1 min annealing at 34ºC and elongation or extension at 72ºC for 2 min. After the last cycle, a final extension of 7 min at 72ºC was added.

Agarose Gel Electrophoresis

Amplified products from each sample were separated electrophoretically on 1.4% agarose gel (Fisher Biotech, USA) containing ethidium bromide in 1XTAE buffer at 120 V for 1½ h. To determine molecular weight a DNA marker (Ø X 174 DNA/Hae III digest and /or 100 bp DNA ladder) was electrophoresed alongside RAPD products. DNA bands were observed on UV-transilluminator and photographed by a Gel Cam Polaroid camera.

Table 1—RAPD primers with corresponding bands scored and their size range together with polymorphic bands observed in rice cultivars

Primer code	Sequence (5´-3´)	Total no. of bands (bp) scored	Size ranges	No. of polymorphic bands
OPD05	TGAGCGGACA	6-10	360-1353	6
OPD06	ACCTGAACGG	5-7	540-1250	3
OPD07	TTGGCACGGG	6-9	580-1550	5

Data Analysis

RAPD bands were scored visually. Their presence was scored with 1 and absence with 0, separately for each cultivar and each primer. For more accuracy, two persons scored the bands. Bands not identified by two scorers were considered as non-scorable. Scores in respect of all primers were pooled for constructing a single data matrix. This was used in estimating polymorphic loci, gene diversity[11], coefficient of population differentiation (G_{ST}), gene flow (N_m), genetic distance (D)[12] and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among populations using POPGENE (version 1.31) computer program[13]. The same program was also used to perform test of homogeneity in different locus between population pairs.

Similarity index (SI) between RAPD profiles of any two individuals on the same gel was calculated from RAPD markers by using following formula:

$$\text{Similarity Index (SI)} = 2N_{AB} / (N_A + N_B)$$

Where, N_{AB} represents total number of RAPD bands shared by individuals A and B. N_A and N_B are the numbers of fragments scored for each individual, respectively[14]. Within population similarity (S_i) was calculated as average of SI across all possible comparisons between individuals within a population. Between population similarity (S_{ij}) was calculated as average similarity between randomly paired individuals from populations i and j[15].

Results and Discussion

Despite most commercially important crop in Bangladesh, research findings on genetic analysis of different rice cultivars using molecular markers are scarce. RAPD revealed ample polymorphisms among six rice cultivars profiled in this study. Three primers displayed appreciable band resolution and substantial variations among different cultivars. Total 26 bands were observed of which 14 (53.85%) were polymorphic (either occurring in or absent in less than 95% of individuals). Primer OPD05 produced maximum bands whereas OPD06 produced minimum bands (Table 1). Banding patterns of different cultivars in respect of three primers showed that primer OPD05 produced maximum polymorphic bands (Fig. 1). Present study also indicated the effectiveness of RAPD in detecting polymorphism among different cultivars of rice. RAPD polymorphism in a set of *O. sativa* accessions was also reported earlier [16, 17].

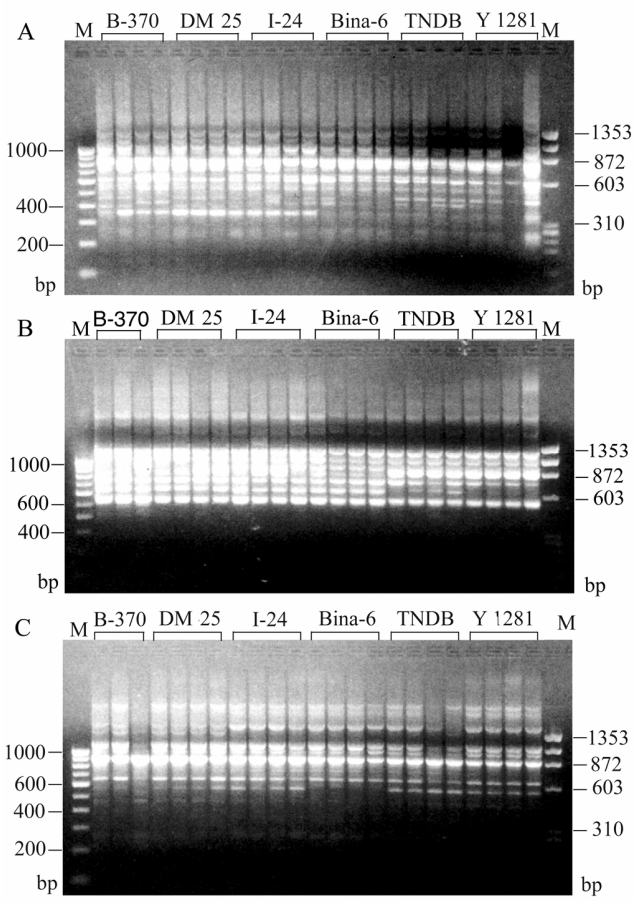

Fig. 1—RAPD profiles of six different cultivars of rice using primer OPD05 (A), OPD06 (B) and OPD06 (C). B-370: Basmati 370, I-24: IRATOM 24, Bina-6: Binadhan 6 and TNDB: TNDB 100. M: Molecular weight marker (100 bp DNA ladder and Ø X 174 DNA/Hae III digest).

Within and between Cultivar Similarity Indices

Intra cultivar similarity index (S_i) for Y 1281 was highest (100%) followed by Binadhan 6, DM 25, IRATOM 24, and TNDB 100 accessions, respectively while S_i value for the Basmati 370 was the lowest (Table 2).

Table 2—Estimates of genetic variation: number and proportion of polymorphic loci and gene diversity values obtained in different cultivars of rice

Cultivar	Number of polymorphic loci	Proportion of polymorphic loci (%)	Gene diversity
Basmati 370	7	26.92	0.09
DM 25	3	11.54	0.04
IRATOM 24	3	11.54	0.05
Binadhan 6	2	7.69	0.02
TNDB 100	6	23.08	0.11
Y 1281	0	0	0

Similarities (S_{ij}) between individuals of different cultivars showed highest value (93%) for TNDB 100 Vs Y 1281. Lowest similarity (S_{ij} = 78%) was observed between Basmati 370 Vs Y 1281 cultivar pair (Table 3). Band-sharing based similarity indices between individuals of same cultivar were higher (average: 95.33%) than similarity indices (average: 86.47%) between individuals among different cultivars. This implies that individuals within each cultivar are genetically more similar to each other, as expected than to individuals from all other cultivars.

Polymorphism in Different Populations

Frequency of polymorphic bands of six different cultivars was highest (26.92%) and lowest (0) across all three primers in Basmati 370 and Y 12814 population, respectively (Table 2). Cultivars showing higher intra-population similarity and lower proportion of polymorphic bands are likely to have less heterozygosity in comparison to those showing less intra cultivar similarity and higher proportion of polymorphic bands. In other words, cultivars having higher similarity are more homogenous. The Basmati-6 and TNDB 100 were found to be more heterozygous compared to other cultivars.

Out of the 14 polymorphic bands 11 were found to cause substantial deviation from homogeneity in the cultivar pairs (Table 4). Basmati 370 Vs Y 1281 cultivar pair was not homogenous at maximum number of bands (9). Basmati 370 Vs TNDB 100 and Basmati 370 Vs IRATOM 24 cultivar pairs were not homogenous at seven bands. Only one band causing significant departure from homogeneity was found in TNDB 100 Vs Y 1281 cultivar pair. Basmati 370 Vs DM 25 as well as IRATOM 24 Vs Binadhan 6 cultivar pairs were not homogenous at three loci.

Nei's Gene Diversity

Intra cultivar gene diversity values showed gene diversity across all populations for all loci was 0.21. High intra cultivar gene diversity was observed in TNDB 100 and Basmati 370 cultivars whereas the gene diversity value for Y 1281 was nil (Table 2). In respect of intra cultivar similarity indices (S_i), the proportion of polymorphic loci and gene diversity[11], Basmati 370 was found as the genetically most diversified cultivar followed by TNDB 100 cultivar.

Population Differentiation and Gene Flow

Overall differentiation among six different cultivars was high (G_{ST} = 0.75). Estimated gene flow (N_m) across all cultivars was low (N_m = 0.16). G_{ST} and N_m values indicated that the cultivars are different from each other. N_m values between cultivar pairs (Table 3) indicate low level of gene flow between each pair of them possibly resulting from low level of cross-pollination between each pair of cultivars. Rice is a self pollinating plant, the pollen of which is short lived (5 min) and there are no known insect pollinators. It is, therefore, highly unlikely that cross-pollination by wind and other rice plants outside of trial area will occur. Another reason behind the self-pollination is likely that since the florets of rice are adichogamous, most of the florets are self-pollinated at the time of floret opening[18]. Synchrony between floret opening and anther dehiscence may contribute to the high rate of self-pollination[19]. Thus, self-pollination reduces the chance of inter mixing of genetic make up of different cultivars resulting in low level of both intra cultivar genetic variation and gene flow in different cultivar pairs.

Genetic Distance

Pair-wise comparisons of genetic distance[12] between cultivars, computed from combined data for three primers, ranged from 0.07 to 0.37 (Table 5). Relatively low genetic distance was observed in TNDB 100 Vs Y 1281, IRATOM 24 Vs Binadhan 6 and Basmati 370 Vs DM 25 cultivar pairs comparative to the other cultivar pairs.

Geographical distance is an important factor that influences the genetic relatedness of populations[20].

Table 3—Inter cultivar similarity indices (%) (above diagonal), intra cultivar similarity indices (%) (in parentheses) and gene flow values (below diagonal) of different cultivar pairs of rice

Cultivar	Basmati 370	DM 25	IRATOM 24	Binadhan 6	TNDB 100	Y 1281
Basmati 370	(88)	91	84	83	81	78
DM 25	0.61	(97)	86	83	88	87
IRATOM 24	0.33	0.22	(96)	92	89	86
Binadhan 6	0.36	0.11	0.34	(98)	88	88
TNDB 100	0.41	0.46	0.47	0.38	(93)	93
Y 1281	0.15	0.10	0.13	0.05	0.83	(100)

Table 4—Chi-square (χ^2) values for the loci causing significant departure from homogeneity in different cultivar pairs (above diagonal) of rice

Cultivars	Loci	DM 25	IRATOM 24	Binadhan 6	TNDB 100	Y 1281
Basmati 370	OPD05-2	6.11*	6.11*	4.8*	6.11*	6.11*
	OPD05-5		4.38*			
	OPD05-7		4.38*		4.38*	4.38*
	OPD05-8				4.38*	4.38*
	OPD05-10			8.00**	8.00**	8.00**
	OPD06-3		6.11*	4.8*		
	OPD06-4	6.11*		4.8*	6.11*	6.11*
	OPD06-6					8.00**
	OPD07-2		8.00**	8.00**		8.00**
	OPD07-4		8.00**	8.00**	8.00**	8.00**
	OPD07-9	8.00**	8.00**		8.00**	8.00**
DM 25	OPD05-5		6.11*	6.11*		
	OPD05-7		6.11*	6.11*	6.00**	6.11*
	OPD05-10			8.00**	8.00**	8.00**
	OPD06-3		8.00**			
	OPD06-4		4.38*	8.00**		
	OPD06-6					8.00**
	OPD07-2		8.00**	8.00**		8.00**
	OPD07-4		8.00**	8.00**	8.00**	8.00**
	OPD07-9			8.00**		
IRATOM 24	OPD05-5					8.00**
	OPD05-8				4.38*	4.38*
	OPD05-10			8.00**	8.00**	8.00**
	OPD06-3				8.00**	8.00**
	OPD06-4			4.38*	4.38*	4.38*
	OPD06-6					8.00**
	OPD07-2				4.38*	
	OPD07-9			8.00**		
Binadhan 6	OPD05-5					6.11*
	OPD05-8				6.11*	8.00**
	OPD06-3				8.00**	8.00**
	OPD06-6					8.00**
	OPD07-2				4.38*	
	OPD07-9				8.00**	8.00**
TNDB 100	OPD07-2					4.38*

* P<0.05; **P<0.01

Table 5—Summary of Nei's (1972) genetic distance (below diagonal) values in different pairs of six rice cultivars

Cultivar	Basmati 370	DM 25	IRATOM 24	Binadhan 6	TNDB 100
DM 25	0.12				
IRATOM 24	0.26	0.24			
Binadhan 6	0.30	0.31	0.11		
TNDB 100	0.32	0.19	0.21	0.20	
Y 1281	0.37	0.22	0.23	0.20	0.07

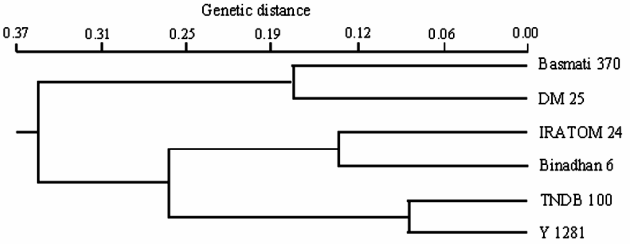

Fig. 2—UPGMA dendrogram based on Nei's (1972) genetic distance, summarizing the data on differentiation between rice cultivars, following RAPD analysis.

UPGMA dendrogram (Fig. 2) based on genetic distance[11] grouped six cultivars of rice into three clusters following geographical proximity. TNDB 100 cultivar was close to Y 1281 cultivar with least genetic distance (0.07) and formed a solitary cluster. IRATOM 24 made a cluster with the Binadhan 6 cultivar with the genetic distance of 0.11 and Basmati 370 grouped with the DM 25 with genetic distance of 0.12. Binadhan 6 is the mutant derived from cross IRATOM 24 × Dular and IRATOM 24 is mutant of

IR-8 and both of them were Bangladeshi cultivars. On the other hand, Basmati 370 and DM 25 were *indica* scented rice cultivars. Moreover, DM 25 was developed from Basmati 370 through mutation. Both Y 1281 (Malaysia) and TNDB 100 (Vietnam) were advanced lines developed through mutation breeding with high yield and long grain characteristics. Therefore, close relatedness between each pair of cultivars mentioned earlier cannot be ruled out. The results are consistent with the band-sharing based similarity indices and χ^2 values for loci causing significant departure from homogeneity.

Genetic variation is important in maintaining the developmental stability and biological potential of an organism. In essence, the present work revealed ample genetic variation and relatedness among the six rice germplasm. Low levels of genetic variation and high levels of genetic relatedness were found in each of the rice cultivars whereas significant levels of genetic variation were observed between each pair of rice cultivars. Significant genetic variation at maximum number of loci between cultivars indicates rich genetic resources in rice. Information on intra and inter cultivar genetic variation from the present study might be useful for breeders in making decision for improvement of rice cultivars through selective breeding and cross breeding programmes. Besides this, breeders could make a strategy for conservation of cultivars having diverse gene pools. As literature on genetic analysis of Bangladeshi rice is very scarce, present study could help the researchers in this regard in future. However, there were some lacunae in the present study e.g., only 24 individuals (four individuals per cultivar) and three primers were used in RAPD analysis that reduces the chance to obtain a reliable knowledge precisely about the genetic structure of each cultivar of rice. Further studies involving large number of samples and primers need to be conducted to get more precise information.

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